Alice Prince *Editor*

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Introduction

Bacterial pneumonia or lower respiratory tract infection is a major cause of morbidity and mortality worldwide, affecting diverse patient populations ranging from infants to the elderly; previously healthy individuals as well as those with major immunological deficiencies—genetic and acquired. Pneumonia results in a tremendous economic burden estimated at 40.2 billion dollars in the United States alone in 2006 (CDC). Recent tabulations list 4, 447,893 reported cases of pneumonia in USA (not influenza) in patients under the age of 18 in 2007, with another 1,576,376 cases in those over 65 years of age (American Lung Assn. 2010 data). Health care statistics attempt to separate the incidence of pneumonia due to influenza virus versus bacterial pneumonias, although many cases of influenza, particularly those that require hospitalization, are complicated by bacterial superinfection (Chien et al. 2009). Despite the prevalence of bacterial pneumonias, there have been few changes in the approach to either prevention or treatment over the past 50 years. While immunization strategies have been successful for a limited number of organisms, reliance upon antimicrobial agents to kill bacteria once infection is established has been the cornerstone of treatment for the past 50 years. Given the continuing high rates of mortality in selected patient groups, identifying specific targets in the host to prevent destructive immune responses, as is the standard in other pathological processes such as autoimmune diseases and cancer, could provide useful therapeutic adjuncts. Understanding the pathogenesis of pneumonia requires both an appreciation of bacterial virulence factors and the genetics underlying bacterial evolution and adaptation to the host, and an appreciation of the complexities of innate immune signaling in the lung; how the mucosal response to inhaled pathogens is activated and regulated.

The respiratory tract is continually exposed to potential pathogens and very frequently colonized by the very organisms, such as *Streptococcus pneumoniae* and *Staphylococcus aureus*, which can initiate severe infection if they gain access to the lower respiratory tract. The normal innate immune defenses of the respiratory tract provide a potent immunological as well as a physical barrier against bacterial penetration. For bacteria to successfully invade requires not only the expression of specific bacterial virulence determinants, but also requires the appropriate host; a patient with enhanced susceptibility to infection or exaggerated response to airway contamination by inhaled bacteria.

Many common pathogens colonize the upper respiratory tract; *S. aureus* often resides in the anterior nares, lined with a squamous cornified epithelial surface—whereas *S. pneumoniae* and the Gram-negative opportunists that often colonize hospitalized patients more often reside in the posterior pharynx, a mucosal surface. The entire respiratory epithelium is well defended against invading bacteria by an active mucociliary escalator and airway lining fluid replete with small peptides and cytokines with potent antimicrobial activities. Organisms that persist in this milieu are occasionally aspirated and gain access to the lower airways where they initiate the recruitment of phagocytes, predominantly neutrophils to eradicate infection. Most of the organisms that cause pneumonia have the genetic flexibility to actively adapt to the milieu within the airway and ongoing selection for mutants able to withstand the antibacterial activities of airway surface fluid and resist phagocytic clearance then cause infection. The major pulmonary pathogens *S. pneumoniae, S. aureus*, and the Gram-negative opportunists *P. aeruginosa* and *K. pneumoniae* have all evolved multiple and sophisticated mechanisms to counter the many effectors of local mucosal immunity.

Pneumonia, caused by the aspiration of upper airway flora, occurs when these mucosal clearance mechanisms are overwhelmed. Activation of innate immune signaling in the respiratory tract varies substantially depending upon the properties of the specific pathogen as well as the experience of the host with these pathogens. The majority of respiratory bacterial pathogens are extracellular, equipped to replicate in the airway lumen and only occasionally to persist intracellularly, within either a phagocyte or epithelial cell. Major airway pathogens express and shed specific gene products or PAMPs (pathogen-associated molecular patterns) that potently activate innate immune signaling in numerous cell types. Many of these PAMPs are recognized by airway epithelial cells, which have a major role in initiating proinflammatory signaling to recruit and activate immune cells in response to the perceived infection. While some of these PAMPs are recognized by surface-associated receptors, some bacterial products are endocytosed by both immune and stromal cells and stimulate intracellular signaling cascades that mediate type I interferon and inflammasome-mediated signaling.

Each of the major components of the mucosal immune system has a scripted response to the presence of bacterial PAMPs at a site in the lung that is normally sterile. It has become apparent that the nature of the immune response that is elicited is highly variable depending upon the repertoire of virulence factors and PAMPs expressed by specific organisms. The extent of immune signaling is determined by the accessibility and distribution of relevant pattern-recognition receptors. Thus in the airway, resident alveolar macrophages have an important function in the initial recognition of perceived pathogens. For example, the ingestion of *P. aeruginosa* and its flagellin stimulates both TLR5-associated NF-κB proinflammatory signaling and also activates the more potent effectors released by the NLRC4 inflammasome, with resultant caspase-1 activation, generation of IL-1β and IL-18 as well as the induction of pyroptosis, itself a proinflammatory form of cell death. Some of the most virulent pulmonary pathogens, such as *Franciscella tularensis*, replicate to incredibly high levels without evoking an immune conversely, much of the pulmonary damage associated with *Staphylococcus aureus* can be attributed to an excessive proinflammatory response that interferes with respiration.

Many cell types are involved in pulmonary clearance mechanisms. While it is apparent that the rapid influx of phagocytes is critical to contain an acute bacterial pneumonia, the regulation of inflammation is equally critical for a successful outcome. Common to many different causes of lung inflammation, the regulation of proinflammatory signaling is critical to enable efficient removal of pathogens without compromising airway function and gas exchange. The specific roles of major components of innate and adaptive immunity in protection of the lung from bacterial pneumonias were characterized in patients with primary and acquired immune deficiencies; those lacking B cells and antibody who were at substantial risk for pneumococcal pneumonia, patients with T cell defects, and especially CD4+ T cell depletion as a consequence of HIV infection who were especially susceptible to such intracellular pathogens as *Pneumocystis jirovecii*. The importance of neutrophil NADPH oxidase (NOX) activity in protection from *S. aureus* infection was well illustrated in the cohorts of patients with chronic granulomatous disease. Many additional correlations between other less profound immunological defects and susceptibility to specific types of lung infection have been made though the availability of genomic and immune function studies in vitro.

Recruited neutrophils are undoubtedly critical for the clearance of bacteria from the airway, as long as their number and state of activation are not excessive. Bacterial evasion of neutrophil clearance is a major factor in the success of pulmonary pathogens. Moreover, the associated oxidative stress and release of active neutrophil proteases in the airways contribute to tissue damage and further facilitate the establishment of foci of infection where bacteria are protected from the normally efficient removal by phagocytes. The recruitment, activation, and clearance of apoptotic neutrophils are all components of mucosal clearance that could potentially be targeted to regulate the amount of inflammation necessary to clear infection without causing pulmonary damage.

The contribution of other types of immune cells in response to acute bacterial infection in the lung has also been well characterized. The surveillance functions of specific subsets of DCs (dendritic cells) are clearly important in pathogen recognition. DCs also have possibly an even more significant function after they have matured and can traffic to local lymph nodes to inform T cells, both in general and in the context of specific infections. T cells, especially CD4+ T cells have long been recognized as critically important in the handling of intracellular pathogens, as was made evident during the HIV epidemic. The role of Th17 cells and the IL-17 family of cytokines in the recruitment of appropriate immune responses to extracellular bacterial infection in the lung has also become well appreciated. There are also substantial data being generated to understand how these immune cells are able to traffic to the site of infection in the airway. PMN, AM, and DC trafficking often involve the modification of epithelial and endothelial junctions to permit the egress of leukocytes in response to local signals.

Epithelial barrier function is modified as a consequence of TLR signaling to facilitate the transmigration of neutrophils and other phagocytes to the airway (Chun and Prince 2009). This may occur to facilitate immune and phagocyte recruitment or as a direct consequence of specific bacterial gene products. Bacteria activate

changes in barrier function and may invade through the epithelial tight junctions to gain access to receptors that are predominantly found at the basolateral aspects of the airway epithelium (Soong 2011). The common opportunistic pathogens of the respiratory tract have evolved sophisticated mechanisms to actively modulate specific targets of the host; the junctional proteins, Rho GTPases as well as ubiquitinylation systems. With large and flexible genomes, these organisms can acquire genes that enable them to evade with phagocytic clearance, and in some cases persist and disseminate systemically within phagocytes (Gresham 2000).

The host response to these airway pathogens causes much of the pathology associated with bacterial pneumonia. Effective mucosal clearance depends upon the coordinated signaling provided by the major components of the innate immune system in the lung. The relative contribution of each of these effectors has been examined in murine models of acute airway infection, with the caveat that the mucosal immune responses of the mouse do not necessarily replicate what occurs in humans. Studies with transgenic and knockout mouse models have provided important insights into the pathological as well as the beneficial consequences of the innate immune response.

In the subsequent chapters, the components of the innate immunity that participate in response to acute bacterial pneumonia will be reviewed, examining the specific contributions of several different types of immune effectors. How common bacterial pathogens cause pneumonia, either by activating immune signaling or by evading the normal clearance mechanisms will be examined. The goal of this volume is to provide an overview of the complexity of host response to specific bacterial pathogens in the lung and to provide some insights into the intricacies of these host–pathogen interactions. As will be evident, not all of the critical immune effectors are as well characterized as others, particularly in the context of even common bacterial pneumonias. Nonetheless, we can extrapolate from relevant studies of asthma, fungal, or viral infection to explain how these cell types participate in mucosal defenses. By providing reviews focused individually on either the host or the specific pathogen, we hope to provide a balanced compendium of the current understanding of how the lung is defended against acute bacterial infection. The ultimate goal is not only to appreciate the complexities of immune protection of the lung, but also to provide insight into which of these cascades are potential targets to modulate pathological responses to bacterial infection, without compromising the efficiency of pathogen eradication.

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Contents

Chapter 1
Alveolar Macrophages

David H. Dockrell, Paul J. Collini, and Helen M. Marriott

1.1 Introduction

Macrophages have been recognized as critical immune effectors since the seminal studies of Élie Metchnikoff (Kaufmann 2008). Macrophages also play distinct but equally important roles in tissue homeostasis (Mosser and Edwards 2008). The capacity of macrophages to respond to specific environmental cues and to initiate specific responses arises because of their plasticity.

Alveolar macrophages (AM) are the specialized tissue macrophages that reside in the alveolar space (Fels and Cohn 1986). They represent the major macrophage population in the lung; one study in mice suggested AM constitute 93% of lung macrophages with interstitial macrophages making up the remainder (van oud Alblas and van Furth 1979). AM adapt to a unique environment characterized by relatively high oxygen tensions but must also ensure that their responses do not compromise the precarious physiological balance that permits gas exchange in the alveolus (Piantadosi and Schwartz 2004). In particular the inflammatory response in the airway must be very tightly controlled. As with all tissue macrophages, AM development reflects the influences of differentiation modified by unique environmental honing. This has equipped the AM to perform its fundamental homeostatic roles in the lung and to clear microorganisms, particulate matter, and environmental toxins. The development of bronchoscopy and the ability to isolate AM by bronchoalveolar lavage (BAL) first increased awareness of the unique characteristics of AM some 50 years ago (Finley et al. 1967; Myrvik et al. 1961). Characterization of the AM transcriptome and proteome in healthy cells has expanded our understanding of these cells' functions (Lehtonen et al. 2007; Zaslona et al. 2009; Jin et al. 2004).

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These studies emphasize that extensive modifications occur as the monocyte differentiates into an AM and that these changes are reflected in differences in the expression of cell surface receptors, cytokines, proteases, metabolic enzymes, and regulators of apoptosis. However, it has also been shown that some disease-specific signatures can be carried independent of differentiation state, for example as evidenced by some chronic obstructive pulmonary disease (COPD) transcriptomic signatures which are detected in both peripheral blood monocytes and AM (Poliska et al. 2011). AM are uniquely susceptible to various physiological or pathological processes. For example, AM from aged mice were found to have selective defects in phagocytosis or generation of reactive oxygen species (ROS) when aged macrophages from other sites or neutrophils did not (Yokota et al. 1988).

In this chapter the focus is on the AM rather than macrophages of the upper airway where the unique environment and stimulation by chronic bacterial colonization produces a distinct macrophage phenotype. The chapter focuses on the role of AM in defending the alveolar space against bacterial infection and does not deal with the important and emerging roles of macrophages in striving to protect the upper airway against bacterial colonization which is a separate topic (Zhang et al. 2009).

1.2 Alveolar Macrophages

1.2.1 Origin and Life Span

Early murine-adoptive transfer experiments suggested both blood and lung cells contributed to the AM pool (Pinkett et al. 1966). Murine studies involving thymidine labeling suggested that only 3% of lung macrophages showed evidence of DNA synthesis and that monocytes could provide a source of AM (van oud Alblas and van Furth 1979). The authors estimated that approximately 15% of monocytes leaving the blood could become lung macrophages and that the turnover period for these in the mouse was approximately 27 days. The same group found monocytes to be the major source of AM following inflammatory stimuli (Blusse van Oud Alblas et al. 1983). Human studies looking at AM turnover in bone marrow transplant recipients also indicated blood monocytes were the major source of AM, which had a life span of 81 days (Thomas et al. 1976). These studies all involved the use of irradiation, which depleted dividing cells in the lung. They also failed to resolve the question as to why it takes so long to repopulate AM after bone marrow transplantation (BMT) in comparison to other hematopoietic cell populations (Tarling et al. 1987). However, with the use of a specialized fractionated irradiation protocol to protect dividing cells in the lungs from the lethal consequences of irradiation, a murine model demonstrated that the majority of mitotic AM are still of recipient origin 45 weeks after transplantation, implying the existence of a population of lung macrophages with the potential to divide and maintain AM. Consistent with this, monocyte depletion in mice did not abolish DNA synthesis in pulmonary macrophages nor alter AM numbers (Sawyer et al. 1982).

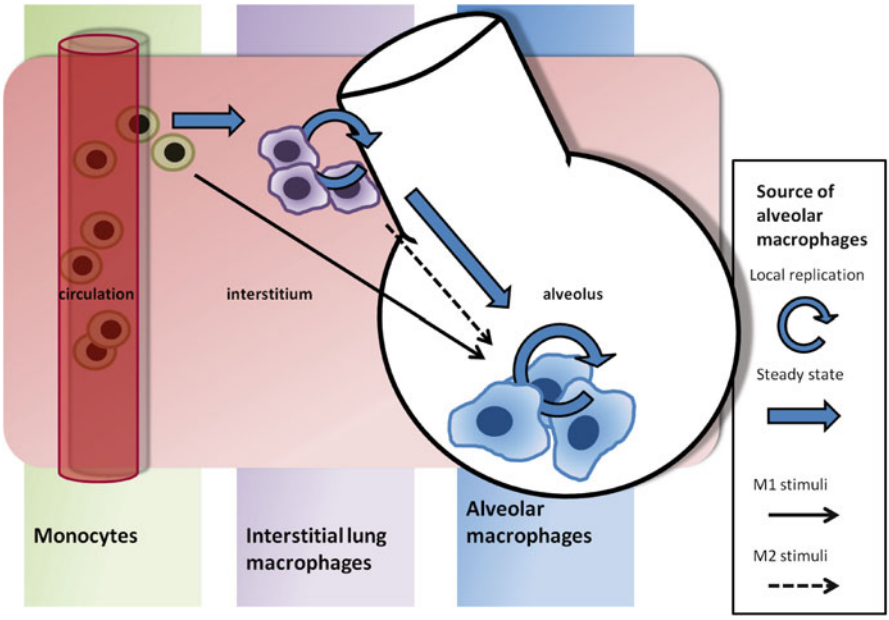

Fig. 1.1 Development and differentiation of the alveolar macrophage. Monocytes from the peripheral blood differentiate first into interstitial (parenchymal) lung macrophages and then further differentiate into alveolar macrophages. Each of these macrophage populations is capable of local replication in the steady state. M1 polarization stimuli favor migration of peripheral blood monocytes into the alveolar space. In contrast inflammatory stimuli, which drive M2 polarization may result in expansion of the pool of tissue intermediates, which form the mature macrophage population, although this has still to be confirmed as happening in the specific environment of the lung

Landsman and colleagues have employed conditional macrophage ablation, using CD11c.diphtheria toxin receptor transgenic mice, and adoptive transfer of bone marrow to demonstrate that monocytes can give rise to AM (Landsman and Jung 2007). They found that rates of reconstitution differed between pulmonary macrophage populations with delayed reconstitution of AM relative to parenchymal lung macrophages that resided in the interstitium. In this chapter these are referred to mainly as interstitial macrophages but the reader should keep in mind that older literature does not distinguish this population from pulmonary dendritic cells. Moreover the authors showed that monocytes must first differentiate into parenchymal (interstitial) macrophages before migration into the alveolar air space. Under steady-state conditions their model proposes that AM numbers will be maintained by replication of both parenchymal macrophages and AM (Fig. 1.1). Under conditions of increased turnover, as induced during inflammation, the rate of monocyte differentiation into parenchymal macrophages and the rate of migration of these into AM increases. Their studies have also clarified the origin of monocytes recruited to the lung. In humans $CD14^{++}/CD16^{-}$ ($CCR2^{+}/CX_3CR1^{int}$) and $CD14^{+}/CD16^{+}$ ($CCR2^{-}/CX_3CR1^{hi}$) populations are distinguishable (Geissmann et al. 2003; Passlick et al. 1989; Gordon and Taylor 2005). The counterpart of the $CD14^{++}/CD16^{-}$

population in mice are the $CCR2^{+}/CX_3CR1^{int}/Gr1^{+}$ cells which are actively recruited to inflamed sites whereas the $CCR2^{-}/CX_3CR1^{hi}/Gr1^{-}$ murine subset is recruited to non-inflamed sites (Geissmann et al. 2003). Both monocyte subsets gave rise to pulmonary dendritic cells but only the $CX_3CR1^{hi}/Gr1^{-}$ monocytes gave rise to parenchymal macrophages and ultimately AM, both in the absence or presence of inflammation (Landsman et al. 2007). However, $CX_3CR1^{int}/Gr1^{+}$ develop the capacity to differentiate into parenchymal macrophages once they have differentiated into $CX_3CR1^{hi}/Gr1^{-}$ monocytes.

The concept that interstitial (parenchymal) macrophages may represent an intermediate stage in the development of resident AM from peripheral blood monocytes has received further support from transcriptomic analysis of different peripheral blood monocyte subsets, interstitial macrophages, AM, and pulmonary dendritic cells in noninflammatory conditions in mice (Zaslona et al. 2009). Interstitial macrophages and AM showed transcriptomic profiles that were more distinct from peripheral blood monocytes than were those from pulmonary dendritic cells, but AM profiles were closer to interstitial macrophages than monocytes. The authors focused on genes involved in trafficking of the macrophages to their final location. CCR2, CCR7, CCL2, the integrins, CD11b and CD49, and matrix metalloprotease (MMP) 12 were all more highly expressed in interstitial macrophages than in AM, suggesting functional differences between the interstitial macrophage and AM with some adaption to their respective locations. During inflammatory conditions there are marked differences in the genes initially expressed by macrophages recruited to the alveolar space with upregulation of TNF-α, neutrophil chemoattractants, CD14, toll like receptor (TLR)4 and the cysteine cathepsins B, L, and K, reflecting different functions during the acute inflammatory response (Srivastava et al. 2005a). Interstitial macrophages, although able to ingest opsonized particles, phagocytose less efficiently and generate less ROS or TNF-α than AM (Johansson et al. 1997; Lavnikova et al. 1993; Prokhorova et al. 1994).

AM differentiation from a pulmonary cell of intermediate differentiation is supported by findings from allogeneic BMT recipients (Thomas et al. 1976; Nakata et al. 1999). The macrophages at this intermediate stage of differentiation found in the lung parenchyma and alveolar space are capable of mitosis. The requirement for this intermediate lung stage explains the long period required to turn over AM from recipient to donor origin in BMT recipients (Tarling et al. 1987; Matute-Bello et al. 2004; Kjellstrom et al. 2000). In practice this means that murine-adoptive transfer experiments need to ensure that the conditioning protocol involves irradiation or alternative strategies to allow turnover of the replication competent pulmonary macrophages and provide sufficient time for maturation of the monocyte-derived cells. The clinical consequence of these observations is the prolonged period of susceptibility to pulmonary infection that results from BMT, an effect that is in large part due to impairment of AM-mediated host defense (Kruger et al. 1999; Lossos et al. 1995; Ojielo et al. 2003). Jenkins et al. (2011) found that Th2 cytokines, which drive alternative activation of macrophages, induce an inflammatory response that requires replication of tissue macrophages rather than accumulation of peripheral blood monocytes as would occur with classical activation. Although it

does not address inflammatory stimuli in the lung, this work suggests these cells of intermediate differentiation could be the source of alternatively activated macrophages in the presence of appropriate inflammatory stimuli in the lung although this possibility needs to be formally assessed.

Estimates of AM life span have also been confounded by the effects of irradiation in conditioning protocols. In one protocol AM half-life was 30 days in mice, comparable with other studies, but when lead shielding of the thorax was employed, to limit the effects of conditioning radiation there was negligible turnover of macrophages at 8 months (Murphy et al. 2008). Moreover there was negligible replication of pulmonary macrophages over this time period, suggesting that AM persistence was due to resident cell survival not due to their replacement by the replication competent pool. It is important to note that such long half-lives are likely to be modified in inflammatory conditions. Another study using adoptive transfer in mice showed significant replacement of AM by monocytes following LPS-mediated lung injury (Maus et al. 2006). The prolonged life span of AM in the steady state reflects the relative resistance of differentiated macrophages to apoptosis (Daigneault et al. 2010). Differentiated macrophages express high levels of antiapoptotic molecules such as the Bcl-2 family members Mcl-1 and A1 or the inhibitor of death receptor signaling FLICE (Fas-associated death domain-like interleukin 1β-converting enzyme) inhibitory protein (FLIP) (Liu et al. 2001; Pagliari et al. 2000; Perlman et al. 1999).

1.2.2 Morphology

AM are a heterogeneous population of cells in BAL. One study found that the majority (70%) of AM isolated from healthy adults were 14–19 μm in size, with 19% 9–11 μm in size, comparable to monocytes and 7% being large multinucleated cells 20–40 μm in size (Reynolds and Newball 1974). Some of the smaller cells have subsequently been identified as dendritic cells while the remainder includes recently recruited monocytes or cells at an intermediate stage of differentiation (van Haarst et al. 1994). The number of these smaller cells will however increase in conditions of chronic inflammation such as during disease states like sarcoidosis. The major population of AM isolated from lung resection specimens show specific ultrastructural features when examined by light or electron microscopy (Cohen and Cline 1971). These include an abundance of cytoplasm with reduced nuclear to cytoplasmic ratios, numerous lysosomes and mitochondria, an extensive network of endoplasmic reticulum, multiple cytoplasmic vacuoles, and an oval or irregular nucleus. These findings reflect adaptation of the macrophage phenotype to perform key AM functions; the capacity to ingest particulate matter, cellular debris, and microorganisms and degrade these in phagolysosomes, the ability to function in an environment of high oxygen tension and therefore to utilize oxidative phosphorylation by mitochondria as a major energy source and their capacity for protein generation (Fels and Cohn 1986). The decrease in nuclear to cytoplasmic ratio and the

associated expansion of cell surface area when combined with the increased irregularity of cell membrane increases the volume of cell membrane available to make up endocytic vacuoles in differentiated macrophages (Sokol et al. 1987). It has been estimated from in vitro experiments that the macrophage will take up its entire surface area as endocytic vesicles every 30 min. Consequently this increase in cell membrane surface area is predicted to increase the capacity of the AM for endocytosis (Steinman et al. 1983). The accumulation of lysosomes and mitochondria is a key feature of the differentiation of monocytes into mature tissue macrophages and emphasizes the highly differentiated state of AM (Cohn and Benson 1965; Cohn et al. 1966). Studies on new born rats have shown that the accumulation of secondary lysosomes, lysosomes that have fused with endocytic vacuoles, a key feature of AM, only occurs after birth with adaptation to the air breathing environment (Kradin et al. 1986).

1.2.3 Metabolism

AM are unique amongst macrophage populations in that they adapt to life in an environment with relatively high oxygen tension. Proteomic analysis of healthy human AM, in comparison to the same donor's monocytes, reveals that AM have upregulated aldehyde dehydrogenase while four components of glycolytic metabolism, glyceraldehyde-3-phosphate dehydrogenase, pyruvate kinase, aldolase and phosphoglycerate mutase 1 are downregulated in AM (Jin et al. 2004). The well-developed mitochondrial system and the availability of oxygen enable AM to rely on oxidative phosphorylation under basal conditions (Oren et al. 1963). AM have a higher rate of respiration than neutrophils or monocytes and metabolism is enhanced by glucose. Phagocytosis results in a smaller oxidative burst than observed in neutrophils or monocytes and the oxidative burst is largely dependent on mitochondrial respiration (Kobzik et al. 1990), although the level of this can vary with species (Hoidal et al. 1978; Papermaster-Bender et al. 1980). In keeping with the capacity to utilize oxidative metabolism AM upregulate the production of several antioxidant enzymes including mitochondrial superoxide dismutase and peroxidredoxin (Jin et al. 2004).

Even so, AM can increase rates of glycolysis under anaerobic conditions or during the response to infection (Cohen and Cline 1971; Oren et al. 1963). This capacity to utilize glycolytic metabolism has also been demonstrated to increase with differentiation for other macrophage populations (Roiniotis et al. 2009). In activated macrophages glycolysis ensures inner mitochondrial transmembrane polarization is maintained and cells are protected from apoptosis (Garedew et al. 2010). In a classic study adaption to normoxia involved high expression of cytochrome oxidase, and low expression of enzymes such as pyruvate kinase involved in glycolytic metabolism but under conditions of hypoxia AM alter this pattern and their metabolic characteristics start to resemble those of macrophages adapted to environments where they must function at lower oxygen tensions, such as peritoneal macrophages

(Simon et al. 1977). Upregulation of glycolytic metabolism during hypoxia or inflammatory responses in macrophages involves stabilization of the transcription factor hypoxia inducible factor 1 subunit alpha (HIF-1α), which leads to upregulation of many factors required for glycolytic metabolism including the GLUT-1 glucose transporter and the glycolytic enzyme phosphoglycerate kinase (Semenza 2001; Cramer et al. 2003). Though the roles of HIF-1α in regulating metabolic responses in AM are still emerging, it has been demonstrated that HIF-1α upregulation can occur under normoxic conditions and regulate transcription of a variety of HIF-1α regulated genes in AM (Ueno et al. 2011).

1.2.4 Specialized Adaptation to Function

1.2.4.1 Regulation of Phagocytosis

AM are professional phagocytes and express a range of receptors required for opsonic and non-opsonic phagocytosis (Aderem and Underhill 1999). Multiple mechanisms regulate AM phagocytosis with inflammatory responses kept in check under basal conditions but with the potential to upregulate these when microorganisms are encountered (Lambrecht 2006). BAL contains immunoglobulins including IgG (Reynolds and Newball 1974) and human macrophages express several FcγR, which stimulate phagocytosis (FcγRI, IIA, and III) and one that provides inhibitory signals to phagocytosis, FcγRIIB (Aderem and Underhill 1999). FcγRI and III express a gamma subunit and TGFβ can downregulate expression of this with associated downregulation of surface expression of FcγRI and III and FcγR-stimulated cytokine responses (Tridandapani et al. 2003). As with macrophages in general, AM also express complement receptors (CR)1, 3, and 4 (Aderem and Underhill 1999) and complement is detectable in BAL (Reynolds and Newball 1974; Reynolds et al. 1975). CR often require activation to become competent for phagocytic uptake and ingestion via CR is not coupled to the generation of ROS or arachidonic acid metabolites (Aderem and Underhill 1999).

Prostaglandins regulate a variety of immune functions and play critical roles in regulating FcγR-mediated phagocytosis in AM. Prostaglandin E_2 (PGE_2) inhibits FcγR-dependent phagocytosis of opsonic targets by increasing cAMP levels (Aronoff et al. 2004). PGE_2 also inhibits bacterial killing, ROS generation (Serezani et al. 2007), and TNF-α release (Aronoff et al. 2005). PGE_2 inhibits phagocytosis through cAMP-mediated activation of exchange protein directly activated by cAMP (Epac-1) and downstream activation of the small GTPase Rap-1, a mechanism distinct from the PGE_2-mediated inhibition of TNF-α release via activation of protein kinase A (PKA) or the inhibition of ROS generation which is mediated both by activation of Epac-1 and by activation of PKA (Aronoff et al. 2005). Epac-1 enhances the activity of a negative regulator of FcγR signaling, phosphatase and tensin homolog deleted on chromosome 10 (PTEN) (Canetti et al. 2007). FcγR engagement results in a signal transduction pathway involving FcγR clustering, phosphorylation of the Fc

receptor immunoreceptor tyrosine-based activation motif (ITAM) by the Src kinases Lyn and Hck and recruitment of spleen tyrosine kinase (Syk) which phosphorylates and activates phosphoinositide 3 kinase (PI3K) (Swanson and Hoppe 2004). PI3K generates phosphatidylinositol-3,4,5-triphosphate (PIP3) which in turn activates the serine/threonine kinase Akt (protein kinase B) and PTEN antagonizes this by dephosphorylating inositol at the D3 position (Maehama and Dixon 1998). PGE_2 can therefore play an important role in detuning the pro-inflammatory response of AM.

In contrast to PGE_2 leukotriene B_4 (LTB_4) and LTD_4 enhance FcγR-dependent phagocytosis with activation of the downstream kinase, Syk (Mancuso et al. 1998; Mancuso and Peters-Golden 2000; Canetti et al. 2006). In the case of LTB_4, but not LTD_4, this involves reversal of PGE_2-stimulated cAMP production, while both leukotrienes inhibit activation of Rap-1 (Lee et al. 2009). These differences reflect differences in the specific G protein subunits following engagement of different leukotriene receptors in AM (Peres et al. 2007). Phagocytosis of IgG-coated targets reduces cAMP levels in an LTB_4-dependent fashion, providing a positive feedback to phagocytosis in AM not observed in peritoneal macrophages or neutrophils. Since AM have much greater capacity to synthesize LTB_4 this likely equips them with a unique mechanism by which to upregulate phagocytosis when required (Peters-Golden et al. 1990; Coffey et al. 1996)

The non-opsonic ingestion of particles by AM involves an array of receptors (Taylor et al. 2005). Scavenger receptors play important roles in ingesting unopsonized particulate matter (Kobzik 1995). AM express a broad range of scavenger receptors including scavenger receptor (SR) AI/II, LOX-1, SRCL, SR-PSOX, and CD68 (SR BIII) (Arredouani et al. 2005). The human class A scavenger receptor MARCO (macrophage receptor with collagenous structure), however, has emerged as a major receptor involved in phagocytosis of environmental dusts and unopsonized bacteria. The mannose receptor is a pattern recognition receptor which contributes to endocytosis and phagocytosis (Allavena et al. 2004). It is a member of the C-type lectin superfamily and recognizes carbohydrate moieties expressed by a variety of structures including microorganisms. Mannose receptors are required for the phagocytosis of zymosan. Alternative activation and IL-4 expression increase mannose receptor expression on AM (Stein et al. 1992). Galectin-3, another carbohydrate-binding lectin, was upregulated in AM as opposed to monocytes in a proteomic screen (Jin et al. 2004). Galectin-3 contributes to phagocytosis of a number of targets including apoptotic cells (Sano et al. 2003). Dectin-1 has emerged as an important receptor for phagocytosis by AM recognizing structures containing β-glucans (Brown and Gordon 2003). It plays a particularly important role in the recognition of yeasts and other fungi (Herre et al. 2004a). Dectin-1 plays a role in the phagocytosis of *Pneumocystis jirovecii* (Steele et al. 2003). Phagocytosis through this receptor occurs through a unique Syk-independent mechanism (Herre et al. 2004b). TLR and dectin-1 synergize in stimulating ROS production and cytokine expression (Gantner et al. 2003).

Surfactant protein A upregulates mannose receptor expression on macrophages by a mechanism, which involves increased cycling of intracytoplasmic pools to the cell surface (Beharka et al. 2002). AM have particularly high levels of mannose receptor and AM from surfactant protein A deficient mice have decreased surface

expression of mannose receptor which results in reduced mannose receptor-dependent phagocytosis. Interestingly surfactant protein A not only increases phagocytosis through mannose receptors in AM but also reduces nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase-dependent production of ROS by reducing assembly of the NADPH complex components on the phagosome (Crowther et al. 2004). Furthermore surfactant protein A, although increasing expression of TLR2 on the surface of macrophages, downregulates signal transduction from TLR including activation of signaling through the transcription factor NF-κB, mitogen-activated protein kinase (MAPK) and Akt (Henning et al. 2008). This illustrates how AM function can involve efficient phagocytosis while dissociating this from a high output inflammatory response.

AM and monocytes show differential regulation of proteins involved in the downstream signaling of phagocytosis and in particular in the regulation of actin polymerization, a central feature of phagocytosis (Aderem and Underhill 1999), in keeping with distinct functional requirements. Heat shock protein (Hsp)27 and macrophage capping protein G (Cap G) are increased in AM (Jin et al. 2004), both proteins implicated in the regulation of phagocytosis, through their role in promoting actin filament assembly via uncapping of the barbed end of preexisting actin filaments (Greenberg 1995). However other proteins regulating actin polymerization during phagocytosis are upregulated in monocytes (Jin et al. 2004).

1.2.4.2 Ensuring Tight Control over Inflammatory Responses to Pathogens

AM have specialized phagocytic machinery which is tightly regulated by environmental factors, including cytokines, prostaglandins, leukotrienes, and surfactant proteins (Fels and Cohn 1986). AM, although efficient phagocytes, have a finite capacity for phagocytosis and digestion of the particles they ingest. This has the advantage of limiting tissue injury by ensuring production of ROS and proteases are not excessive, but also necessitates mechanisms to scale up responsiveness during infection. AM do not generate the more potent forms of ROS and possess efficient antioxidant mechanisms. Production of nitric oxide (NO) via inducible nitric oxide synthase (iNOS) in human AM has been recognized to occur at lower levels than in rodent macrophages (Jesch et al. 1997) and to occur at lower levels in differentiated tissue macrophages (Daigneault et al. 2010). Although human AM can produce iNOS with important consequences for the control of ingested pathogens, including *Mycobacterium tuberculosis*, production is clearly tightly regulated (Nicholson et al. 1996). Thus the AM emerges as a cell with tremendous phagocytic potential but in which the level and consequences of phagocytosis are tightly regulated by activation status. This means that AM are adapted to their homeostatic roles in the airway and have the capacity to clear finite numbers of microorganisms in the steady state. AM are characterized by an extensive capacity to digest or store ingested particles in their large lysosomal compartment. Proteomic analysis comparing healthy human monocytes to AM has revealed that several of the most marked differences influence the expression of proteins involved in degradation of

ingested materials, including lysosomal proteases. (Jin et al. 2004). These include the abundantly expressed aspartic cathepsin D (Kato et al. 1972) and cysteine cathepsins such as cathepsin B (Burnett et al. 1983), H, and X. There were also increases in other proteases such as the serine protease tripeptide peptidase I and the aspartic protease napsin A, as well as the serine protease inhibitor, leukocyte elastase inhibitor.

When AM encounter significant numbers of bacteria their capacity for phagocytosis and clearance will become overwhelmed (Dockrell et al. 2003; Knapp et al. 2003). Under these circumstances the capacity of AM to sense microbial particles becomes critical and they will utilize surface or endosomal expressed TLR and intracellular pattern recognition systems such as the nucleotide-binding oligomerization domain (NOD)-like receptor (NLR) containing inflammasome system to activate pro-inflammatory signaling (Sabroe et al. 2003a; Franchi et al. 2009). These mechanisms will not only upregulate the intrinsic capacity for the AM to ingest and kill pathogens but will also enable recruitment of other immune cells to aid pathogen control. The level of control exerted over pro-inflammatory cytokine secretion is nicely exemplified by IL-1β release. Classic studies have illustrated that AM produce much less IL-1 after LPS stimulation than monocytes (Wewers et al. 1984). IL-1β release requires processing of pro-IL-1β by a caspase-1 containing inflammasome (Martinon et al. 2002). In contrast to monocytes differentiated macrophages including AM require a second signal, provided by endogenous adenosine triphosphate (ATP) to activate a caspase-1 inflammasome and to allow IL-1β release (Netea et al. 2009). Activation of macrophages with interferon gamma (IFN-γ) increases endogenous ATP production and upregulates IL-1β release by differentiated macrophages. This illustrates how pattern recognition receptors and cytokines can act in combination to activate AM along pathways, which will remove the intrinsic inhibition to pro-inflammatory responses. While this will enhance microbial clearance, it runs the risk of exposing the lung to an inflammatory response which will compromise its physiological function. This again emphasizes how these inflammatory responses must be tightly regulated to ensure appropriate initiation and cessation.

Macrophages also have a well-developed system of receptors which will downregulate inflammatory responses (Taylor et al. 2005). These include those like human FcγRIIB which trigger inhibitory signals when they bind opsonized targets (Aderem and Underhill 1999) and those which modify inflammatory signals produced by pattern recognition receptors such as an inhibitory member of the Triggering Receptors Expressed by Myeloid cells (TREM) family, TREM-like transcript 1 (Taylor et al. 2005).

1.2.4.3 Efferocytosis

AM also contribute to the downregulation of the inflammatory response by ingesting effete cells that have become apoptotic in a process termed efferocytosis. Some studies have shown lower levels of ingestion by AM compared to peritoneal macrophages

(Hu et al. 2004). Surfactant protein A enhanced ingestion by AM but levels were still less than peritoneal macrophages. In another study C1q, surfactant protein A and D treatment of apoptotic cells all enhanced phagocytosis by AM in vitro, via CD91/calreticulin complex-mediated uptake, with surfactant protein D being the major collectin influencing efferocytosis in vivo in the murine lung under basal conditions (Vandivier et al. 2002a). In mouse lungs a higher percentage of resident AM efferocytosed apoptotic thymocytes than did recruited monocytes under inflamed conditions (Jennings et al. 2005). Another study showed that following inflammatory stimuli AM are able to ingest apoptotic cells at comparable levels to other macrophage populations (Newman et al. 1982). Thus although the efficiency of efferocytosis has varied with experimental conditions, AM are able to ingest apoptotic cells.

Surfactant proteins have been shown to exert a dual role on inflammatory signaling from macrophages; when the carbohydrate binding domain interacts with signal inhibitory regulatory protein (SIRP)α inflammatory signaling is suppressed, via activation of the tyrosine phosphatase Src homology 2 domain-containing protein (SHP)-1, while engagement of the carbohydrate recognition domain by microorganisms or other targets leaves the N-terminal collagen domain free to interact with the calreticulin/CD91 complex and results in pro-inflammatory signaling (Gardai et al. 2003). Since surfactant proteins can also bind apoptotic cells it has been proposed that surfactant protein A or D may enhance phagocytosis of apoptotic cells when the N-terminal collagen domain binds the calreticulin/CD91 complex but may limit phagocytosis when the carbohydrate binding domain binds SIRPα (Janssen et al. 2008). In this model the surfactant proteins constitutively block phagocytic uptake of apoptotic cells through engagement of SIRPα, stimulating a pathway previously shown to inhibit FcγR- and CR-mediated phagocytosis (Oldenborg et al. 2001). SIRPα mediated inhibition required activation of SHP-1, the GTPase RhoA and Rho kinase, which negatively regulate phagocytosis (Janssen et al. 2008). Inflammatory stimuli resulted in accumulation of a population of apoptotic cells which preferentially bind surfactant proteins A and D and potentially recruitment of macrophages to the lung with a greater capacity to clear apoptotic cells than the resident AM in this model (although resident AM also showed enhanced clearance of apoptotic cells following inflammatory stimuli).

These findings suggest surfactant can allow low-level efferocytosis by AM under basal conditions but facilitate increased efferocytosis under inflammatory conditions. While different models have found varying contributions of the resident as compared to the recruited cells to efferocytosis, these may reflect differences in types of apoptotic cells or in models of inflammation (Jennings et al. 2005; Janssen et al. 2008). However, it has been demonstrated at other inflammatory sites that apoptotic cells can release ATP and UTP to recruit monocytes/macrophages, providing a signal for the recruited macrophages that contributes to clearance of apoptotic cells (Elliott et al. 2009). Regardless of any concerns over the relative contributions of resident and recruited cells to efferocytosis in the lung the fact that following inflammatory events there are only low percentages of apoptotic neutrophils in human BAL fluid, despite the influx of large numbers of inflammatory cells, attests to the competence of efferocytosis to clear apoptotic cells following inflammatory

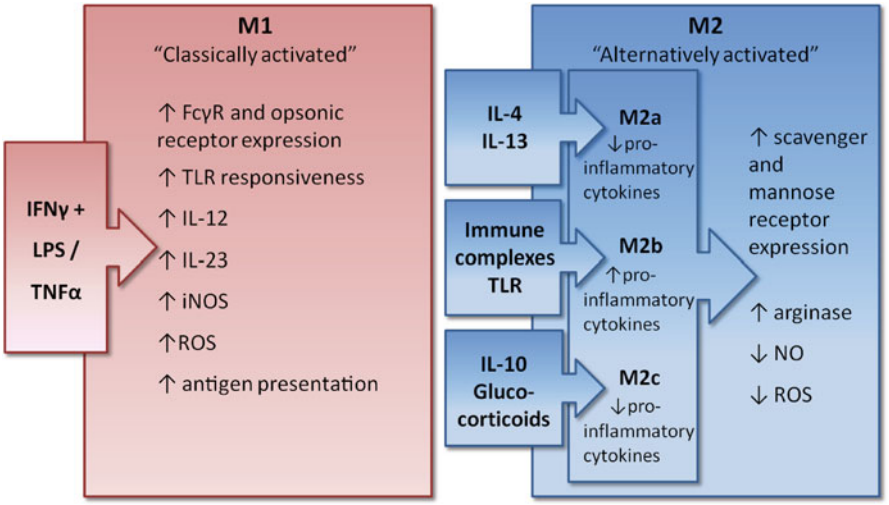

Fig. 1.2 Macrophage polarization. M1 or classical activation of macrophages is engendered by interferon γ (IFN-γ) plus either lipopolysaccharide (LPS) or tumor necrosis factor-α (TNF-α). M2 or alternatively activated macrophages can be subdivided according to the activating stimuli into M2a, M2b, and M2c (*small boxes*). M2a and M2c are relatively poor producers of pro-inflammatory cytokines, unlike M2b-polarized cells (*medium sized box*). All M2 macrophages often share similar trends in non-opsonic receptor expression and arginase, nitric oxide (NO) and reactive oxygen species (ROS) activity though the extent varies (*large box*). *iNOS* inducible nitric oxide synthase; *IL* interleukin; *TLR* toll-like receptor

episodes such as pneumonia (Droemann et al. 2000). Ingestion of the apoptotic cells not only removes the effete cells but also downregulates pro-inflammatory cytokine production and upregulates important anti-inflammatory cytokines such as TGFβ (Fadok et al. 1998; Huynh et al. 2002).

1.2.5 Polarization

A key attribute of macrophage populations is their plasticity. Macrophages respond to a variety of environmental cues, including microbial stimulation, by changing phenotype. The activation of macrophages has been described as being M1 ("classically activated") or M2 ("alternatively activated") (Mosser and Edwards 2008) (Fig. 1.2). Classical activation arises following stimulation with IFN-γ and LPS or TNF-α and is associated with characteristic features. These include increased expression of receptors for opsonized particles such as FcγRs, TLR responsiveness, IL-12 and IL-23 production, specific patterns of chemokine and chemokine receptor, iNOS and ROS generation, as well as increased capacity for antigen presentation (Piantadosi and Schwartz 2004; MacMicking et al. 1997; Mantovani et al. 2004; Mosser 2003; Verreck et al. 2004). Alternatively activated macrophages

comprise several groups. In one proposed schema subtypes M2a are induced by IL-4 or IL-13, M2b by immune complexes and TLR stimuli and M2c by IL-10 and glucocorticoids (Mantovani et al. 2004). M2 macrophages usually show upregulation of IL-10 and variably manifest features such as upregulation of non-opsonic receptors (scavenger and mannose receptors), distinct patterns of chemokine or chemokine receptor expression, limited production of NO and ROS with upregulation of arginase, which competes with iNOS for arginine and therefore limits the availability of arginine to generate NO. M2a and M2c macrophages downregulate pro-inflammatory cytokines but these are still expressed at high levels in M2b-activated macrophages. There are many variations in these features and human and murine cells do not show identical responses. Responses show a high degree of plasticity and Mosser and Edwards have suggested that classifying macrophages by their physiological functions, e.g., host defense, wound healing, or immune regulation is more informative with recognition that cells may show phenotypes that reflect a mix of these extremes (Mosser and Edwards 2008). Although it has long been recognized that classically activated macrophages play a role in host defense against intracellular pathogens, the role of macrophages activated with other stimuli is emerging, for example macrophages activated via IL-4 will contribute to host defense against helminthes and nematodes (Anthony et al. 2006; Zhao et al. 2008).

Most studies that have examined expression of M1 or M2 markers have found low levels of M1 markers in AM from healthy controls under basal conditions (Wiken et al. 2010). Methodology will, however, confound results since polarization may alter rapidly following ex vivo culture. Since AM are in intimate contact with the external environment considerable attention has focused on the polarization of AM in various disease states. These studies have revealed some surprises. For example, COPD is associated with airway inflammation and this leads to the prediction that there would be an increase in M1 polarization in this condition but surprisingly transcriptional profiling suggested M2 polarization was accentuated in cigarette smokers with an even greater accentuation in patients with COPD (Shaykhiev et al. 2009). This might explain the greater susceptibility to bacterial infection in the airways of these patients. It is important to note though that the results of different polarization markers are quite variable and that diseases like COPD are quite heterogeneous, raising the possibility that results may only apply to a subset of patients and that other factors may have confounded findings, as the authors have alluded to, such as the frequency of viral infections. In general macrophage responses to acute bacterial infections are characterized by M1 polarization and M2 polarization has been associated with subversion of the immune response to bacteria and impairment of the host response (Benoit et al. 2008). M1 responses do, however, expose the lung to potential tissue injury and M2 responses are required during the resolution phase of infection. The pattern of polarization to infections may also be mixed. For example although *Streptococcus pyogenes* stimulates many genes typical of an M1 response, there are some M2 genes induced, including IL-10 and the enzyme arginase (Goldmann et al. 2007).

1.2.6 Models of Alveolar Macrophages

The information included in this chapter relies on studies using AM either of human or animal (usually rodent) origin. There is also extensive reference to animal models of infection mostly obtained from mouse studies. While this has been hugely informative and underpins the bulk of the information included in this chapter, it is important to remember that important species-dependent differences in immune function can occur and validation of findings requires a combination of approaches. Unfortunately there is much less information available from studies of AM obtained from patients with acute bacterial infection and there is a need for more translational studies which confirm key in vitro findings, or those derived from mouse studies, in well-phenotyped patient samples. One of the difficulties in obtaining accurate information about AM is that macrophage cell lines usually do not exhibit the degree of differentiation seen in tissue macrophages such as AM. However, with careful characterization differentiation protocols can allow the development of a cell that approximates a differentiated macrophage (Daigneault et al. 2010). Where a key observation has been made in a cell line this may be presented but caution is urged in extrapolating the evidence to AM and until additional approaches are performed these observations should be regarded as preliminary. It is clear from the above that AM are a distinct macrophage type whose unique phenotype is honed by development in the lung. Nevertheless because of ease of access monocyte-derived macrophages (MDM) are often used as a model of AM and prior studies suggest they share many features with AM justifying their use (Gantner et al. 1997). Where data has been derived from MDM this will be specified.

1.3 Phagocytic Function

Microaspiration is a frequent event; 50% of healthy young adults microaspirated nasopharyngeal contents over two nights of follow-up in one study (Gleeson et al. 1997), with higher rates reported in those with medical comorbidities (Lee et al. 2010). Although the lower airway has traditionally been regarded as a sterile environment, it is likely that the AM are frequently exposed to low numbers of bacteria, which need to be ingested and cleared. AM represent only one component of pulmonary host defense, which also comprises humoral factors, epithelial cells and a variety of immune cells. The initial challenge for resident AM is that their initial activation state in the distal airway does not favor high-levels of phagocytosis and killing; sensing of bacteria and activation of AM to upregulate mechanisms of phagocytosis are important to enable bacterial clearance. In addition bacteria possess a range of adaptations to prevent phagocytosis, in particular several successful respiratory pathogens evade phagocytosis by means of polysaccharide capsules. In one study human AM ingested nontypeable *Haemophilus influenzae* (NTHi), unencapsulated *Streptococcus pneumoniae*, and *Staphylococcus aureus* efficiently after opsonization in human serum but were much less efficient at ingesting polysaccharide

capsule expressing bacteria such as *H. influenzae* type b (Hib) and serotype 3, 6, or 14 *S. pneumoniae* (Jonsson et al. 1985).

1.3.1 Phagocytosis of Streptococcus pneumoniae

1.3.1.1 Microbial Resistance to Phagocytosis

S. pneumoniae remains the commonest cause of community acquired pneumonia (CAP). Phagocytosis is inhibited by the polysaccharide capsule and opsonization which enhances phagocytosis is hindered by several pneumococcal proteins (Preston and Dockrell 2008). These include factors limiting complement or antibody deposition, including the cholesterol-dependent cytolysin, pneumolysin, pneumococcal surface proteins A or C, and the IgA protease. In addition the pneumococcal serine-rich repeat protein expressed by some strains has been shown to be important for biofilm formation in the lung in a murine model, which also enables the organism to resist phagocytosis (Sanchez et al. 2010). The degree of inhibition to phagocytosis varies by capsule type with strains that are able to colonize efficiently having thicker capsule and being more resistant to non-opsonic phagocytosis by neutrophils, although these strains much less commonly cause invasive disease, suggesting they may be primarily adapted to the ecological niche required for colonization (Weinberger et al. 2009). Despite these adaptations AM ingest *S. pneumoniae* in murine models and when AM are depleted bacterial clearance and the threshold at which neutrophils are recruited are lowered (Dockrell et al. 2003; Bewley et al. 2011a). In a mathematical model based on murine models of infection the AM contributed to the first stage of host defense rapidly clearing small initial numbers of bacteria (Smith et al. 2011).

1.3.1.2 Role of Opsonization

Opsonization enhances phagocytosis of *S. pneumoniae* in vitro (Jonsson et al. 1985; Hof et al. 1980). Ingestion of serotype 1 *S. pneumoniae* by AM requires opsonization and the combined effect of complement and immunoglobulin maximizes internalization to phagolysosomes (Gordon et al. 2000). Once bacteria are attached to the cell surface both complement and immunoglobulin-opsonized bacteria are internalized at similar rates and there is a good correlation between phagocytosis and killing, suggesting internalization is required for a significant amount of killing. Detectable levels of anti-pneumococcal IgG are found in BAL in many healthy individuals (Eagan et al. 2007). In MDM FcγRIIA, FcγRIII, CR1, and CR3 all contribute to the internalization of pneumococci (Ali et al. 2003) (Fig. 1.3). Complement deposition reduces the burden of bacteria in the lung parenchyma in mice and deficiency in C1q or in natural IgM antibody, both of which impair complement activation by the classical pathway, result in higher bacterial counts in the lung

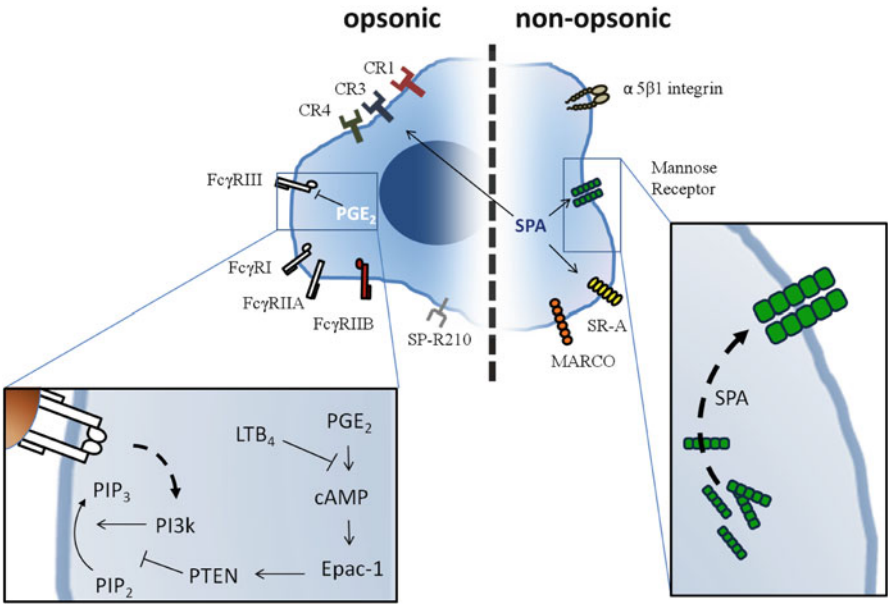

Fig. 1.3 Major phagocytic receptors of the AM. Major phagocytic receptors expressed by alveolar macrophages that are involved in recognition of extracellular bacteria (*Streptococcus pneumoniae, Staphylococcus aureus, Pseudomonas aeruginosa, Haemophilus influenzae and Klebsiella pneumoniae*) discussed in this chapter. Alveolar macrophages phagocytose all five pathogens using Fcγ receptors. Complement receptors are also important for *S. pneumoniae* (CR1 and CR3) and *H. influenzae* (CR3). Additionally AM phagocytose surfactant protein A (SPA) opsonized *S. aureus* via the SP-R210 receptor and *K. pneumoniae* via the mannose receptor. There is evidence that non-opsonic phagocytosis of *S. pneumoniae, S. aureus*, and *H. influenzae* involves the scavenger receptors SR-A and MARCO. Additionally, AM utilize Fcγ, via Fc binding to surface protein A and α5β1 integrin for unopsonized *S. aureus*. Inset: Engagement by bacteria lead to Fcγ clustering and activation of the PI3 kinase system (PI3K) which is differentially regulated by PGE_2 and leukotrienes. SPA additionally upregulates surface expression of mannose receptors, SR-A and CR3

parenchyma and blood though not in BAL (Brown et al. 2002). While these differences could, as the authors suggest, reflect the greater level of systemic infection in the absence of complement activation, the association with reduced levels of macrophage activation in the lung also suggests the potential for decreased bacterial clearance in the lung by AM in the absence of the classical pathway. Importantly capsular serotypes associated with more efficient complement deposition are associated with greater degrees of AM phagocytosis and bacterial clearance from the airway in murine models (Hyams et al. 2010; Sanchez et al. 2011). Nevertheless some older studies have suggested that complement is not essential for the early stages of pneumococcal clearance in the rat lung since decomplemented rats cleared bacteria as efficiently as normal rats, albeit in a model where bacteria were being efficiently controlled (Rehm and Coonrod 1982), emphasizing that CR-mediated uptake is only one mechanism of uptake.

Opsonic uptake of *S. pneumoniae* by AM is reduced during HIV infection (Eagan et al. 2007), by cigarette smoke (Phipps et al. 2010) and by fasting which

reduces levels of the adipocyte-derived hormone leptin, LTB_4 expression, and extracellular signal-related kinase (ERK)1/2 activation (Mancuso et al. 2006, 2011). FcγRIIB-deficient mice, which lack the inhibitory FcγR, have enhanced *S. pneumoniae* clearance in a peritoneal infection model when challenged with lower inocula although clearance of bacteria from the lung was not assessed (Clatworthy and Smith 2004). Conversely at higher dose challenges in the context of immunization the greater phagocytosis in the FcγRIIB-deficient mice comes at a cost of enhanced inflammation and death. Pulmonary efferocytosis increases PGE_2 levels and impairs phagocytosis of *S. pneumoniae* (Medeiros et al. 2009). While this also has the beneficial effect of downregulating the inflammatory response as infection starts to be controlled, it could also result in a potentially immunosuppressive effect in settings where numbers of apoptotic cells are high as might occur during some inflammatory lung processes.

1.3.1.3 Mechanisms of Non-opsonic Uptake

MARCO is a key receptor for phagocytosis of unopsonized particles in the lung (Arredouani et al. 2005) and $MARCO^{-/-}$ AM phagocytose *S. pneumoniae* less efficiently, resulting in impaired bacterial clearance in the early stages of infection, increased neutrophil recruitment, and decreased survival (Arredouani et al. 2004). Concentrated ambient particles reduce non-opsonic uptake of *S. pneumoniae* (Zhou and Kobzik 2007). Soluble metal ions in the ambient particles induce oxidant-dependent inhibition of phagocytosis. Welders are known to have much greater mortality following pneumococcal pneumonia (Coggon et al. 1994). Welding fumes contain soluble metal ions which can cause oxidant injury and less toxic fumes containing metals, such as iron, inhibit phagocytosis while the more toxic chromium containing fumes cause macrophage apoptosis, mimicking the phenotype of the AM depletion experiments in mice (Dockrell et al. 2003; Antonini et al. 1999, 2005). This provides an important environmental example of the importance of AM phagocytosis to bacterial clearance.

SR-A also contributes to uptake of *S. pneumoniae* in murine models (Kuronuma et al. 2004). Surfactant protein A enhances the surface expression of SR-A via a mechanism that requires casein kinase 2 activity. Enhancement of phagocytosis is independent of surfactant protein A binding to bacteria, is not observed with surfactant protein D and does not require the interaction of the surfactant collagen tail with the calreticulin/CD91 complex reported to enhance phagocytosis in other models (Janssen et al. 2008)

1.3.2 Phagocytosis of Staphylococcus aureus

S. aureus is a major cause of metastatic infection and causes pneumonia both in the community and the intensive care unit. Rodent lungs efficiently clear inhaled *S. aureus* and AM phagocytosis and killing is the major clearance mechanism

(Green and Kass 1964; Goldstein et al. 1974). In comparison to *S. pneumoniae, S. aureus* are more readily phagocytosed by AM and unopsonized bacteria are more efficiently internalized than pneumococci in vitro (Jonsson et al. 1985; Hof et al. 1980). Phagocytosis of *S. aureus* in mouse lungs is comparable to unencapsulated *S. pneumoniae* (Esposito et al. 1988), suggesting the primary factor driving better rates of internalization is lack of the inhibitory influence provided by pneumococcal capsule. Despite this opsonization modestly increases uptake of *S. aureus* by AM in some studies (Lee et al. 1984). Antibody can contribute to ingestion but it is suggested this is not just through FcγR-mediated uptake since surface-expressed IgG can bind the protein A component of the *S. aureus* cell wall via its Fc region and mediate non-opsonic ingestion (Verbrugh et al. 1982). Both classical and alternative complement pathways are activated by *S. aureus* and in the presence of low levels of other opsonins the alternative complement pathway enhances phagocytosis (Murphey et al. 1979). However, microbial mechanisms, such as the expression of the metalloprotease aureolysin, cleaves C3 and limits C3b deposition on the bacterial cell wall (Laarman et al. 2011). A very high proportion of surface bound bacteria are internalized (Lee et al. 1984).

As compared to neutrophils the mechanisms of uptake by AM of unopsonized *S. aureus* are distinct with AM extending lamellipodia to phagocytose unopsonized *S. aureus*. Experimental approaches using in vitro binding assays and heat killed *S. aureus* demonstrate interactions between SR-A or MARCO and *S. aureus* (Arredouani et al. 2005; Dunne et al. 1994), while SR-A-deficient mice were less able to clear *S. aureus* from the peritoneum (Thomas et al. 2000). However, scavenger receptor inhibition did not block ingestion of a range of *S. aureus* by MDM (DeLoid et al. 2009). This study showed that there were differences in the mechanisms of phagocytosis for different unopsonized strains though all involved actin polymerization. Surfactant protein A can opsonize *S. aureus* (McNeely and Coonrod 1993). The surfactant protein A receptor SP-R210 has been demonstrated to internalize surfactant protein A-coated *S. aureus* (Sever-Chroneos et al. 2011). Moreover surfactant protein A opsonization was found to be dependent on expression of the adhesion Eap by *S. aureus*. In this model SR-A contributes to ingestion of bacteria in the absence of opsonization by surfactant and also helps keep the inflammatory response triggered by phagocytosis via the SP-R210$_L$ isoform in check. The class B scavenger receptor CD36 was identified as important for phagocytosis of *S. aureus* by macrophages and shown to contribute to bacterial clearance following systemic infection but there is less information on its role in the lung (Stuart et al. 2005). Recently the α5β1 integrin was found to mediate phagocytosis of heat killed *S. aureus* by AM via interaction with fibrinogen-binding protein (Kapetanovic et al. 2011)

1.3.3 Ingestion of Pseudomonas aeruginosa

P. aeruginosa function as an opportunistic infection causing chronic infection in patients with cystic fibrosis (CF) and other groups with structural lung disease or acute infections in immunocompromised patient groups, hospitalized or ventilated patients or in some of those with chronic lung disease (Lyczak et al. 2000). A variety

of factors enable evasion of innate host defense and prevent AM phagocytosis including expression of a mucoid exopolysaccharide and biofilm formation or loss of flagella and motility (Lyczak et al. 2000; Chmiel and Davis 2003; Luzar et al. 1985; Amiel et al. 2010; Mahenthiralingam and Speert 1995; Leid et al. 2005). *P. aeruginosa* elastase degrades complement, IgG, and surfactant proteins (Sever-Chroneos et al. 2011; Wretlind and Pavlovskis 1983; Mariencheck et al. 2003; Alcorn and Wright 2004). Murine AM depletion experiments highlight that AM contribute to very early clearance of bacteria but at later stages their principle role is to regulate inflammatory cell recruitment (Cheung et al. 2000; Kooguchi et al. 1998). Opsonization enhances AM phagocytosis and unopsonized bacteria are ingested at a lower level than *S. aureus* (Lee et al. 1984). Immunization enhances AM clearance suggesting a contribution by FcγR-mediated uptake (Buret et al. 1994). However, antibodies from CF patient serum may have impaired interaction with FcγR (Fick et al. 1981), likely in part due to the effect of *P. aeruginosa* elastase-mediated cleavage (Wretlind and Pavlovskis 1983). Since AM from CF patients may be more dependent on FcγR- as opposed to CR-mediated uptake these findings may be particularly important for CF patients (Berger et al. 1994). Both classical and alternative pathway complement enhance phagocytosis when other opsonins are limited but are not essential for ingestion when bacteria are opsonized by immune serum (Murphey et al. 1979). *P. aeruginosa* expresses an alkaline protease (AprA), which limits C3b deposition, via the classical and lectin pathways of complement activation, but does not affect the alternative pathway of complement activation. (Laarman et al. 2012). The impaired C3b deposition appears to be the direct result of AprA-mediated cleavage of the C2 complement component

Surfactant proteins A and D opsonize *P. aeruginosa*, including mucoid strains (Mariencheck et al. 1999; Restrepo et al. 1999) and enhance AM phagocytosis and bacterial clearance in a murine model of infection (Giannoni et al. 2006). Surfactant protein A2 variants appear more effective at enhancing phagocytosis than A1 variants (Mikerov et al. 2007). Surfactant protein C may also contribute to AM clearance and AM from surfactant protein C deficient mice had enhanced markers of alternative activation, although surfactant protein C does not opsonize *P. aeruginosa* (Glasser et al. 2008). Pseudomonas elastase also cleaves surfactant protein A and D (Mariencheck et al. 2003; Alcorn and Wright 2004). A novel mechanism of *P. aeruginosa* phagocytosis by AM has recently been described involving internalization via lipid rafts (Kannan et al. 2008). In this model activation of the Src family kinase Lyn mediates lamellipodia formation and subsequent PI3K/Akt activation to enhance ingestion and killing.

Overall the combination of microbial factors preventing phagocytosis and potentially also the more limited capacity of AM to ingest *P. aeruginosa*, as evidenced by the fact that AM are only able to control bacterial clearance over the first 4–8 h after infection (Kooguchi et al. 1998), suggest *P. aeruginosa* represent a particular challenge to AM. AM benefit from additional stimulation, such as TNF-α treatment, to enhance protein kinase C activation and phagocytosis of *P. aeruginosa* (Heale and Speert 2001), emphasizing the fact, as suggested by the experiments in surfactant protein C-deficient mice, that there may be a defect in activation. NKT-cell help may be important in priming AM function and activation of CD1d-restricted T-cells

results in enhanced IFN-γ production and increased AM phagocytosis and clearance of *P. aeruginosa* in a murine model (Nieuwenhuis et al. 2002). Increased PGE_2 enhanced PTEN activity and impaired clearance of opsonized *P. aeruginosa* (Hubbard et al. 2011), but PGE_2 also inhibited phagocytosis of unopsonized bacteria via enhanced IL-1R-associated kinase M (IRAK-M) activity, an inhibitor of MyD88-dependent TLR-signaling (Hubbard et al. 2010). This also emphasizes the importance of ensuring adequate cytokine production and associated activation to ensure AM are able to clear *P. aeruginosa* in the face of the considerable challenges this microorganism presents.

1.3.4 Ingestion of Haemophilus influenzae and Klebsiella pneumoniae

Other acute bacterial pathogens have been less studied with regard to mechanisms of phagocytosis by AM. *H. influenzae* type b (Hib) also possess a capsule that inhibits phagocytosis and requires opsonization for optimal ingestion; however, the degree of inhibition was less than that observed for a serotype 3 pneumococcus (Jonsson et al. 1985). *H. influenzae* also produce an IgA protease (St Geme et al. 1994). The unencapsulated non typeable *H. influenzae* (NTHi) are phagocytosed to a comparable extent as *S. aureus* or unencapsulated *S. pneumoniae*. Complement and IgG both opsonize Hib and one study showed complement enhanced binding but IgG1 enhanced internalization of bound bacteria to macrophages (Noel et al. 1990). NTHi represents a particular challenge for patients with COPD and AM from these patients are impaired in their capacity to phagocytose NTHi (Berenson et al. 2006). Although murine models of COPD do not replicate all the features of human disease, a model of COPD involving LPS and elastase treatment demonstrated AM were less able to clear NTHi (Ganesan et al. 2012). Using this model the investigators established AM from these mice had absent surface expression of SR-A and reduced expression of MARCO and mannose receptors. They showed that blockade of SR-A uptake in AM from mice without COPD blocked NTHi uptake and therefore concluded reduced SR-A expression underlined the defect in COPD and by implication suggested SR-A is a key receptor for phagocytosis of NTHi. In one study surfactant protein A opsonized *H. influenzae* with strain-specific differences and opsonization of *H. influenzae* type a but not Hib (McNeely and Coonrod 1994). Surfactant protein A enhances *H. influenzae* phagocytosis (Tino and Wright 1996) and another study showed surfactant protein A enhanced CR3 trafficking to the cell surface, enhancing CR3-dependent phagocytosis (Gil et al. 2009). Studies with surfactant-deficient mice reveal impaired phagocytosis by AM in both surfactant A- and D-deficient mice though in only surfactant A-deficient mice is this associated with impaired killing and reduced clearance (LeVine et al. 2000).

Klebsiella pneumoniae causes pneumonia in a variety of settings including the hospital environment (Bartlett et al. 1986). Resistance to phagocytosis is an

important virulence factor for *K. pneumoniae* and several of the loci governing resistance to phagocytosis in a screen of *K. pneumoniae* mutants are capsular synthesis genes (Pan et al. 2011). Unencapsulated mutants failed to induce pneumonia and were more avidly ingested by AM in another study which showed that *K. pneumoniae* capsule significantly impedes C3 complement deposition (Cortes et al. 2002). AM depletion experiments confirm a role for AM in the clearance of AM from mice (Cheung et al. 2000). Immune serum enables FcγR-mediated uptake and PGE_2 can inhibit while LTB_4 and LTC_4 enhance phagocytosis (Aronoff et al. 2004; Mancuso et al. 1998). In addition to a role for immunoglobulin and complement, surfactant protein A enhances bacterial killing by AM (Hickman-Davis et al. 2002). A further study showed the ability of surfactant protein A to opsonize *K. pneumoniae* and enhance AM phagocytosis varied by capsular type with some strains showing little surfactant protein A-dependent phagocytosis (Kabha et al. 1997). Surfactant D is unable to enhance phagocytosis of encapsulated strains but can increase ingestion of spontaneous unencapsulated *K. pneumoniae* phase variants which occur early in infection (Ofek et al. 2001). The mannose receptor also contributes to phagocytosis of *K. pneumoniae* by AM (Athamna et al. 1991) and is involved in uptake of surfactant protein A opsonized bacteria (Kabha et al. 1997).

1.4 Intracellular Killing

The challenge for AM is to ensure effective killing of the bacteria they ingest. As stated, their activation status under basal conditions does not favor high-level production of microbicidal molecules and therefore AM need to couple bacterial phagocytosis with bacterial sensing via pattern recognition receptors to ensure their activation status allows effective generation of microbicidal molecules (Mosser and Edwards 2008; Mantovani et al. 2004). In addition AM do not have the same intrinsic capacity to generate certain antimicrobial molecules as other phagocytes such as neutrophils even when appropriately activated. For example, they are unable to create the more toxic antimicrobial products generated by the myeloperoxidase system such as HOCl, since they have negligible levels of this enzyme in comparison to neutrophils (Cohen and Cline 1971; Hampton et al. 1998). They are therefore more reliant on combining ROS with NO to produce reactive nitrogen species (RNS) but as mentioned above this requires appropriate activation to ensure NO generation via iNOS (MacMicking et al. 1997). Macrophages may also employ novel TLR-mediated mechanisms of ROS-dependent killing such as utilizing mitochondrial ROS to enhance phagolysosomal killing (West et al. 2011). This can result in slower kinetics of killing than in neutrophils (Cohen and Cline 1971). They also must contend with microbial adaptations, which resist intracellular killing or favor escape from the phagolysosomal compartment, a well-recognized feature of bacteria that are adapted to intracellular survival (McDonough et al. 1993; Lindgren et al. 2004).

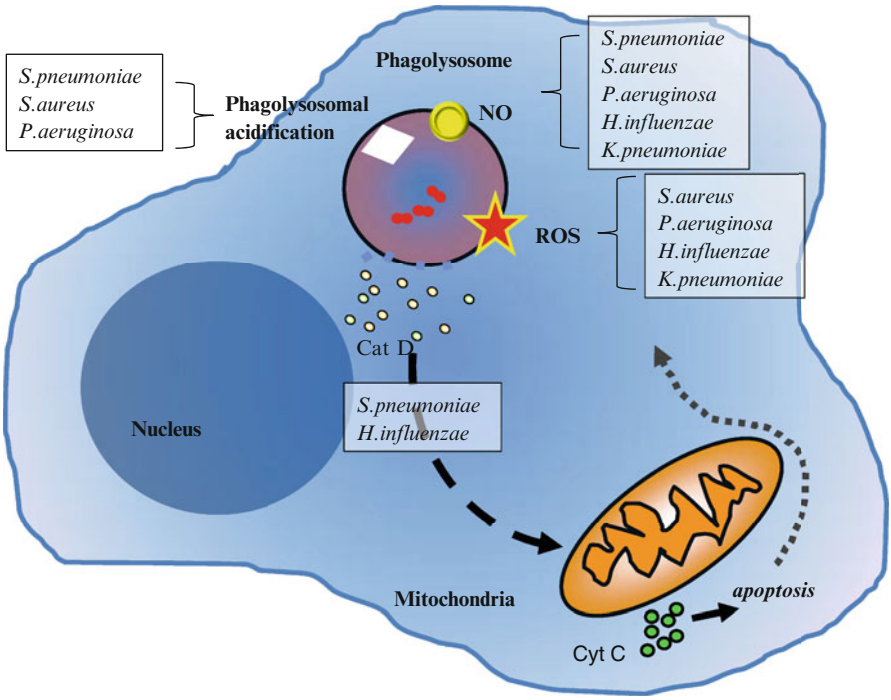

Fig. 1.4 Alveolar macrophage killing mechanisms for extracellular bacteria. Nitric oxide (NO), reactive oxygen species (ROS) and reactive nitrogen species (RNS) are generated in the AM in response to different pathogens as indicated. Phagolysosomal acidification provides an additional level of degradation for some bacteria acting via lysosomal proteases. In addition *S. pneumoniae* and *H. influenzae* lead to the release of phagolysosomal proteases, such as cathepsin D (Cat D) which activate an apoptotic cascade (*bold hashed arrow*) culminating in mitochondrial outer membrane permeabilization, cytochrome C (Cyt C) release and additional ROS-dependent killing (*grey hashed arrow*)

1.4.1 Intracellular Killing of Streptococcus pneumoniae

Ingestion of pneumococci by AM results in recruitment to phagosomes that fuse with lysosomes (Gordon et al. 2000). Opsonization enhances trafficking to the phagolysosome and killing. Once ingested pneumococci are efficiently killed by AM (Jonsson et al. 1985), see Fig. 1.4. Pneumococci produce ROS as a result of their own metabolism and have their own antioxidant mechanisms (Preston and Dockrell 2008). NADPH generation of ROS is not required for killing of pneumococci by neutrophils (Marriott et al. 2008a; Standish and Weiser 2009) and, similarly, mice with deficient NADPH oxidase-dependent ROS production do not have a significant defect in AM-mediated bacterial clearance (Marriott et al. 2007). Incubation of AM with catalase to degrade hydrogen peroxide also does not impair killing of pneumococci (Biggar et al. 1976). MDM increase production of iNOS

and NO after exposure to pneumococci, NO contributes to early and late phases of bacterial killing and iNOS-deficient mice need to recruit greater numbers of neutrophils and generate higher levels of pro-inflammatory cytokines to control early stages of bacterial replication in the lung, suggesting NO contributes to bacterial killing either alone or as RNS (Bewley et al. 2011a; Marriott et al. 2007, 2004). However, AM have a finite killing capacity as evidenced by the fact that although they can control low inocula of bacteria in mouse experiments, once the infecting dose is increased above a threshold, additional host responses are required including the recruitment of neutrophils (Dockrell et al. 2003).

An additional AM killing strategy for pneumococci is induction of apoptosis. Macrophages and AM undergo apoptosis in vitro and in vivo (Dockrell et al. 2001, 2003; Srivastava et al. 2005b). The process is dependent on the production of pneumolysin by the bacteria and appears to involve an immune recognition pathway involving pattern recognition receptors such as TLR4 and possibly other receptors that sense pneumolysin and trigger cell death (Dockrell et al. 2001; Srivastava et al. 2005b). Although pneumolysin can induce apoptosis in a variety of cell types (Braun et al. 2002; Bermpohl et al. 2005; Marriott et al. 2008b), in this particular case apoptosis appears to be part of a host defense strategy as rates are higher with less virulent strains (e.g., unencapsulated strains), are enhanced by bacterial opsonization in immune serum, and inhibition of apoptosis reduces antimicrobial killing (Ali et al. 2003; Marriott et al. 2004; Dockrell et al. 2001). Apoptosis correlates with the intracellular bacterial burden and is a delayed process, suggesting that it complements conventional methods of killing in the phagolysosome and may be activated when the cell has exhausted its intrinsic capacity for phagolysosomal killing (Ali et al. 2003; Marriott et al. 2005). The molecular pathway that leads to apoptosis involves phagolysosomal permeabilization, activation of cathepsin D, increased degradation of the antiapoptotic Mcl-1 protein via ubiquitination, mitochondrial outer membrane permeabilization, and caspase activation (Bewley et al. 2011a, b; Marriott et al. 2004, 2005). The mechanism by which macrophage apoptosis leads to bacterial killing appears to involve the release of NO and ROS during apoptotic cell death (Bewley et al. 2011a). In animal models cathepsin D inhibition, Mcl-1 overexpression or caspase inhibition all inhibit bacterial clearance from the lung in association with reduction in AM apoptosis (Dockrell et al. 2003; Bewley et al. 2011a; Marriott et al. 2005). AM apoptosis also appears to have an important role in preventing bacterial translocation into the blood stream and in raising the threshold at which neutrophil recruitment is required to control bacterial infection (Dockrell et al. 2003; Bewley et al. 2011a; Marriott et al. 2005).

1.4.2 Intracellular Killing of Staphylococcus aureus

In contrast to the response to pneumococci, *S. aureus* are more readily ingested but less efficiently killed after internalization by AM (Jonsson et al. 1985; Nibbering et al. 1989). In neutrophils the importance of ROS-based killing and/or the

dependence on the neutrophil cationic granule proteases, including neutrophil elastase and cathepsin G, activated in association with NADPH oxidase activity is well established. This is evidenced by the susceptibility of patients with defects in the NADPH oxidase system which characterizes chronic granulomatous disease (CGD) to *S. aureus* infection (Reeves et al. 2002; Lekstrom-Himes and Gallin 2000). AM respond to ingestion of *S. aureus* with lower levels of ROS generation than other phagocytes and lack the neutrophil expressed cationic granule proteases (Nibbering et al. 1989), but still require ROS to kill *S. aureus* (Watanabe et al. 2007). It is therefore not surprising that AM killing of *S. aureus* is less effective than that of neutrophils (Lee et al. 1984). AM produce superoxide and not the products of the myeloperoxidase-hydrogen peroxide-halide system after ingestion of *S. aureus* (Devalon et al. 1987). Importantly the superoxide does not produce DNA denaturation to the same extent that the ROS produced by the myeloperoxidase achieves. Bacterial catalase also helps protect *S. aureus* against AM ROS (Das and Bishayi 2009). ROS production against *S. aureus* is inhibited by surfactant but stimulated by IFN-γ, hence IFN-γ is used in management of CGD (Geertsma et al. 1993; Speert and Thorson 1991; Goldblatt and Thrasher 2000). The NO system also provides protection against systemic infection with *S. aureus* although while there is little information on its specific role in the lung it is reasonable to speculate that it will contribute to the antimicrobial defenses of AM, albeit that its activity will be dependent on the activation state of the macrophage as discussed above (Mantovani et al. 2004; Sakiniene et al. 1997). Histone deacetylase (HDAC) inhibitors, which modulate gene transcription, have been shown to reduce expression of NADPH oxidase subunits and iNOS with associated decreased killing of *S. aureus* by macrophages, illustrating the importance of active gene transcription (Mombelli et al. 2011). Successful killing of *S. aureus* in the AM requires acidification of the phagolysosome, via the action of the vacuolar ATPase, which enhances ROS production (Bidani et al. 2000). Recently it has emerged that acidification of the phagosome is required for bacterial degradation by enzymes including lysozyme and unidentified serine, cysteine, and acid proteases such that the necessary TLR ligands and TLR-dependent cytokines can be released (Ip et al. 2010). Linkage of phagocytosis to activation of the appropriate phagosomal or other intracellular pattern recognition system is potentially important to enhance the killing capacity of AM for *S. aureus*, either directly or indirectly through the paracrine effects of the cytokines released. However the exact details of this are still to be clarified and TLR activation may, under some circumstances, inhibit ROS production such as when TLR2-mediated activation of c-Jun N-terminal kinases (JNKs), members of the MAPK family, were observed to inhibit ROS and *S. aureus* killing in murine macrophages (Watanabe et al. 2007). This emphasizes that the regulation of ROS in AM during *S. aureus* infection is complex, with many inhibitory circuits preventing excessive generation of ROS in the lung.

S. aureus has the capacity over time to escape from phagolysosomes in MDM (Kubica et al. 2008), which will compromise the host response (Ip et al. 2010). This process required a variety of factors including the global regulator, accessory gene regulator (agr) and an agr-regulated toxin, α-hemolysin, a factor previously

implicated in mediating phagosomal escape of *S. aureus* from a CF epithelial cell line (Jarry et al. 2008). Mutants that lacked α-hemolysin were readily killed and did not escape the MDM phagosome (Kubica et al. 2008). Previously α-hemolysin has been shown to cause apoptosis (Bantel et al. 2001) but in the MDM model apoptosis does not occur (Koziel et al. 2009). Moreover there is an absence of cathepsin D activation and both Mcl-1 and Bcl-2 (a further antiapoptotic Bcl-2 family member) are upregulated, while mitochondrial outer membrane permeabilization does not occur (Bewley et al. 2011a; Koziel et al. 2009). Although the relevance of these findings to AM and clearance of *S. aureus* in the lung remains to be established, it is interesting to speculate that *S. aureus* which stresses conventional phagolysosomal antimicrobicidal mechanisms may also subvert the late phase killing provided by macrophage apoptosis-associated killing. In the face of these challenges AM may have to engage additional mechanisms to kill *S. aureus*. Recently it has been observed that macrophages may release DNA-based extracellular traps as an antimicrobial strategy against *S. aureus*, a process the authors found was enhanced by the cholesterol lowering statin class of drugs (Chow et al. 2010). Utilization of additional extracellular killing strategies may reflect the relative inefficiency of the intracellular mechanisms of macrophage killing.

1.4.3 Intracellular Killing of Pseudomonas aeruginosa

AM killing of *P. aeruginosa* involves NADPH oxidase-dependent ROS and NO (Zhang et al. 2010; Zhang et al. 2011). IFN-γ and TNF-α prime macrophages enhancing their antibactericidal activity against *P. aeruginosa* (Pierangeli and Sonnenfeld 1993). Lysosomal acidification is required for optimal intracellular killing and is compromised in the face of common mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) such as ΔF508 (Deriy et al. 2009). Appropriate acidification of a population of secretory lysosomes is required to activate acid sphingomyelinase during ingestion of *P. aeruginosa* by AM (Zhang et al. 2010). This results in ceramide generation and accumulation of ceramide in membrane platforms, which cluster and activate NADPH oxidase, resulting in generation of ROS and bacterial killing. Ceramide also activates Ksr a serine-threonine kinase that activates Raf-1 (Zhang et al. 2011). Ksr also forms a scaffold for the Raf-1 mitogen-activated protein kinase (MEK)-extracellular signal-regulated kinase (ERK)-MAPK signaling pathway but this does not modulate *P. aeruginosa* killing. Instead Ksr mediates iNOS activation and production of both NO and of peroxynitrite, formed by the reaction of NO and ROS. The authors found that Ksr did not alter iNOS expression but formed a multiprotein complex with iNOS and Hsp90. Hsp90 is a factor implicated in iNOS activation, resulting in generation of NO and RNS, which diffuse into the phagosome and mediate bacterial killing. They also suggested that since NO can upregulate Fas and Fas-mediated apoptosis of epithelial cells can represent a mechanism controlling *P. aeruginosa* infection, AM production of NO would also have additional indirect extracellular effects on killing

(Assis et al. 2006; Grassme et al. 2000). Although the generation of ceramide via lysosomal acidification is coupled to generation of antimicrobicidal molecules, there is also evidence that phagolysosomal acidification is important for effective *P. aeruginosa* digestion in the phagolysosomes of AM as described above for *S. aureus* and that CFTR is required for this activity (Di et al. 2006). Of interest in this model, and in contrast to other investigators (Zhang et al. 2010), the effect of phagolysosomal acidification was not mediated by alteration of NADPH oxidase-dependent ROS generation, which was unaffected, and the authors speculated it was likely due to impaired activation of lysosomal proteases though this possibility was not further tested (Di et al. 2006). The factors required to prime AM killing of *P. aeruginosa* are incompletely defined. In one study nucleotide oligomerization domain (NOD)2, a pattern recognition receptor which recognizes muramyl dipeptide, a motif found in peptidoglycan, enhanced bacterial killing in association with NF-κB activation and upregulation of IL-1β and TNF-α (Tsay et al. 2009). In the same study loss of TLR4 signaling did not alter bacterial killing.

1.4.4 Intracellular Killing of Haemophilus influenzae and Klebsiella pneumoniae

There is less data on the mechanisms of intracellular killing of *H. influenzae*. Most studies suggest prompt intracellular killing once ingested (Jonsson et al. 1985). One study indicated a relative deficiency in killing of the majority of NTHi strains tested but others using single strains have not confirmed this (Jonsson et al. 1985, 1987; Craig et al. 2001). Intracellular killing has been linked to surfactant protein A stimulated ROS production (LeVine et al. 2000) and *Haemophilus* spp. can stimulate NO production in AM (Gomis et al. 1997). Although evidence for AM apoptosis-associated killing has not been published, we have preliminary evidence that several of the molecular events linked to AM apoptosis-associated killing of *S. pneumoniae* such as cathepsin D activation and Mcl-1 downregulation (Dockrell et al. 2003; Bewley et al. 2011a; Marriott et al. 2005), also occur following Hib infection and that inhibition of AM apoptosis impairs bacterial clearance in vivo, suggesting apoptosis-associated killing may also play a role in *H. influenzae* killing.

K. pneumoniae intracellular killing in AM has also been linked to ROS, NO, and RNS production (Yokota et al. 1988; Hickman-Davis et al. 2002; Tsai et al. 1997). AM from elderly mice were found to have impaired ROS expression in association with reduced bacterial killing but the functional consequences of impairing ROS were not examined to determine if they reproduced the bacterial killing defect of aging (Yokota et al. 1988). PGE_2 inhibits AM assembly of NADPH oxidase, via cAMP-dependent effects on the recruitment of the p47 phox subunit to the mature NADPH complex (Serezani et al. 2007) in a pathway that involves PTEN activation (Canetti et al. 2007). This pathway reduces ROS generation and *K. pneumoniae* killing by mice (Serezani et al. 2007). Conversely LTB_4 enhances NADPH oxidase

assembly and ROS-mediated killing of *K. pneumoniae*, emphasizing that the balance of prostaglandins and leukotrienes not only determine the efficiency of phagocytosis but also the level of ROS generation by AM in response to bacterial stimuli including *K. pneumoniae* (Serezani et al. 2005). An NO inhibitor reduced AM killing of *K. pneumoniae* in vitro and reduced pulmonary clearance in a mouse model (Tsai et al. 1997). Surfactant protein A was found to enhance bacterial killing, NO and RNS in transplant patients, though not healthy donors AM (Hickman-Davis et al. 2002).

1.5 Regulation of Inflammation

1.5.1 Role of Pattern Receptor Recognition of Bacteria by Alveolar Macrophages

In addition to the ingestion and killing of microorganisms, AM also play a critical role in sensing bacteria through their signaling pattern recognition receptors such as TLR, NOD, and NALP (Sabroe et al. 2003a; Franchi et al. 2009; Inohara and Nunez 2003). TLR for example contribute to activation of phagocytosis and killing of bacteria, enhancing generation of ROS and NO, ensuring maturation of the phagosome and cytokine generation (Sabroe et al. 2003b; Sato et al. 2000; Blander and Medzhitov 2004). TLR are important in the containment of subclinical infection and the early stages of pneumonia. Pattern recognition receptors can synergize in these responses, for example co-stimulation with TLR2 and TLR4 agonists amplifies responses (Sato et al. 2000), and whole microorganisms will activate multiple different receptors. In view of the finite capacity of AM to phagocytose and kill bacteria, even after appropriate stimulation, pattern recognition receptors will also be critical to the AM's role in coordinating the immune response when their capacity to clear the bacterial inoculum is overwhelmed. Under these circumstances AM function as part of a cellular network producing cytokines and chemokines, both functioning as a source of key chemoattractants, but also by releasing cytokines that stimulate the epithelium or other cells to release the critical chemokines (Krakauer 2002; Standiford et al. 1991; Morris et al. 2005, 2006). This is essential for the recruitment of other immune cells such as neutrophils.

Since pattern recognition receptors are frequently expressed by a range of other cell types including epithelial cells, endothelial cells, and smooth muscle cells (Krakauer 2002; Standiford et al. 1991; Morris et al. 2005, 2006), it is often difficult to determine to what extent the activation of pattern receptors in AM, as opposed to other cells in the lung, contributes to host defense. Equally many of the cytokines produced that are used as markers of pattern recognition receptor engagement can have multiple sources and most of the experiments with knockout mice have not been conducted with knockouts on a macrophage-specific background. Nevertheless there are important examples of macrophage-derived factors contributing to host defense. For example, production of the neutrophil chemokine CXCL8 (IL-8)

involves expression by macrophages, epithelial and other cells. In vitro *S. pneumoniae* induce relatively modest CXCL8 responses from epithelial cells, in particular when encapsulated (Marriott et al. 2012). Macrophages, however, enhance epithelial cell production by producing IL-1β, which stimulates epithelial cells to produce CXCL8, and in mice genetic manipulation of IL-1 signaling reduces CXC chemokine release and neutrophil levels in BAL during pneumonia. Depletion of AM in lung explants also confirms a critical role for AM in TLR-dependent pro-inflammatory cytokine generation in response to pneumococci (Xu et al. 2008). Epithelial cells are a major target of pro-inflammatory cytokines produced in the lung (Quinton et al. 2007). Early response cytokines in murine BAL, isolated during pneumococcal pneumonia, include IL-1 and TNF-α, which stimulate NF-κB activation, pro-inflammatory cytokine and chemokine generation, and neutrophil recruitment. Although several cell types can contribute to the generation of these early response cytokines, AM are a key source. In keeping with the important role of AM in releasing early response cytokines, to stimulate chemokine production and neutrophil recruitment, AM depletion in mice during *P. aeruginosa* infection reduces neutrophil numbers in the lung (Kooguchi et al. 1998; Hashimoto et al. 1996).

1.5.2 Pathogen Recognition Receptors Involved in Recognition of Bacteria by AM

There are multiple potential pattern recognition receptors involved in the response to each pulmonary pathogen. Host defense against *S. pneumoniae* involves TLR2, 4, and 9. TLR2 deficiency does not reduce bacterial clearance overall but modifies the early response of some cytokines (Knapp et al. 2004). TLR4 recognizes pneumolysin and TLR2 and 4 responses synergize in the activation of macrophages after exposure to microbial components of pneumococci. TLR4 knockout mice were more susceptible to infection with pneumolysin expressing *S. pneumoniae* (Malley et al. 2003). A further study, however, suggested TLR4-deficient mice only had a worse outcome following low-dose challenge with *S. pneumoniae* and that TLR4 played less role in the protection than against *K. pneumoniae* (Branger et al. 2004). TLR9 recognizes unmethylated CpG dinucleotides found in prokaryotic DNA and one group found TLR9 played a greater role in protection against pulmonary *S. pneumoniae* than did lack of TLR2, TLR4 (or TLR1 or TLR6 which heterodimerize with TLR2) (Albiger et al. 2007). Nevertheless the effects of single TLR knockouts are in most cases modest and restricted to early outcomes (Knapp et al. 2004; Branger et al. 2004; Albiger et al. 2007). The impact of deletion is greater for TLR2/TLR9 or TLR4/TLR9 double knockouts and greater for mice lacking the MyD88 adapter molecule that is involved in generating multiple TLR-dependent signals from a variety of TLR (Lee et al. 2007). These results speak to the considerable redundancy in TLR signaling. Nevertheless there can be subtle differences in the consequences of engagement of different TLR. For example although both TLR2 and TLR4 signaling increased neutrophil recruitment to the lung in healthy

volunteers challenged with lipoteichoic acid or LPS, only LPS was associated with AM activation (Hoogerwerf et al. 2008).

NOD1 and 2 represent the cytoplasmic system involved in peptidoglycan recognition and are upregulated following *S. pneumoniae* infection, with NOD2 involved in NF-κB activation during pneumococcal infection (Opitz et al. 2004). Recently it has been demonstrated that lysozyme M is involved in the digestion of the pneumococcal cell wall resulting in the release of peptidoglycan for recognition by NOD2 in macrophages and stimulating production of the macrophage chemokine CCL2 (Davis et al. 2011). While this was shown to play a role in the recruitment of macrophages to the upper airway it is possible that NOD2 recognition systems could also influence CCL2-dependent recruitment in the alveolar space. The authors had previously shown that TLR2-dependent macrophage responses protected naïve mice against upper airway colonization in a Th17-dependent process (Zhang et al. 2009).

The NLR inflammasome system is also emerging as an important pattern recognition system in macrophages. The NLRP3 inflammasome recognizes pneumolysin and triggers IL-1β release, resulting in improved clearance of bacteria in murine infection models (Witzenrath et al. 2011). Of interest certain strains of pneumolysin that have point mutations that abrogate their pore forming capacity are not able to activate the NLRP3 inflammasome and it has been suggested that failure to activate this recognition system may contribute to the increased invasiveness of these strains. Another group found that mice lacking the inflammasome receptor adaptor protein, apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) had a more impaired response to pneumococci than the NALP3 knockouts and found a role for an additional inflammasome receptor, absent in melanoma 2 (AIM2), in the response to pneumococci (Fang et al. 2011).

Many other receptors may also be involved in the inflammatory response to pneumococci in macrophages and excessive stimulation may be as harmful as an absence of inflammatory stimulation. Thus an optimal response requires an appropriate balance of pro-and anti-inflammatory signals. Retuning these inflammatory signals may have therapeutic potential. For example, deficiency of PTEN and the associated enhancement of PI3K activity enhanced AM phagocytosis, bacterial clearance, and prolonged survival even though it decreased neutrophil recruitment and inflammation during pneumococcal pneumonia, suggesting PTEN signaling may be harmful during pneumonia and favor excessive inflammation (Schabbauer et al. 2010). Conversely engagement of TREM-1 amplifies early inflammatory signals but downregulates inflammatory responses at later time points in murine models of pneumococcal pneumonia. This aids resolution of inflammation and is achieved via upregulation of IL-1 receptor associated kinase (IRAK)-M, a negative regulator of TLR signaling (Lagler et al. 2009).

In comparison to *S. pneumoniae* other bacteria show many similarities but also some differences in the pattern recognition receptors they engage. Cytokine responses to *S. aureus* were not altered by TLR2, TLR2/TLR4 or TLR9 deletion in peritoneal macrophages but were reduced in MyD88-deficient cells (Kapetanovic et al. 2007). In AM the same group found that cytokine production required phagocytosis and phagosomal maturation but was independent of NOD2, implicating additional intracellular pattern recognition receptors (Kapetanovic et al. 2011).

Another group found that the pore forming toxin Panton-Valentine-Leukocidin could bind TLR2 and activate signaling (Zivkovic et al. 2011). *Pseudomonas aeruginosa* can activate several additional pattern recognition receptors. TLR5 contributes to host defense via recognition of flagellin (Feuillet et al. 2006). Some studies show an additional component from TLR4, in addition to TLR5, while others do not and the importance of TLR4 may vary dependent on the cytokine measured (Ramphal et al. 2008; Raoust et al. 2009; Skerrett et al. 2007). In addition the NLRC4 inflammasome has been implicated in the recognition of the basal rod component (Pscl) of the pseudomonal type 3 secretion system (Miao et al. 2010). For other Gram negative bacteria TLR2 and/or 4 may be activated to a greater extent than for the gram positive bacteria (Kapetanovic et al. 2007). Upper airway clearance of Hib involved TLR2, TLR4, and NOD1 while NTHi in the lower airway required MyD88 dependent but not independent responses and also featured a role for TLR4 (Zola et al. 2008; Wieland et al. 2005). In the case of *K. pneumoniae* roles were found for MyD88-dependent and MyD88-independent (Toll-IL-1 receptor domain-containing adaptor inducing IFN-β (TRIF)-dependent) signaling (Cai et al. 2009), as well as a specific role for TLR4 (Branger et al. 2004). These findings suggest that although specific pathogens may use different recognition systems, there is a broad reliance on the combined effects of TLR, NOD, and NLR recognition systems in AM sensing of bacteria, with the need to counterbalance signals using a variety of additional signaling systems, as exemplified for pneumococci to ensure optimal but not excessive inflammation.

1.5.3 Efferocytosis

Ingestion of apoptotic cells is an important part of resolving an acute inflammatory response to infection and AM play essential roles both as key phagocytic cells involved in the process and also by the fact that ingestion of apoptotic cells downregulates the production of pro-inflammatory cytokines by macrophages. MDM can induce Fas-dependent apoptosis in monocytes following exposure to *S. pneumoniae* in vitro, raising the possibility that AM may regulate the induction of apoptosis in recruited inflammatory cells (Dockrell et al. 2001). In a murine model of *S. pneumoniae* infection efferocytosis of apoptotic AM downregulated pro-inflammatory cytokines and chemokines and reduced neutrophilic inflammation (Marriott et al. 2006). In particular this reduced rates of invasive disease. However, ingestion of apoptotic cells, although helping downregulate inflammatory responses and aiding resolution, does compromise the AM's own competence at clearing bacteria via upregulation of PGE_2, emphasizing that this important role is carried out at the expense of a primary role in bacterial clearance (Medeiros et al. 2009). It has been shown however, that although AM are key to initial bacterial clearance and control of low dose of bacteria, at higher doses their role as primary effectors of bacterial clearance is overwhelmed (Dockrell et al. 2003; Knapp et al. 2003). Thus it seems that in the face of higher bacterial challenges and a more established inflammatory response AM's key role becomes regulating the inflammatory response. In line with

this AM depletion in a murine model of high-dose pneumococcal challenge did not alter bacterial clearance but adversely influenced the clearance of effete neutrophils (Knapp et al. 2003). AM depletion resulted in failure to clear apoptotic neutrophils, which became secondarily necrotic, resulting in release of pro-inflammatory mediators and also increased mortality. In keeping with the importance of an AM M2 phenotype to enhance efferocytosis, it has been shown that lack of galectin-3, a β-galactoside-binding lectin expressed by macrophages, a feature of impaired M2 polarization, results in reduced efferocytosis of apoptotic neutrophils with enhanced pulmonary inflammation in a murine model of pneumococcal pneumonia (Farnworth et al. 2008; MacKinnon et al. 2008). Although efferocytosis in general is regarded as anti-inflammatory, the ingestion of cells rendered apoptotic after exposure to bacteria may be less anti-inflammatory than cells that are apoptotic in the absence of infection (Zheng et al. 2004). This may represent a mechanism by which the inflammatory response is downgraded but not turned off by efferocytosis in the context of infection.

The *P. aeruginosa* toxin pyocyanin reduces efferocytosis of apoptotic cells by macrophages (Bianchi et al. 2008). Moreover pyocyanin-expressing bacteria are associated with a reduction in AM clearance of apoptotic cells in mouse models of *P. aeruginosa* infection. The reduced efferocytosis is dependent on the production of ROS, which enhances activity of Rho kinase and a small GTPase RhoA. The balance of GTPase activity between Rac-1 and RhoA determines the efficiency of efferocytosis by macrophages with RhoA inhibiting efferocytosis. Hence RhoA activation by pyocyanin results in inhibition of efferocytosis in the lungs of mice exposed to *P. aeruginosa*. These observations are noteworthy because the sputum of patients with CF is known to contain high levels apoptotic or necrotic neutrophils that have not undergone clearance by efferocytosis and *P. aeruginosa* is a frequent infection in the lungs of these patients (Vandivier et al. 2002b). Efferocytosis is therefore an important component of the resolution phase of acute bacterial infection and microbial strategies which inhibit this will favor chronic inflammation.

1.6 Linkage to Adaptive Immunity

Macrophages are considered one of the three main antigen-presenting cells, together with dendritic cells and B-cells. However AM, in keeping with their anti-inflammatory phenotype, differ from macrophages at other sites, such as splenic macrophages, in their ability to present antigen. Various studies have reported that AM are inefficient at presenting antigen to T-cells and indeed may reduce T-cell activation (Chelen et al. 1995; Blumenthal et al. 2001). This has been shown to be due to reduced co-stimulation as AM have lower expression of CD80/CD86 than peripheral blood monocytes and antigen presentation could be restored with anti-CD28 treatment (Chelen et al. 1995; Blumenthal et al. 2001). AM can also reduce the ability of dendritic cells to act as antigen presenting cells (Holt et al. 1993). The capacity of AM to present antigen is also influenced by respiratory disease. For example, the expression of HLA-DR and CD80 was lower in AM from patients

with COPD than those from either healthy smokers or nonsmokers (Pons et al. 2005). In some inflammatory diseases, such as asthma, suppression of antigen presentation is removed and AM have an increased ability to present antigen and show increased expression of HLA DR (Viksman et al. 1997). Infections are often able to subvert antigen presentation; this is commonly seen with viruses as a strategy to avoid the removal of infected host cells by cytotoxic T-cells. However, this strategy is also employed by bacterial infections. For example, the expression of HLA-DR is downregulated in AM from patients with a clinical diagnosis of pneumonia and although microbiological details on the specific pathogens were not provided in this study the majority of these infections would be anticipated to be caused by the bacteria discussed in this chapter (Bühling et al. 2000). As well as direct antigen presentation macrophages are able to regulate other immune cells. In addition to the interaction between lymphocytes and AM which results in regulation of macrophage activation and macrophage plasticity, AM are able to produce cytokines that regulate the adaptive immune response of lymphocytes. The ability of AM to downregulate the activity of lymphocytes has long been recognized (Holt 1986).

In order to evoke adaptive immunity antigen presenting cells traffic to local draining lymph nodes. Dendritic cells are found throughout the airway but in much smaller numbers than AM in normal bronchial alveolar lavage and are more capable of trafficking to lymph nodes to present antigen (Jakubzick et al. 2006). Kirby et al. (2009) found that some AM migrated to draining lymph nodes after exposure to *S. pneumoniae*. They found the number of AM in the draining lymph nodes were small but appeared before dendritic cells and were contained to the lung draining lymph nodes (Kirby et al. 2009). This rapid appearance of AM in draining lymph nodes suggested an early role for AM in transporting antigen to regional lymph nodes and in playing an important role in linking innate and adaptive immunity during bacterial infection in the lung.

1.7 Microbial Subversion of Macrophage Host Defense

From the discussion above it is apparent that bacteria can alter AM function by multiple mechanisms involving alteration in the phagocytosis, killing or recognition of bacteria as well as by modulating efferocytosis. Bacteria may also influence AM function by indirect effects on the lung environment which influence epithelial cells, other immune cells or soluble factors expressed in the lung. They may however have other direct effects on AM through modulation of cell viability. There are multiple examples of bacterial toxins or other components inducing macrophage death in vitro. For example, the *S. aureus* leukocidin LukA/B and the *P. aeruginosa* cytotoxin ExoU have both been implicated in the induction of macrophage necrosis as have *P. aeruginosa* ATP utilizing enzymes such as nucleoside diphosphate kinase which leads to the formation of adenine nucleotides, activating the macrophage P2X7 purinergic receptor (Dumont et al. 2011; Hauser and Engel 1999; Zaborina et al. 2000). Bacterial factors may also induce apoptosis which, although is less

inflammatory than necrosis, will remove critical immune effectors if it occurs before the AM have performed their key functions in host defense, as demonstrated for *P. aeruginosa* (Zaborina et al. 2000). In the case of those infections for which apoptosis-associated killing by AM contributes to late phase bacteria killing premature induction of apoptosis by the pathogen may prevent this strategy. Nevertheless the role of most of these microbial factors in vivo is usually not well established. In addition to its role as a factor recognized by pattern recognition receptors (Malley et al. 2003; Witzenrath et al. 2011) and involved in the initiation of AM apoptosis-associated microbial killing (Bewley et al. 2011a), the pneumococcal toxin pneumolysin may cause direct cytotoxicity in the lung causing AM necrosis (Maus et al. 2004). Thus it remains plausible that microbial factors can deplete AM by induction of necrosis, apoptosis, or other death mechanisms and thereby directly subvert AMs roles in host defense.

1.8 Conclusions

AM are specialized tissue macrophages adapted to a unique environment and a specific homeostatic role. The ability of AM to function effectively as the sentinels of innate immunity in the lower respiratory tract requires them to function effectively as the resident phagocytes. Their capacity to ingest and clear microorganisms is, however, finite and they also play key roles coordinating and downregulating the inflammatory response. The capacity of AM to function efficiently requires highly regulated mechanisms to reverse the intrinsic resistance to the production of pro-inflammatory responses, when these are required by bacterial challenge, but also necessitates that pro-inflammatory responses are matched tightly to the minimum amount required to clear the pathogen. Ensuring this occurs efficiently in the face of varied microbial adaptations that subvert innate immunity is a challenge but the success of this component of innate host defense is illustrated by the relative rarity of pneumonia in comparison with the frequency with which the lower airway is exposed to bacteria.

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Chapter 2
Lung Dendritic Cells and Pulmonary Defence Mechanisms to Bacteria

Bart N. Lambrecht, Katrijn Neyt, Corine H. GeurtsvanKessel, and Hamida Hammad

2.1 General Function of Dendritic Cells in the Lungs

Dendritic cells (DCs) are potent antigen-presenting cells (APCs) that have emerged as key regulators of adaptive immunity (Lambrecht and Hammad 2009). The general function of lung DCs is to recognize and pick up foreign antigens at the outskirts of the body, and subsequently migrate with their cargo to the draining mediastinal lymph nodes where antigen is processed into immunogenic peptides and displayed onto MHCI and MHCII molecules for presentation to naïve T cells. In fact these cells should be seen as specialized cells of the mononuclear phagocyte system that have evolved from the cells of the innate immune system to control adaptive immunity that came later in evolution (Banchereau and Steinman 1998). Dendritic cells express all the pattern recognitions receptors shared with phagocytes of the innate immune system, yet at the same time also have the machinery to talk to T cells and B cells and relay information about the type of antigen to these cells, so that a tailormade adaptive response is induced and long-term memory is initiated.

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As these cells respond to many noxious stimuli from both the outside world (pathogen associated molecular patterns or PAMPs) and from within (danger associated molecular patterns or DAMPS) and at the same time closely communicate with lung structural cells like alveolar epithelial cells (AECs), endothelial cells and fibroblasts, it has been proposed that they could be crucial players in many lung diseases, particularly where T cell responses are involved in initiation of maintenance of the disease (van Rijt et al. 2005). Only very recently, there have been the first case reports of patients that display defects in the DC system. These DC-deficient patients are at risk of severe viral skin infections and pulmonary infections with atypical mycobacteria (Vinh et al. 2010 #13365; Bigley et al. 2011 #13358). Our own experiments employing DC-deficient mice have elucidated a crucial role for these cells in the induction of protective immunity to influenza virus, via induction of both CD4 and CD8 T cell responses (GeurtsvanKessel et al. 2008). Similar conclusions have been reached in models of tuberculosis and bacterial lung infections with Staphylococci and *Bordetella pertussis* (Dunne et al. 2009; Jiao et al. 2002; Martin et al. 2011). Conversely, DCs are also heavily involved in maintaining immunopathology in which T cells play a predominant role, the best example being the mucosal inflammation seen in asthma and COPD. An emerging concept, that we cover in this chapter, is that there are several subsets of DCs in the lungs of mice, rats and humans that share many functional features among species. The most simple discrimination is based on a division of two subsets of conventional $CD11c^{hi}$ DCs (separated into a CD11b+ subset and a CD11b− subset, the latter also expressing CD103 and langerin), a $CD11c^{dim}$ plasmacytoid DC subset, and a fourth class of inflammatory DCs that derive from monocytes under conditions of inflammation and also express CD11b as well as some inflammatory monocyte markers like Ly6C, FcεRI and CD64 (see Fig. 2.1 for an overview of mouse DC subsets).

2.2 Brief Overview of Pulmonary Innate Defence Mechanisms Needed to Understand DC Biology

2.2.1 Mechanical and Physical Pulmonary Defence Mechanisms

The inspired air is contaminated with toxic gases, particulates and microbes. The first line of defence of the lung is made up of the complex physical shape of the conducting upper and lower airways causing a highly turbulent airflow that facilitates the impaction, sedimentation and deposition of particulate matter and microorganisms on the mucosa, followed by the removal of these deposited particles by the mucociliary blanket and/or the physical expulsion from the respiratory tract by sneezing, coughing or swallowing (Barber et al. 2003). The action of the mucociliary blanket is a dynamic and complexly regulated escalator for bringing inhaled particles to the throat so that they can be swallowed. The conducting airways are lined with ciliated epithelium and the structure and function of the cilia in propulsing mucus have been extensively studied (Cowan et al. 2001; de Iongh and Rutland 1995; Santamaria

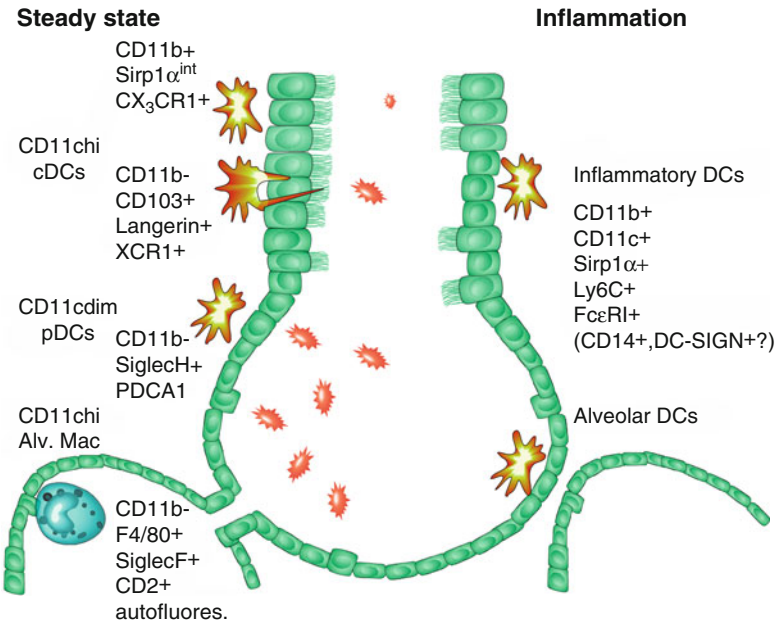

Fig. 2.1 In the left of the diagram the subsets of DCs found in steady state are depicted. These include conventional (c)DCs and plasmacytoid (p)DCs. Conventional DCs are still subdivided in two large families, one being CD11b positive, the other negative for CD11b. Under conditions of inflammation there is immediate recruitment of monocytes that can also give rise to inflammatory type DCs. These are also CD11b positive, yet still express discrete set of markers to discriminate them from CD11b+ cDCs

et al. 2008). The correct movement of cilia and function of the mucociliary escalator also depends on the low viscosity of the periciliary fluid layer, physically a hydrated sol layer, allowing sufficient separation between the apical side of the epithelium and the viscous mucous blanket covering the cilia (Matsui et al. 1998a, b). Any discussion on the biology of dendritic cells, such as their potential to take up antigen, needs to be seen in the light of this complex mucociliary escalator. Dual photon live imaging studies on oxygenated tracheal explants have shown that DCs can extend long dendrites into the lumen of the trachea, and these movements seem to follow the direction of the mucociliary blanket (Hammad et al. 2009).

2.2.2 Humoral Innate Immune Mechanisms in the Lung

Innate immune defences are evolutionary conserved pathways of defence that kill microbes in a generic pathway, often relying on the recognition and antagonism of common motifs in microbial proteins or lectins, the so-called pathogen-associated molecular patterns that are so crucial for the function of the microbe that their

antagonism leads to loss of pathogenicity. Just like acquired or adaptive immunity, innate immunity consists of a humoral and a cellular part.

Humoral innate defence mechanisms are elaborate in the lung and consist of lactoferrin, lyzozyme, defensins, complement, cathelicidins and collectins (Bals and Hiemstra 2004). These molecules can be produced by airway structural cells or by recruited innate immune cells like neutrophils and macrophages (see below). Surfactant protein A and D are collectins that opsonize bacteria and viruses like influenza. As humoral innate immunity developed early in evolution, many of its component mediators like defensins, surfactant proteins and complement have the potential to influence the function of dendritic cells in the airways (Awasthi et al. 2011; Brinker et al. 2001; Castellano et al. 2004; Ryan et al. 2011; Yang et al. 1999).

2.2.3 Cellular Innate Immune Mechanisms in the Lung

The cellular arm of innate immunity in the lung is primarily made up of alveolar macrophages and recruited neutrophils (Fig. 2.1). Alveolar macrophages serve an important function in the phagocytosis, killing and/or neutralization of inhaled particulate antigens. Resident alveolar macrophages continuously encounter inhaled substances due to their exposed position in the alveolar lumen. These cells are packed with enzymes, metabolic products and cytokines that are vital to defence of the alveolar space but can potentially damage the alveolo-capillary membrane. To avoid collateral damage to type I and type II AECs in response to harmless antigens, they are kept in a quiescent state, producing little inflammatory cytokines (Holt 1978). Alveolar macrophages also actively suppress the function of interstitial lung DCs that are situated in the alveolar septa (Holt et al. 1988). It has been estimated previously that the pool of alveolar macrophages can handle up to 10^9 intratracheally injected bacteria before there is spillover of bacteria to DCs and before adaptive immunity is induced (MacLean et al. 1996). Elegant studies have demonstrated that in vivo elimination of alveolar macrophages using clodronate-filled liposomes lead not only to overt inflammatory reactions to otherwise harmless particulate and soluble antigens (Thepen et al. 1989), but also to an increased sensitivity to bacterial, fungal and viral infection. In their exposed position, alveolar macrophages serve the first line of defence against inhaled pathogens not only by directly acting as the main phagocytes, but also as an important producer of pro-inflammatory chemokines, cytokines, lipid mediators bioactive mediators that recruit other cell types to the lung (see Fig. 2.2).

In contrast to alveolar macrophages that reside in the lung and serve an immediate line of innate defence against inhaled pathogens, neutrophils are recruited within minutes after inoculation of microbes into the lung. The main function of neutrophils is phagocytosis and killing of microbes, particularly fungi like *Aspergillus* sp. and *Pneumocystis jeroveci*. They can also kill microorganisms through release of alfa-defensins and lyzozyme. Neutrophil killing function depends on oxidative

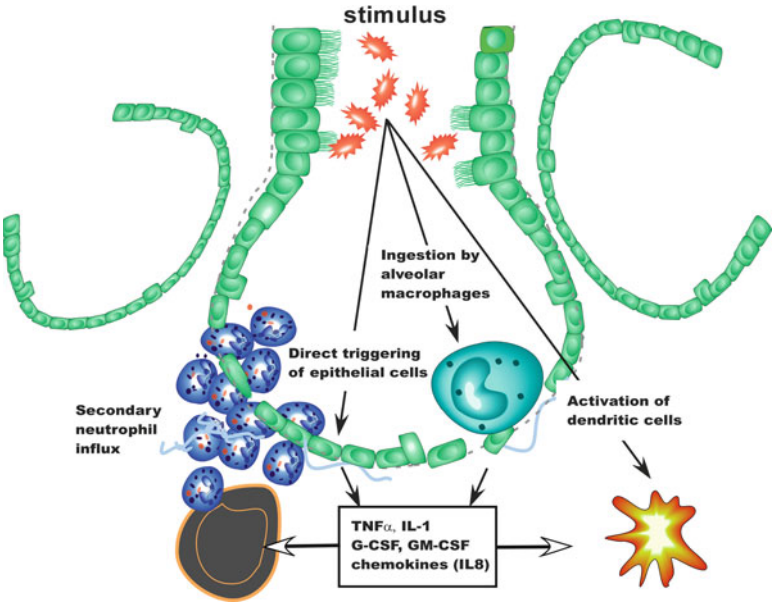

Fig. 2.2 When a pathogen is inhaled, it will directly trigger the lung epithelial cells to produce chemokines and cytokines that attract and activate neutrophils, monocytes and DCs. Additionally, pathogens will also trigger a response from alveolar macrophages that first phagocytose the pathogen, but also induce an innate immune response, to further recruit neutrophils and monocytes. The entire inflammatory milieu with its cytokines, and endogenous danger signals will act to activate the DC network

enzymes like those of the NADPH oxidase system and myeloperoxidase. Once recruited, neutrophils can also further enhance more neutrophil recruitment through the production of cytokines (IL-1, TNFα, IL-6) as well as through release of calcium-binding proteins of the S100 family (S100A8, A9 and A12) that act on the receptor for advanced glycation end products (RAGE receptor). Neutrophils also communicate directly with DCs through Mac-1 or CD11b expressed on their surface interacting with DC-SIGN on DCs (Cheung et al. 2010; van Gisbergen et al. 2005b), as well as through release of cytokines, chemokines, neutrophil elastase, S100 family members and possibly through ROS production (Cheung et al. 2010; Tang et al. 2012). In this way, neutrophils might function as danger sensors that communicate the presence of infection to DCs and instruct them to tailor ensuing immune responses to the type of pathogen (van Gisbergen et al. 2005a). As only one example, neutrophils are recruited early after infection of mice with *Mycobacterium tuberculosis*. Depletion of neutrophils using monoclonal antibodies leads to reduced migration of DCs to the mediastinal lymph nodes and delayed antimycobacterial defence mechanisms (Blomgran and Ernst 2011). As with alveolar macrophage depletion, depletion of neutrophils lowers the threshold by which lung DCs seem to capture bacteria and fungi (Park et al. 2010).

2.3 Induction of Innate Immune Responses in the Lung Also Leads to DC Recruitment

The above mechanisms of innate defence act in a coordinated fashion. Although a single aspect of the innate defence system can be triggered directly through recognition of foreign PAMPs, the innate defence mechanisms are often induced simultaneously via triggering of common receptors on both phagocytes (for cellular defences) and epithelial cells (for inducing the production of humoral innate defence mechanisms). The most famous pattern recognition receptors (PRRs) belong to the family of toll-like receptors (TLRs, TLR1–11), NOD-like receptors, RIG-I like receptors and C-type lectin receptors (Kawai and Akira 2010). These receptors recognize particular conserved PAMPs on specific groups of microbes. The archetypical TLR4 is expressed at the cell surface and recognizes the Gram-negative cell wall component LPS, whereas TLR2 recognizes peptidoglycan and TLR5 recognizes bacterial flagellin. The endosomal TLR receptors TLR3 recognize double-stranded RNA, TLR7 and TLR8 single-stranded RNA and TLR9 unmethylated CpG motifs (Kawai and Akira 2010). The exact cellular localization and downstream signalling pathways of these pathways have been studied extensively over the last few years and several clinical primary immunodeficiency syndromes have been brought back to deficiencies in one of the signalling intermediates of these pathways (Ku et al. 2007).

Many studies have shown that administration to the lung of either purified individual TLR ligands or whole (inactivated) bacteria or viruses leads to massive recruitment of DCs to the lungs, through the production of chemokines that can attract DCs (McWilliam et al. 1994; Stumbles et al. 2001). DCs reside in an immature state in the periphery of the lung, where they are located strategically to detect inhaled particulate and soluble antigen. Within the DC population, cDCs and pDCs differ in their TLR expression pattern, but relatively little is known about this in the lung. The expression profile of TLRs on DCs seems however to be organ-specific. A study has compared the expression of TLR4 and TLR9 on lung and spleen cDCs, and found that lung DCs expressed higher levels of TLR4 but only very low levels of TLR9, whereas spleen DCs had the opposite pattern (Chen et al. 2006). Immgen array gene expression data have shown that mouse $CD103^{+}$ DCs mainly express TLR3, whereas $CD11b^{+}CD103^{-}$ DCs mainly express TLR2 and TLR7 (Desch et al. 2011). Both subsets express low levels of TLR4 in steady state.

In human lung, type 1 mDC and BDCA3+ type 2 mDC express mRNA transcripts for TLR1, TLR2, TLR3, TLR4, TLR6 and TLR8. In response to TLR2 and TLR4 ligands mDC type 1 and mDC type 2 release proinflammatory cytokines (TNF-α, IL-1β, IL-6 and IL-8), whereas only type 1 mDCs produce proinflammatory cytokines in response to TLR3 triggering. Human lung pDC express TLR7 and TLR9 and release proinflammatory cytokines and type I interferon in response to imiquimod and CpG oligonucleotides (Demedts et al. 2006).

2.4 Indirect Activation of the DC Network by Epithelial Cells

Although direct recognition of foreign PAMPs by PRRs is the most likely explanation how DCs respond to foreign antigen, it is now clear that recognition of PAMPs by the closeby epithelial cells is at least as important in activating the lung DC network. This conclusion was reached by studying the in vivo response of lung DCs to the TLR4 agonist endotoxin (LPS), in bone marrow chimeric mice that lacked TLR4 exclusively on either radiosensitive hematopoietic cells or radioresistant epithelial cells. In the absence of TLR ligation, lung DCs demonstrated a sessile behaviour. Provision of LPS led to a dramatic increase in motility and antigen sampling behavior that led to crawling of DCs in between basal epithelial cells. Strikingly, instruction for this pattern of motility required TLR4 triggering of epithelial cells and not on DCs directly (Hammad et al. 2009). Lung epithelial cells also produce the essential chemokines that chemoattract immature cDCs and inflammatory monocytes to the site of antigen exposure. For lung DC recruitment to inflammatory stimuli, several chemokines and cytokines have been implicated. The chemokine CCL20 and epithelial β-defensin are ligands for CCR6 expressed by immature (lung) DCs, and bronchial epithelial cells produce these factors in response to TLR ligation, C-type lectin triggering, allergen inhalation, virus infection, as well as exposure to environmental pollutants (Hammad et al. 2009; Kallal et al. 2010; Nathan et al. 2009; Reibman et al. 2003). However, a careful study of the contribution of the CCR6 pathway to steady-state cDC lung biology has not been performed. It seems that under conditions of inflammation, other chemokine receptor interactions come into play. When sensitized mice receive an aerosol challenge with the relevant protein antigen, CCR2, and not CCR5 or CCR6 seems to be the relevant chemokine receptor for causing the accumulation of lung DCs, although this might be predominantly through its capacity to release monocytic precursors from the bone marrow (Robays et al. 2007). When a challenge with sheep red blood cells is given in the lungs, CCR2 directs DC precursors from the blood to the lung interstitium, whereas CCR6 directs their transit from the interstitium to the airway (Osterholzer et al. 2005). In rats, a CCR1/CCR5 antagonist however blocks bacteria-induced DC recruitment to the lung (Stumbles et al. 2001). Freshly isolated respiratory mucosal DC respond to different CC chemokines, (MCP-1, -4, RANTES and eotaxin), complement cleavage products and *N*-formyl-peptides (McWilliam et al. 1996).

The precise role of the CX3CR1 receptor in lung DC recruitment following inflammation is currently unknown. Exposure of mouse lungs with cigarette smoke leads to CX3CL1 upregulation in the lungs. As the receptor CX3CR1 is present on many inflammatory cells like monocytes and CD11b+ cDCs, it is likely that this pathway could also contribute to recruitment of inflammatory type DCs to the lung (Jakubzick et al. 2008; McComb et al. 2008). Another trigger for recruitment of DCs to the lungs under inflammatory conditions is the production by bronchial epithelial cells of a homodimer of the p40 subunit of IL-12 (Walter et al. 2001). Viral as well as mycobacterial infection of the lung as well as allergic inflammation induces this p80 form of IL-12, and it was shown recently that lung DCs infected

with *M. tuberculosis* use an alternatively spliced variant of the *IL-12rb1* gene to generate a shorter IL-12Rβ1 isoform (IL-12Rβ1ΔTM) that promotes the responsiveness of the classical IL-12Rβ1 to IL-12 p80 (Robinson et al. 2010). Whether different DC subsets would employ this mechanism of migration to the lung differentially and how this receptor is regulated is currently unknown.

Epithelial cells not only make chemokines that attract immature monocytes or DCs, they also produce critical maturation cytokines like IL-1, GM-CSF and TSLP that can activate the recruited monocytes to differentiate in DCs and induce their maturation into fully competent APCs capable of interacting with naïve T cells (for more detailed discussion, see Lambrecht and Hammad 2012).

2.5 Induction of Adaptive Cellular Antimicrobial Immunity by DCs

Dendritic cells are potent APCs that have emerged as key regulators of adaptive immunity (Lambrecht and Hammad 2009). The general function of lung DCs is to recognize and pick up foreign antigens at the outskirts of the body, and subsequently migrate with their cargo to the draining mediastinal lymph nodes where antigen is processed into immunogenic peptides and displayed onto MHCI and MHCII molecules for presentation to naïve T cells. Once DCs transport their antigenic cargo to the draining lymph nodes, they induce the proliferation and differentiation of naïve T cells into particular types of T cell responses (see Fig. 2.3). Discrete types of T helper cells provide crucial help for different parts of the innate and adaptive immune response (Zhu et al. 2010). Th1 cells make IFNγ and mainly provide help to monocytic cells, including macrophages and dendritic cells, and thus enforce killing of intracellular pathogens, and at the same time enforce opsonization of these through provision of B cell help. Conversely, Th2 cells make IL-4, IL-5 and IL-13 to provide help to eosinophils, mast cells and basophils to eliminate complex helminths, and at the same time induce IgG1 and IgE from B cells to arm the basophils and mast cells with effector potential. For a long time since the original description of the Th1/Th2 concept, it has been unclear which subtype of T cell help was important for inducing neutrophilic responses and protection from extracellular pathogens like fungi. This gap has been breached recently by the discovery of the cytokines IL-17 and IL-22 that are produced by Th17 cells that induce neutrophilic inflammation, production of defensins by epithelial cells and are important for clearance of fungi and extracellular bacteria (Ouyang et al. 2008).

The precise signals that induce different types of Th lineage commitment of naïve T cells have been intensely studied (Zhu et al. 2010). APCs can provide different levels and quality of signal 1 (peptide-MHC), signal 2 (costimulatory molecules) and signal 3 (instructive cytokines) to naïve T lymphocytes upon antigen encounter and triggering of their PPRs (Banchereau and Steinman 1998) (see Fig. 2.4). When stimulated through the unique TCR, naive $CD4^+$ T cells differentiate into Th1 cells in the presence of high amounts of IL-12. IL-12 instructs Th1 development via

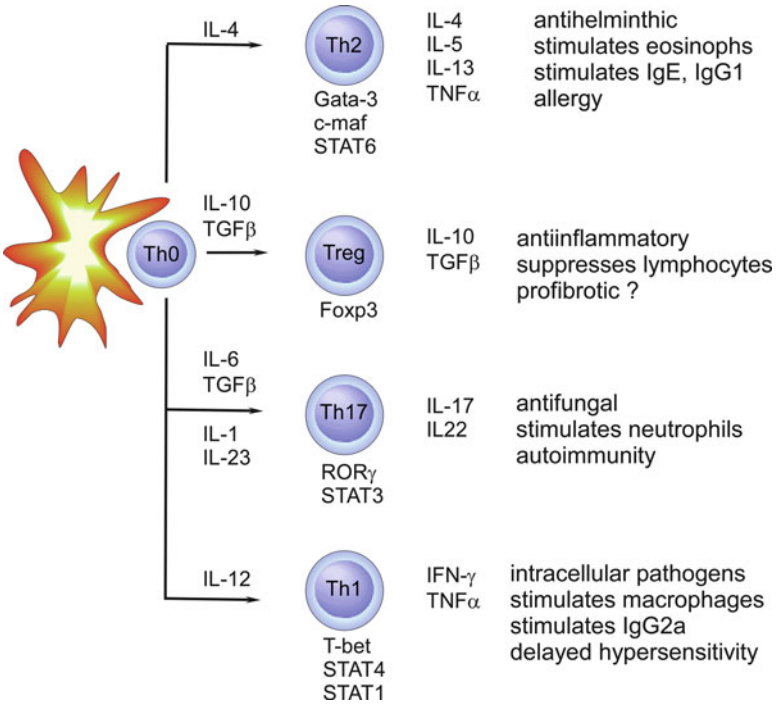

Fig. 2.3 When faced with a pathogen, DCs can induce many types of T helper responses based on their potential to produce polarizing cytokines. The functions of the ensuing T cells and the cytokines mediating effector functions are depicted

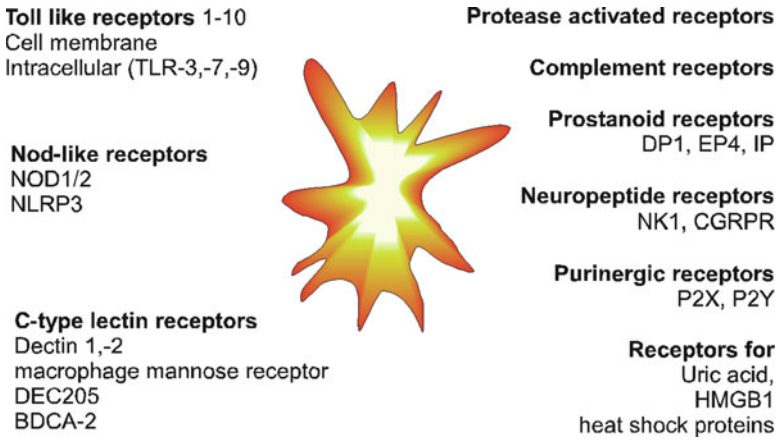

Fig. 2.4 Expression of 'danger' receptors by dendritic cells (DCs). DCs express the highly conserved receptors of the innate immune system also expressed by macrophages such as Toll-like receptors (TLRs), Nucleotide Oligomerization Domain (NOD-like) receptors and C-type selectin receptors (Left side of figure). These receptors react to foreign pathogen-associated molecular patterns (PAMPs). In addition, DCs express numerous receptors for inflammatory mediators and necrotic debris, the so-called damage-associated molecular patterns (DAMPs) (right side of figure).

activation of STAT4 and the lineage instructing transcription factor T-bet. IL-17-producing cells are induced when exposed to a cocktail of cytokines including TGFβ, IL-6 and IL-1α/β, while IL-23 further enhances the proliferation of these cells. The Th17 lineage-specific transcription factor RORγt enforces Th17 characteristics in naïve T cells, and is induced by the cocktail of cytokines instructive to their development. The mechanisms leading to Th2 cell differentiation in vivo are still poorly understood, but in most instances require a source of IL-4 to activate the transcription factors STAT6 and GATA-3, and a source of IL-2, IL-7 or TSLP to activate the transcription factor STAT-5 (Kopf et al. 1993; Le Gros et al. 1990; Paul and Zhu 2010; Seder et al. 1992; Zheng and Flavell 1997). Despite the overwhelming evidence that IL-4 is necessary for most Th2 responses, DCs were however never found to produce IL-4 and it was therefore long assumed that Th2 responses would occur by default, in the absence of strong Th1- or Th17-instructive cytokines in the immunological DC-T cell synapse, or when the strength of the MHCII-TCR interaction, or the degree of costimulation offered to naïve T cells is weak (Constant et al. 1995; Jankovic et al. 2004; Lambrecht et al. 2000; Stumbles et al. 1998). In this model, naïve CD4 T cells were the source of instructive IL-4. In an alternative view, IL-4 is secreted by an accessory innate immune cell type, like NKT cells, eosinophils, mast cells or basophils that provide IL-4 in trans to activate the Th2-differentiation program (Ben-Sasson et al. 1990). In the lung allergic response to house dust mite allergen, we have recently found that basophils help DCs to induce Th2 immunity by providing an important, but not essential source of IL-4 (Hammad et al. 2010).

Lung DCs are also essential in instructing the selection and expansion of CD8 cytotoxic T cells that recognize virus-infected cells, cells infected with intracellular bacteria and tumorally transformed cells via presentation of endogenous cellular antigen on the MHCI complex (GeurtsvanKessel et al. 2008). An important conceptual point is that DCs do not have to be infected themselves to perform this task but can phagocytose virally infected or transformed cells and use the process of cross-presentation to present the exogenous antigen into their MHC-I loading machinery. In the lungs, antigen cross-presentation seems to be a unique feature of the subset of CD103+ DCs (Desch et al. 2011; GeurtsvanKessel et al. 2008). Once activated by DCs and CD4 T cell help, cytotoxic T cells can lyse and kill infected cells in a process requiring granzyme and/or perforin, or kill target cells in an FasL and/or TRAIL-dependent manner, causing apoptotic cell death in targets (Hufford et al. 2011).

The potential of DCs to boost very effective antibacterial immunity might be applied clinically in the future. One such example could be in the eradication of hard-to-eliminate pathogens from colonized airways, such as seen in *Pseudomonas* infection in CF or bronchiectasis patients. In a recent study, it was shown that DCs pulsed with the *Pseudomonas aeruginosa* major constitutive outer membrane porin protein F (OprF) protected mice against *P. aeruginosa* infection and inflammation. Upon adoptive transfer in vivo, porin-pulsed dendritic cells (DCs) induced Th1-mediated resistance to infection and associated inflammatory pathology caused by either the PAO1 strain or a clinically isolated mucoid strain, highlighting the pivotal contribution of DCs to vaccine-induced protection (Peluso et al. 2010). Expansion of local DC numbers by cytokines that stimulate their growth and or function might also be

a feasible strategy. In mice, overexpression of GM-CSF from the BCG vaccine strain led to enhanced protection from mycobacterial infection by accelerated priming of antigen-specific CD4+ T cells in the mediastinal lymph nodes and increased migration of activated CD4+ T cells into the lung (Nambiar et al. 2010).

2.6 Dendritic Cells and Humoral Immune Mechanisms in the Lung

Humoral immunity plays a predominant role in protecting from severe infections with encapsulated bacterial strains. Antibodies are well known for their neutralizing effects on secondary infections and this is the principle of most vaccinations against childhood infections. During a primary infection however, antibodies, some of which have broad spectrum specificity (the so-called natural antibodies) also have the capacity to activate complement and opsonize bacterial cell walls and capsules, hence facilitating clearance of the bugs. Antibodies of the IgA and IgG class are actively secreted into the airway lumen via the action of the polymeric Ig receptor. Airway luminal IgA is an important defence against viral entry. More and more evidence suggests that DCs also control crucial aspects of humoral immunity, by directly interacting with B cells or by providing crucial T cell help of immunoglobulin class switching through stimulation of T follicular helper cells (T_{FH}). Lung DCs can promote the production of IgA in a process dependent on TGFβ (Naito et al. 2008). Intratracheal injection of the mucosal adjuvant cholera toxin B subunit also induces DC-dependent IgA class switching (Smits et al. 2009). In contrast to gut epithelial DCs, a recent study on lung DCs identified both RALDH1 and RALDH2 enzymes that promote retinoic acid production, involved in IgA class switching. Both subsets of lung cDCs had equal levels of RALDH activity (Guilliams et al. 2010).

Elegant studies by Snapper et al. have demonstrated that dendritic cells pulsed with pneumococcal antigens can induce antibodies directed against pneumococcal polysaccharides, and lead to neutralizing immunity upon adoptive transfer (Colino et al. 2002, 2009). Along the same lines, but potentially more clinically applicable, adenoviral overexpression of the DC growth factor Flt3L was able to boost antipneumococcal antibody responses, leading to elimination of nasal carriage rates in mice (Kataoka et al. 2011).

2.7 Organized Lymphoid Structures and Bronchiectasis

The organized accumulation of lymphocytes in lymphoid organs serves to optimize both homeostatic immune surveillance, as well as chronic responses to pathogenic stimuli (Cupedo and Mebius 2005). During embryonic development, circulating hemopoietic cells gather at predestined sites throughout the body, where they are subsequently arranged in T and B cell-specific areas, thus leading to the formation

of secondary lymphoid organs (SLOs) like lymph nodes and spleen. In contrast, the body has a limited second set of selected sites that support neo-formation of organized lymphoid aggregates in adult life. However, these are only revealed at times of local, chronic inflammation when the so-called tertiary lymphoid organs (TLOs) appear. Just like in lymph nodes and spleen, areas of TLOs are characterized by the formation of specialized high endothelial venules and the organized production of chemokines leads to cellular organization of T cells and B cells in discrete area. In humans, TLOs have been observed in the joint and lung of rheumatoid arthritis (Rangel-Moreno et al. 2006), around the airways of COPD patients (Hogg et al. 2004) and in the thyroid (Marinkovic et al. 2006). Certain infectious diseases are also accompanied by formation of TLO. Influenza virus infection of the respiratory tract leads to the formation of inducible bronchus-associated lymphoid tissue (iBALT) that supports T and B cell proliferation and productive immunoglobulin class switching in germinal centres (GCs) (GeurtsvanKessel et al. 2009; Moyron-Quiroz et al. 2004). Tertiary lymphoid follicles or iBALT is very frequently seen in tubular bronchiectasis, and the close association with bronchi might explain the obstruction of small bronchioles and airway obstruction often seen. Formation of TLOs could be the result of chronic colonization of bronchiectatic airways by microbes, and indeed it has been proposed that latent adenoviral infection is a cause of follicular bronchiectasis (Bateman et al. 1995 #13360). However, in one school of thought, TLO formation can also be seen as a source of self-specific autoantibodies and a reflection of an underlying auto-immune component to disease. In TLO associated with RA bronchiectasis, one has indeed seen the production of pathogenic antibodies to citrullinated proteins (Rangel-Moreno et al. 2006). Whatever the mechanism of induction or the pathogenic role of TLO structures, it has been shown that DCs are necessary for their maintenance in response to influenza virus infection, vaccinia virus infection and chronic LPS administration. The reasons for this is that DCs express lymphotoxin $\alpha 1\beta 2$ that stimulates local stromal cells to produce chemokines that keep T and B cell together in a logical context as also seen in SLOs like spleen and lymph nodes.

2.8 Antiinflammatory Pathways

With its large surface area, the lung is a portal of entry for many pathogens as inhaled air is contaminated with infectious agents, toxic gases and (fine) particulate matter. At the same time, inhaled microbes and toxic substances can gain easy access to the bloodstream across the delicate alveolar-capillary membrane. Innate and adaptive immune defence of this vulnerable barrier is not easy and needs to be tightly controlled as too much edema, inflammation and cellular recruitment will lead to thickening of the alveolar wall and will jeopardize the diffusion of oxygen, vital to life. Considering the large surface area of the respiratory epithelium and the volume of air inspired on a daily basis, it is remarkable that there is so little inflammation under normal conditions, suggesting the presence of regulatory mechanisms that act to protect the gas-exchange mechanism. Following even severe

bacterial or viral infection, a return to homeostasis is the usual outcome. Understanding the conditions by which lung immune homeostasis is regulated might be crucial to advance our insight into the pathogenesis of inflammatory lung diseases caused by chronic bacterial colonization (as seen in bronchiectasis) or chronic infections (as seen in TB). One type of cell that has received particular attention in suppressing immune responses in the lung is the alveolar macrophage. Alveolar macrophages adhere closely to AECs at the alveolar wall and are separated by only 0.2–0.5 μm from interstitial DCs. In macrophage-depleted mice, the DCs have a clearly enhanced antigen-presenting function (Holt et al. 1993). When mixed with DCs in vitro, alveolar macrophages suppress T cell activation through release of NO (mainly in rodents), prostaglandins, IL-10 and TGFβ. Alveolar macrophages also express CD200R, an inhibitory receptor that regulates the strength of innate immunity to inhaled pathogens. Another cell type that has received a lot of attention is the regulatory T cell or Treg. Natural Tregs express high levels of CD25 and express the lineage-specific transcription factor Foxp3 (Hori et al. 2003). These cells are generated in the thymus and have a natural reactivity for self-antigens as well as some foreign antigens, and mainly suppress autoimmunity (Watanabe et al. 2005). Induced Tregs are generated when DCs encounter self-antigen in the periphery or upon chronic immune stimulation. It is assumed that these induced Tregs serve to dampen overt immune activation to stimuli that cannot be fully eliminated, a typical example being chronic helminth infections or mycobacterial infections (Grainger et al. 2010). It is also possible that failure of Treg function at a certain stage of the disease contributes to ongoing inflammation that might ultimately progress to fibrosis. In this regard it is a striking observation that Tregs also make TGFβ as part of their suppressive program. TGFβ might be at the crossroads of immunoregulation and fibrosis initiation. Dendritic cells from mycobacteria-infected mice seem to induce large numbers of Treg cells that have a broad anti-inflammatory function, even to inert allergens (Leepiyasakulchai et al. 2012).

Finally, immune regulation might also stem from changes in stromal cells of the airways, such as epithelial cells. Airway epithelial cells play a predominant role in deciding whether or not an acute or chronic stimulus like endotoxin is recognized or not (Hammad et al. 2009). Epithelial cells express many PRRs and the sensitivity of these can be regulated through negative regulators of signalling. Finally, some epithelial-derived cytokines like IL-37 have an intrinsically anti-inflammatory effect on innate immunity in the lung (Nold et al. 2010). It is currently unknown if defects in these counterregulatory mechanisms are involved in maintenance of inflammation in patients with lung infections, and whether these pathways mainly work by affecting the function of lung DCs.

2.9 Conclusion

There has been great progress in our knowledge of innate and adaptive immune responses in the lung, it is increasingly clear that DCs control many aspects of the innate and adaptive immune response to bacterial lung infection.

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Chapter 3
CD4 T-Cell Immunity in the Lung

Jay K. Kolls

3.1 Introduction

CD4+ T helper cells are critical cells that mediate adaptive immune responses in the lung (Fig. 3.1). These cells are initially primed by antigen-presenting cells (APCs) that present peptide antigen in the context of class II major histocompatibility complexes engaged with the T-cell receptor (termed signal 1). Both APCs and T-cells express co-stimulatory molecules and receptors (termed signal 2) and this second signal is critical for both generating antigen-specific effector T-cells as well as memory cells. Antigen presentation without signal 2 can lead to T-cell anergy. CD4+ T-cell differentiation into specific effector lineages occurs under the influence of lineage-specific cytokines (signal 3) that can control both chromatin remodeling as well as the induction of lineage specific-transcription factors in CD4+ T-cells.

The profound role that CD4+ T-cells play in pulmonary host defense has clearly been demonstrated by the types of pulmonary infections that complicate congenital deficiencies in these cells or by their depletion in AIDS. Pulmonary infection with Pneumocystis was one of the first AIDS defining illnesses followed by bacteremic pneumococcal pneumonia (Centers for Disease Control (CDC) 1981; Gottlieb et al. 1981; Masur et al. 1981).

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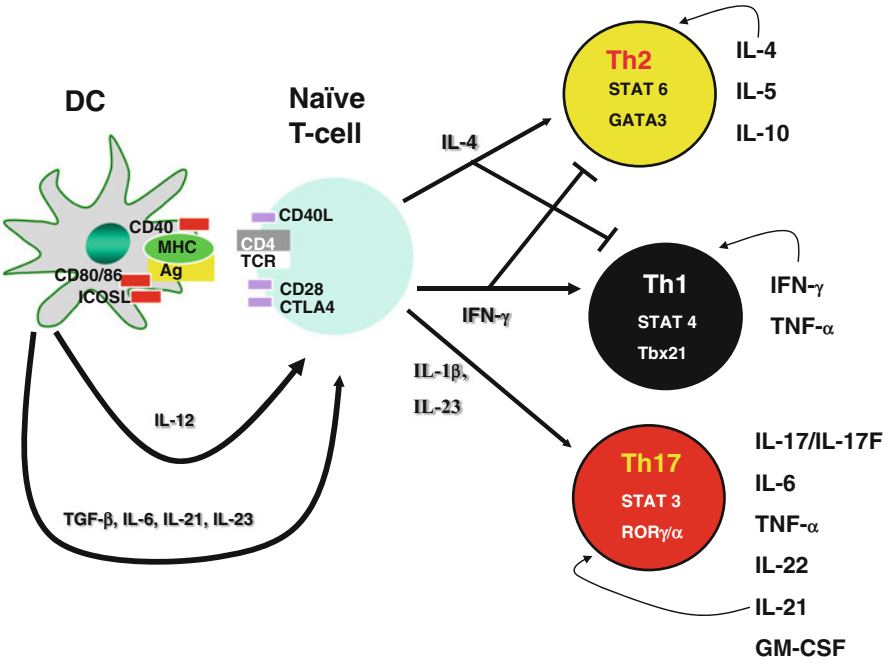

Fig. 3.1 T-helper cell differentiation. Dendritic cells (DCs) present antigen in the context of class II MHC to naïve CD4+ T cells. Signal 1 consists of activation through the T-cell receptor followed by signal 2 which is mediated through co-stimulatory molecules such as ICOSL, CD28, and cytotoxic T lymphocyte-associated protein (CTLA-4). T-cell differentiation occurs under specific cytokine signals. IFNγ can induce T-bet via STAT1 signaling which in turn can regulate IL-12bR2 expression making Th1 cells responsive to IL-12p70. IL-4 induces Gata3 which augments IL-4 producing which supports the differentiation of Th2 cells. Th2 cells produce IL-4, IL-5, and IL-13 as their effector cytokines. TGFβ and IL-6 can induce RORγT expression as well as activation of STAT3 that induces IL-23 receptor expression and the combination of these cytokines supports the differentiation and proliferation of Th17 cells. These cells produce the cytokines IL-17/IL-17F, IL-22, and IL-21. IL-21 can signal in an autocrine fashion to reinforce the differentiation of the Th17 lineage

3.2 CD4+ T Helper Subsets

3.2.1 Th1

Th1 cells were first described by Mossman et al. (1986) by their ability to express interferon-gamma (IFN-γ). Th1 cell development requires the transcription factors T-bet (Szabo et al. 2000) and these cells can differentiate from naïve T-cell precursors in the presence of Th1-polarizing cytokines such as IL-12p70 which is a heterodimeric cytokine consisting of IL-12p35 and IL-12p40 (Wolf et al. 1991). Th1 responses can also occur independent of IL-12 and type I interferons have been

shown to facilitate Th1 differentiation in certain situations (Longhi et al. 2009). IFN-γ once produced by differentiating Th1 cells can signal in an autocrine–paracrine fashion to further amplify Th1 differentiation and lineage commitment. IFN-γ signals via a receptor complex consisting of two IFN-γR1 and two IFN-γR2 chains which are widely expressed on myeloid-derived cells such as macrophages and dendritic cells as well as structural cells in the lung such as epithelial cells and fibroblasts (Gough et al. 2008). These receptors can activate Janus-associated kinases 1 and 2 which phosphorylate STAT1 which allows STAT1 homodimerization and translocation to the nucleus, followed by binding to gamma-activated sequences (GAS) that regulate gene transcription (Gough et al. 2008). IFN-γ regulates cellular immunity to many intracellular infections including *Mycobacterium tuberculosis*, *Listeria monocytogenes*, and *Salmonella typhimurium*. Patients with IL-12p40 mutations can develop BCG or S. typhimurium infection but some patients can be managed with antibiotics and theoretically they can respond to interferon (Picard et al. 2002). For example, patients with IFN-γ receptor mutations can develop disseminated infection with bacillus Calmette-Guerin (BCG) that is resistant to antibiotics and IFN-γ therapy (Dorman et al. 2004; Sologuren et al. 2011). Patients with IL-12p40 mutations can develop BCG or *S. typhimurium* infection but some patients can be managed with antibiotics and theoretically they can respond to interferon (Picard et al. 2002). Thus, there is strong evidence that this pathway is essential for human control of these intracellular pathogens.

3.2.2 Th2

Th2 cells differentiation requires the transcription factor GATA3 and STAT5 (Paul 2010), although initial differentiation and activation of these transcription factors can occur independently of IL-4. GATA3 binds to the *Il4* locus and IL-4 and signaling via STAT6 is critical for further TH2 proliferation and lineage commitment (Paul 2010). Th2 cells produce interleukin (IL)-4, IL-5, and IL-13 and mediate immunity against infections with helminths (Fig. 3.2). These cells also facilitate B-cell differentiation and antibody responses to T-cell dependent protein antigens (Willart and Hammad 2011). Deletion of *Gata3* in mice results in embryonic lethality but conditional deletion in T-cell confirms its essential role in Th2 differentiation and the expulsion of helminths from the gastrointestinal tract (Zhu et al. 2004). IL-5 is the principal growth factor that regulates eosinophilopoiesis and IL-5 deleted mice show marked reduction of peripheral and bone marrow eosinophils (Kopf et al. 1996; Fallon et al. 2002). Transgenic over-expression of IL-5 results in eosinophilia (Dent et al. 1990). IL-13 signaling via STAT6 in airway smooth muscle and in airway epithelium leads to airways hyperresponsiveness to methacholine (Wills-Karp et al. 1998; Grunig et al. 1998). Moreover IL-13 is a major factor in mucous production and goblet cell differentiation in the airway (Wills-Karp et al. 1998; Grunig et al. 1998). Th2 cell and their effector cytokines have been widely implicated in atopic diseases such as

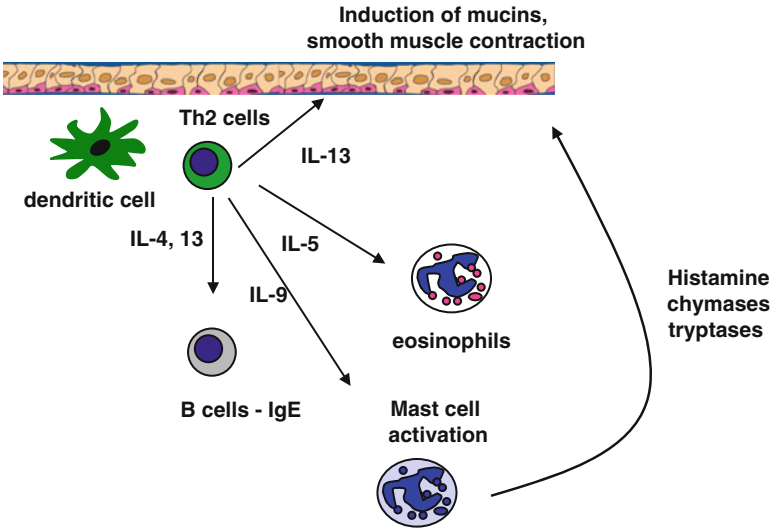

Fig. 3.2 Th2 cells and immunity at the mucosa. Parasitic or helminth infection can induce TSLP and IL-25 in the epithelium which can support early IL-4 production leading to the differentiation of Th2 cells. IL-4 and IL-13 can support the induction of IgE as well as stimulate epithelial cells to increase mucous production. IL-5 induces eosinophilopoiesis. The combination of IgE-mediated degranulation of mast cells, the recruitment of eosinophils, and the changes in gene expression in epithelium can lead to host control of helminth infection

allergic rhinitis, atopic dermatitis, and asthma (Barnes 2008). Furthermore, IL-13 has been implicated in fibrotic processes in the lung in response to drugs such as Bleomycin (Belperio et al. 2002; Liu et al. 2004; Jakubzick et al. 2003). It has been recently recognized that Th2 cell priming can be driven by a number of cytokines produced by lung epithelium including TSLP, IL-25, and IL-33 (Willart and Hammad 2011).

3.2.3 Th17

The initial dichotomy of T-cell subsets however could not explain all of the infections complications of CD4+ T-cell deficiency. For example, CD4+ T-cells are essential for host resistance to *Pneumocystis* pneumonia, however, mice deficient in IL-4 (Garvy et al. 1997a), IFN-γ (Garvy et al. 1997b), STAT4, and/or STAT6 (unpublished observations) all clear *Pneumocystis* suggesting additional CD4+ T effector populations. IL-17 was cloned in 1993 (Rouvier et al. 1993) and the first IL-17 receptor (IL-17RA) was cloned in 1996 (Yao et al. 1995). IL-17 mRNA was largely restricted to CD4 memory cells and early studies

using intracellular cytokine staining showed that T-cell that produced IL-17 were divergent than those that produced IFN-γ but often co-expressed TNF and GM-CSF (Infante-Duarte et al. 2000). Pivotal studies published in 2005 showed that these cells develop independently of STAT4 or STAT6 and the canonical Th transcription factors T-bet or GATA3 demonstrating that Th17 cells are a distinct CD4+ T-cell lineage (Harrington et al. 2005; Park et al. 2005). Subsequently it was demonstrated that Th17 development requires STAT3, and two nuclear hormone receptors RORA and RORC for development from naïve CD4+ T-cells (Yang et al. 2008; Ivanov et al. 2006). It was initially believed that one of the critical instructional cytokines for Th17 differentiation was IL-23 (Aggarwal et al. 2003); however, IL-23 receptor is not expressed on naïve CD4 T-cells. Several groups showed that a critical first step in Th17 differentiation is stimulation with TGF-β and IL-6 which allows induction of IL-23R (Veldhoen et al. 2006; Bettelli et al. 2006; Mangan et al. 2006). Signaling via IL-23 allows terminal differentiation and expansion of Th17 cells (McGeachy et al. 2009). Another critical effector cytokine produced by Th17 cells is IL-22 which is controlled by IL-23 as well as the transcription factor aryl hydrocarbon receptor (Ahr) (Veldhoen et al. 2008). Th17 cells also produce IL-21 (Nurieva et al. 2007; Korn et al. 2007) which can function in an autocrine manner to further expand Th17 differentiation (Fig. 3.1).

3.2.3.1 T-Follicular Helper Cells

T_{FH} cells are a subgroup of CD4+ T-cells that are found in the B-cell follicle region in secondary lymphoid tissues such as bronchial associated lymphoid tissues or lymph nodes in the lung. These cells are critical for T-cell-dependent B-cell activation through the expression of CD40L and IL-21 (Crotty 2011). Their transcriptional program is distinct from other CD4+ T-cell lineages and they develop after stimulation with ICOS. These cells also require the transcription factor Bcl-6 for development as well (Crotty 2011).

3.2.3.2 T-Regulatory Cells

Tregs develop under the transcription factors Foxp3 and STAT5 and are critical for mediating tolerance to inhaled antigen in the lung and preventing or reducing allergic inflammation (Josefowicz et al. 2012). They can suppress the effector activity of many T-helper subsets and can be thymically derived (natural Tregs) or induced in the periphery (iTregs). An exhaustive review of these cells is beyond the scope of this chapter but the reader is referred to an excellent thorough review of these cells if they seek a more in-depth description of these cells (Josefowicz et al. 2012; Ray et al. 2010).

3.3 Other Sources of Th1/Th2/Th17 Effector Cytokines in the Lung

3.3.1 γδ T-Cells

γδ T-cells are resident in lung tissue and produce a variety of effector cytokine including IFN-γ, IL-4, Il-17, and IL-22 (Bonneville et al. 2010). In the context of pulmonary infection with bacteria these cells provide a substantial amount of early IL-17 and are regulated by IL-23 and IL-1β (Lockhart et al. 2006; Shibata et al. 2007a; Sutton et al. 2009; Martin et al. 2009; Chen et al. 2011). As these cells express the γδ T-cell receptor, it is unclear if these cells are responding directly to cytokine stimulation alone or whether they also require endogenous TCR-dependent signals as well in the lung. γδ T-cells have recently been shown to limit pathology in RSV infection (Dodd et al. 2009). These cells have also been shown to produce IL-10 and can play a regulatory role in other pulmonary infections such as *Pneumocystis* infection (Steele et al. 2000).

3.3.2 NKT-Cells

NKT-cells are another source of multiple effector cytokines in the lung including IL-4, IFNγ, and IL-17 (Michel et al. 2007). One population that has extensively studied is a population that expresses an invariant T-cell receptor (iNKT cells) that recognizes a galactolipid Sphingomonas, alpha-galactosylceramide (Brossay et al. 1998; Burdin et al. 1998). These cells have been found to be elevated in the bronchial alveolar lavage fluid of patients with asthma (Akbari et al. 2006; Pettersson et al. 1985). These cells can also produce IFNγ in response to *Streptococcus pneumoniae* pulmonary infection (Nakamatsu et al. 2007). These cells also produce IL-17 in response to *Escherichia coli* LPS (Michel et al. 2007) as well as ozone (Pichavant et al. 2008). NK cells can develop under the control of IL-15 and express antiviral molecules such as IFN-γ as well as cytotoxic molecules (Steel et al. 2012).

3.3.3 Innate Lymphoid Cells

Another cell population that produces effector cytokines in the lung is innate lymphoid cell. These cells are defined by lacking lineage markers, a lack of T-cell receptors but require IL-7 signaling for their development. Thus these cells are present in RAG1 or RAG2$^{-/-}$ mice but are lacking in RAG2, yC double-deleted mice (Halim et al. 2012; Spits and Cupedo 2012). RORγT expressing cells are critical for the formation of secondary lymphoid tissues (via regulation of lymphotoxin expression)

and play critical roles in mucosal immunology in the gastrointestinal tract through the production of IL-17 and IL-22 (Ouyang et al. 2008). Type 2 ILCs produce IL-5 and IL-13 and participate in the clearance of helminths from the GI tract (Neill and McKenzie 2011). These cells appear to be regulated by IL-25 (IL-17E) as well as IL-33, a member of the IL-1 family. Recently it has been demonstrated that a population of ILCs prices IL-13 in response to IL-33 induced by viral infection and these cells mediate in part, viral-induced exacerbation of allergic disease in the lung (Kim et al. 2012). In addition to viruses, these cells can also be activated by protease allergens to drive eosinophilic airways inflammation as well as airways hyperresponsiveness (Halim et al. 2012). In this allergen setting the activation of these cells required IL-33 and TSLP (Halim et al. 2012). Thus these cells recapitulate many aspects of CD4+ T-cell immunity in that there are subsets that express similar effector molecules, yet these cells are activated early and their activation is independent of TCR stimulation.

3.4 Effector Mechanisms of CD4+ T-Cell Effector Cytokines in the Lung

3.4.1 Type 1 Effectors

As mentioned above, receptors for IFNγ are expressed on a variety of lung cells including alveolar macrophages, dendritic cells, fibroblasts, and lung epithelial cells. There are several mechanisms of action by which IFN-γ is thought to be critical for control of lung immunity against intracellular pathogens. The first is through macrophage priming and the induction of intracellular microbicidal activity of macrophages (Murray 1988). IFN-γ priming of macrophages results in significantly augmented TLR signaling (Schroder et al. 2006). IFN-γ also increases microbicidal activity in part, through the induction of inducible nitric oxide synthase which can increase the production of reactive nitrogen intermediates (Xie et al. 1992, 1993) as well as by increasing the production of reactive oxygen species. These activities may explain the therapeutic benefit of IFN-γ in patients with chronic granulomatous disease due to mutations in NADPH oxidase (Naderi et al. 2012; Segal et al. 2011; Fernandez-Boyanapalli et al. 2010). This increase in microbicidal activity has been termed classically activation of macrophages (Gordon 2003).

IFN-γ markedly upregulates class II MHC molecules as well as co-stimulatory molecules such as CD80 and CD86 which can augment antigen presentation to naïve T-cells. Of course, one of the IFN-γ's first observed activities was its ability to suppress viral replication in many target cells including macrophages, fibroblasts, and lung epithelial cells (Hovanessian et al. 1980). This occurs in part though the induction of many anti-viral genes such as MxA (Ronni et al. 1995); however, other respiratory viruses such as SARS coronavirus are controlled by IFNγ via MxA independent mechanisms (Spiegel et al. 2004).

IFN-γ also induces chemokines such as CXCL9, CXCL10, and CXCL10 which are all ligands for CXCR3 which is expressed on Th1 cells and thus, IFN-γ can increase the recruitment of Th1 cells and this is critical for granuloma formation which is essential for control of many intracellular pathogens such as *M. tuberculosis* (Aly et al. 2007; Chakravarty et al. 2007). In fact both systemic and aerosolized IFN-γ have been investigated for the potential adjunctive treatment of TB. A recent meta-analysis showed that IFN-γ was well tolerated and associated with higher sputum sterilization rates (Gao et al. 2011); however, definitive randomized control trials are lacking to make firm conclusions on the efficacy of this cytokine.

3.4.2 Type 2 Effectors

Both IL-4 and IL-13 can activate STAT6 signaling in a variety of lung cells including alveolar macrophages, fibroblasts, airway smooth muscle, and airway epithelium. Activation of the STAT6 pathway leads to alternative macrophage activation characterized by the expression of arginase 1, YM1, YM2, and the macrophage mannose receptor (Gordon and Martinez 2010). IL-4 treatment of macrophages reduces their phagocytic ability although it increases the clearance of apoptotic neutrophils (Gordon and Martinez 2010). It has been suggested that AAMs have regulatory roles in helminth infection and can reduce immunopathology (Gordon and Martinez 2010). AAMs also have increased expression of Dectin-1 in addition to macrophage mannose receptor and thus they may have greater fungicidal activity. Recently it has been shown that *Francisella tularensis*, a virulent pathogen in the lung can subvert classical activation of macrophages and thus prolong its intracellular survival (Shirey et al. 2008).

IL-13 has profound effects on airway epithelium by increasing the expression of several mucin genes including *Muc5ac* and *Muc5b* as well as inducing goblet cell hyperplasia, by activation of STAT6 (Rose et al. 2000). These effects are critical for host defenses against helminths such as *Nippostrongylus brasiliensis* (Price et al. 2010) but contribute to pathology in allergy/asthma. During viral infection of the airways mucins can not only prevent viral spread but also may contribute to airway obstruction. Moreover IL-13 has recently been shown to increase the susceptibility of epithelial cells to infection with rhinovirus (Lachowicz-Scroggins et al. 2010).

3.4.3 Type 17 Effectors

Human bronchial epithelium express IL-17RA and IL-17RC which allow these cells to respond to IL-17A and IL-17F as well as IL-22R and IL-10R2, the receptors for IL-22 (Fig. 3.3) (McAllister et al. 2005; Aujla et al. 2008). Both IL-17A and IL-17F can induce ligands for CXCR2 such as IL-8 and granulopoietic growth

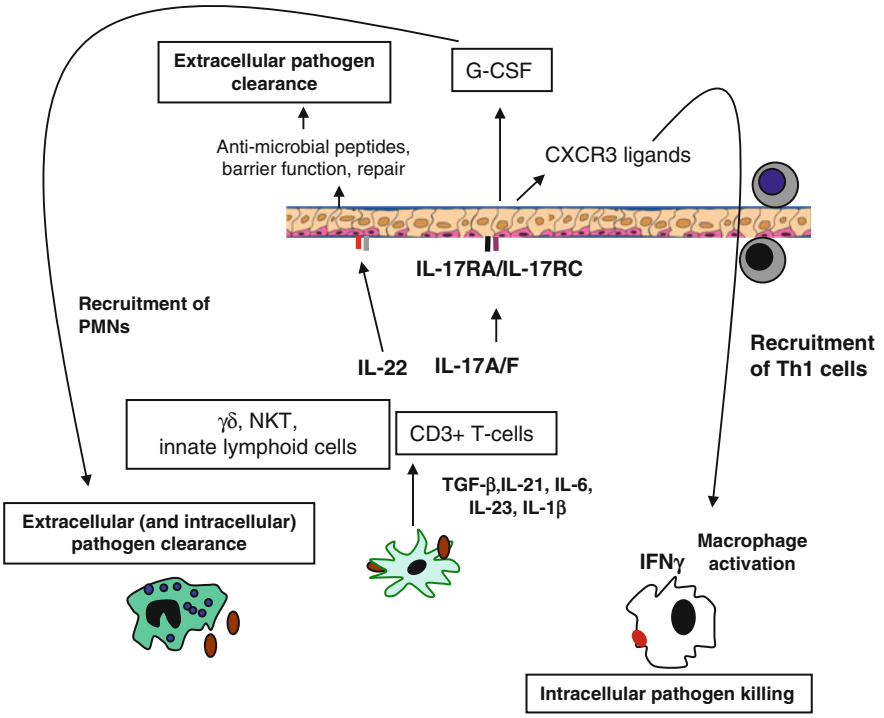

Fig. 3.3 Type 17 cytokines in mucosal immunity. Infections can activate dendritic cells and macrophages to produce IL-6, IL-23 as well as IL-1β that can induce IL-17 and IL-22 production by several immune cells in the mucosa including innate lymphoid cells, NK cells, NKT cells, and γδ T-cells. IL-17 and IL-22 can signal to the epithelium to augment G-CSF as well as ligands for CXR2 that mediate the recruitment of neutrophils. IL-22 in conjunction with IL-17 augments antimicrobial peptides and epithelial repair function important for control of extracellular fungal pathogens. Upon vaccination with certain adjuvants Th17 cells through the production of IL-17 can also induce ligands for CXCR3 that increase the recruitment of IFNγ–producing Th1 cells that can also help control intracellular pathogen growth

factors such as G-CSF which can be augmented in the presence of TNF-α (McAllister et al. 2005; Jones and Chan 2002). Since IL-17A and IL-17F can be co-expressed in the same cell, it has been reported that these two IL-17 family members can form three cytokines including IL-17A homodimers, IL-17A/F heterodimers which has intermediate activity compared to IL-17A homodimers, and IL-17F homodimers which has the least potent activity (Wright et al. 2007). IL-17 receptors are also expressed on lung fibroblasts, pulmonary vascular endothelium, and bronchial smooth muscle. Using CXCL1 as a model CXCR2 ligand, it has been shown that a major effect of IL-17 signaling is increasing mRNA stability of this transcript (Hartupee et al. 2007; Sun et al. 2011) resulting in augmented protein production. A similar activity has also been reported for IL-17-mediated increases in G-CSF production (Cai et al. 1998). IL-17RA is also abundantly expressed on myeloid

cells; however, these cells express very little IL-17RC and thus IL-17A and IL-17F have limited activity on myeloid cells. It has been reported that IL-17 can enhance IL-12p70 in alveolar macrophages (Lin et al. 2009) as well CCL2, CCL3, GM-CSF, IL-1β, and IL-9 in CD4+ T-cells (Ishigame et al. 2009). It has recently been shown that Th17 cells express IL-17RA and IL-17RE, the receptor for IL-17C and IL-17C, and increase the production of IL-17 by these cells (Song et al. 2011; Ramirez-Carrozzi et al. 2011; Chang et al. 2011). IL-17C can be expressed in lung epithelium (unpublished observations) and thus can serve as a feed forward mechanism by which the epithelium could influence interstitial T-cell responses. IL-17A can also augment apical bicarbonate anion transport in polarized NHBE cells and this activity may be important in mediated IL-17A's antimicrobial effect in the lung as bicarbonate anion has been shown to greatly augment the bioactivity of human defensins (Kreindler et al. 2009). IL-17RA is required not only for host resistance to the extracellular pathogens *Klebsiella pneumoniae* (Ye et al. 2001) but also for the intracellular pathogen *F. tularensis* (Lin et al. 2009). In the former scenario, IL-17 regulates G-CSF and neutrophil recruitment (Ye et al. 2001) and dominant sources in primary infection are lung γδ T-cells (Chen et al. 2011). In the case of *F. tularensis*, IL-17 regulates IL-12p70 production by macrophages which are required for Th1 immunity which is ultimately the effector cell required for control of the pathogen (Lin et al. 2009).

IL-22 activates STAT3 in NHBE cells as well as increases their clonogenic potential in colony assays (Aujla et al. 2008). Moreover in mature epithelium IL-22 augments epithelial repair in response to mechanical injury (Fig. 3.3) (Aujla and Kolls 2009). IL-22 also induces antimicrobial genes in lung epithelium including lipocalin 2 (Aujla et al. 2008) and regenerating islet-derived protein three-γ (Zheng et al. 2008). Blockade of IL-22 during experimental *K. pneumoniae* lung infection results in rapid dissemination of bacteria from the lung and increased mortality due to bacteremia. In this model IL-22 is regulated by IL-23 and recombinant IL-22 can rescue IL-23-deficient mice (Aujla et al. 2008). IL-22 has also been shown to decrease lung leak in response to ventilator-induced lung injury (Hoegl et al. 2011). These data support a potential therapeutic role of IL-22 in diseases such as severe pneumonia or acute respiratory distress syndrome.

As stated above in primary infection, early sources of IL-17 and IL-22 can be from innate lymphoid cells (Crellin et al. 2010), NK or NKT cells (Crellin et al. 2010; Cella et al. 2009), or γδ T-cells (Lockhart et al. 2006; Shibata et al. 2007b; Simonian et al. 2009). Classic Th17 cells can also be elicited in the lung in the setting of vaccination and in this setting they play roles in protection against a diverse set of organisms including both intracellular and extracellular bacteria as well as fungi. Using an ESAT-6 peptide Khader et al. showed that Th17 cells are primed in the lung prior to robust Th1 responses and that Th17 cells regulated local expression of ligands for CXCR3 (expressed on Th1 cells) and through this mechanism, Th17 cells could augment the recruitment of Th1 cells into the lung (Khader et al. 2007). Fungal-specific Th17 cells have also been shown to be critical for vaccine-induced protection against *Coccidioides posadasii*, *Histoplasma capsulatum*, and *Blastomyces dermatitidis* infection (Wuthrich et al. 2011). This protection was also

dependent on neutrophils. IL-17 has also been shown to mediate serotype-independent immunity using a whole cell polysaccharide vaccine from *S. pneumoniae* (Malley et al. 2006). Recently, Chen et al. showed that Th17 cells elicited by *K. pneumoniae* vaccination recognize conserved outer membrane proteins in the cell wall of the bacteria and these antigens could also provide serotype-independent immunity through Th17 cells that conferred heterologous protection against multiple serotypes of the organism (Chen et al. 2011). Again this protection was meditated by neutrophils. What is unclear from these studies to date are the specific aspects of Th17 function that are required for protection. For examples, what homing receptors are required to elicit these cells efficiently in the lung? Is only IL-17A required or is there a role for IL-17F or IL-17A/F heterodimers? Is IL-22 also important? What are the critical target cells that IL-17 is signaling in the lung to afford vaccine-induced immunity? What are the best Th17 adjuvants? Are there truly memory Th17 cells that can persist in the host or will these cells require periodic boosting to recall these responses? What is the role of plasticity in the function and fate of these cells?

3.5 Conclusions

CD4 T-cells play critical roles in lung immunity and when these cells are impacted by HIV or other modes of immunosuppression, the host is susceptible to many opportunistic infections by bacteria, fungi, and viruses. These cells will be critical targets to achieve therapeutic vaccines against intracellular pathogens such as *M. tuberculosis*. Furthermore these cells are now becoming attractive targets for other pathogens such as fungi and encapsulated bacteria. To this end, much work lies ahead to understand the generation of these cellular responses and how they can be manipulated therapeutically.

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Chapter 4
Neutrophils in Acute Bacterial Pneumonia

John C. Gomez, Qin Wang, and Claire M. Doerschuk

4.1 Introduction

During bacterial pneumonias, neutrophils are usually the first leukocytes recruited from the circulation to the lung, where they protect the host by killing microbial pathogens through phagocytosis and release of antimicrobial products. In addition to their role in eliminating pathogens, the role of neutrophils in shaping the immune response and resolution of inflammation is now increasingly appreciated. Patients with defects in neutrophil production or function suffer from recurrent microbial infections, thus illustrating the critical role of neutrophils in host defense. However, the very characteristics and functions that make neutrophils useful to the host can also injure host tissues, and neutrophil mediated tissue damage has been implicated in the pathogenesis of a number of serious disorders, including ALI and ARDS, in which both these beneficial and harmful effects are integrated. This article focuses on the role of neutrophils during bacterial pneumonias (Fig. 4.1). The first sections focus on recruitment of neutrophils from the bone marrow and from the blood. The later sections focus on neutrophil functions in the lung. There is clearly much work remaining to understand these processes, in order to find and develop ways to treat disease and modulate the inflammatory and immune response.

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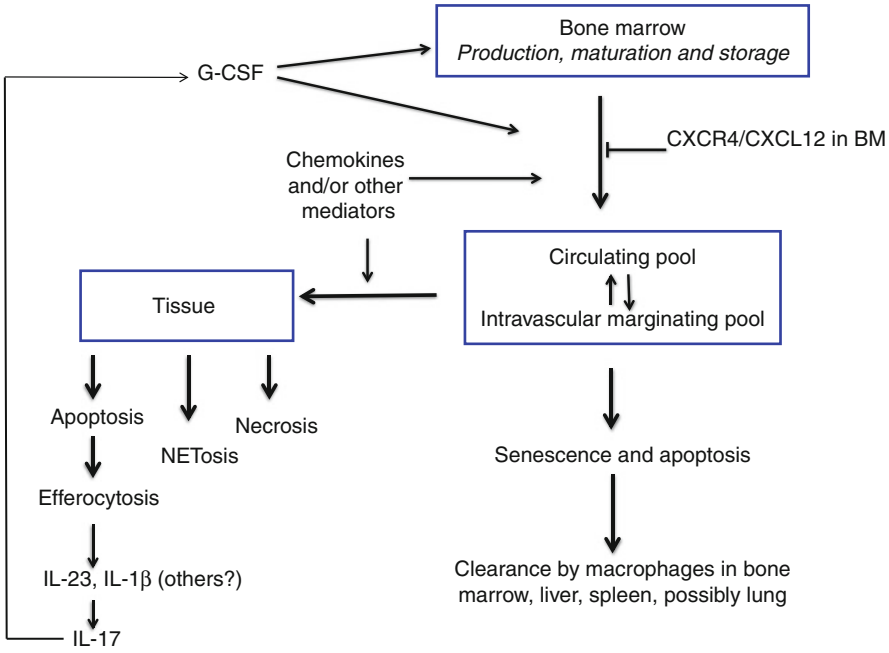

Fig. 4.1 Neutrophil kinetics at steady state and during inflammation. In the bone marrow, neutrophils are produced from precursors, mature and are stored until release. G-CSF is a major cytokine that regulates both neutrophil production and release from the bone marrow. CXCR4/CXCL12 (SDF-1) signaling retains cells within the bone marrow. Upon release into the circulation, intravascular neutrophils may circulate or enter the marginating pool in the liver, lungs, and other organs. In the absence of inflammation, intravascular neutrophils senesce and become apoptotic and are cleared by the reticuloendothelial system in the liver, spleen, bone marrow, and possibly the lung. During inflammation, chemokines and other mediators induce neutrophil migration into tissue as well as enhancing release from the bone marrow. Emigrated neutrophils can die through one of the several death pathways. Apoptotic neutrophils are taken up by tissue macrophages, which then release IL-23, inducing IL-17 which in turn induces G-CSF

4.2 Neutrophil Production, Maturation, and Release from the Bone Marrow and Trafficking

Interactions between the lungs and the bone marrow are important in neutrophil homeostasis in healthy individuals and during many lung diseases. During homeostasis, communication between the lungs and the bone marrow contributes to regulating the circulating neutrophil count, as illustrated by the changes in circulating neutrophil numbers in response to chronic smoking and the inhalation of air pollutants (van Eeden and Hogg 2000, 2002; Corre et al. 1971). During the inflammatory response, mediators produced in the lungs play a critical role in controlling neutrophil production and release from the bone marrow. For example, the time required for maturation of neutrophils, as measured by their transit time in the bone marrow, is shortened during pneumonia induced by *Streptococcus pneumoniae* (Terashima

et al. 1996). GM-CSF, granulocyte-colony stimulating factor (G-CSF), chemokines, and cytokines are produced in the lungs during inflammation and have effects on neutrophil production and release from the bone marrow, as will be described in subsequent sections. Thus, the bone marrow has an important impact on the inflammatory response, and the interaction between the lungs and the bone marrow is critical in the number and maturity of neutrophils that reach the lungs, both of which influence the effects of neutrophils in pulmonary inflammation.

Neutrophil release from the bone marrow is particularly significant for the lungs because the pulmonary microvasculature is the first capillary bed that the newly released neutrophils will traverse. Newly released neutrophils were preferentially retained in the lung capillaries during endotoxemia and pneumococcal infections, and migrated more slowly into the lung parenchyma (van Eeden et al. 1997; Sato et al. 1998a, b; Lawrence et al. 1996). Newly released circulating neutrophils had decreased deformability as measured by filter assays in vitro, which may contribute to their preferential retention in the lung for reasons discussed in subsequent sections (van Eeden et al. 1997, 2000). The accumulation of immature neutrophils in the lungs is exacerbated by shortened transit time of neutrophils in the bone marrow, for example, in *S. pneumoniae* pneumonia (Terashima et al. 1996). Morphologically mature neutrophils and band cells in human bone marrow exhibit functional deficiencies in degranulation, respiratory burst, and phagocytosis compared with blood neutrophils (Berkow and Dodson 1986), and may thus be less capable of defending the host against infection. Furthermore, prolonged sequestration and activation of these immature neutrophils within the vasculature may result in damage to the endothelium.

4.2.1 Neutrophil Production and Circulation in Health

The bone marrow is the site of granulopoiesis in mature animals. Neutrophils are ultimately derived from a multipotent hematopoietic stem cell (HSC) that gives rise to all lineages of hematopoietic cells. Neutrophil progenitors are able to divide up to the myelocyte stage; post-mitotic neutrophil precursors undergo a process of maturation that lasts several days and involves changes in cell surface molecule expression, biomechanical and structural properties, and granule content (Bainton 1999). A large number of mature neutrophils reside in the bone marrow stroma presumably ready for release into the circulation, and there is a marginating pool of neutrophils within the vasculature of the bone marrow (Fig. 4.2).

The cytokine granulocyte-colony stimulating factor (G-CSF) is a major regulator of neutrophil production and release from the bone marrow. G-CSF acts by binding to its receptor G-CSFR, which induces Jak/STAT signaling through sequential phosphorylation of the Janus family of tyrosine kinases (Jaks), phosphorylation of the G-CSFR by Jaks, and recruitment of STATs to the phosphorylated G-CSFR and phosphorylation of STATs by Jaks. Jak/STAT signaling, as well as other signaling cascades including Ras/MAP kinase and PI3K/Akt, promote proliferation and differentiation toward mature neutrophils and regulate neutrophil release and

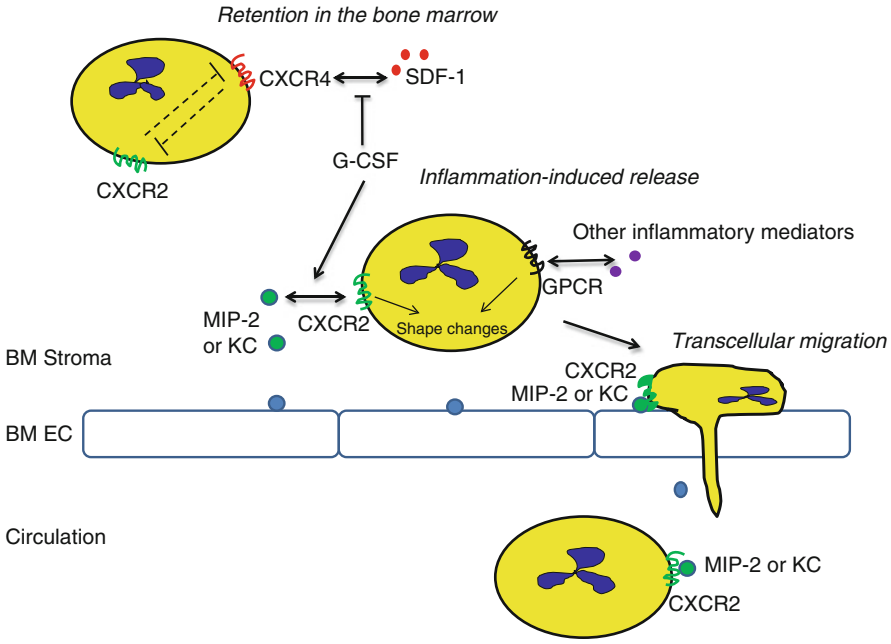

Fig. 4.2 Complex interplay of signals mediated by G protein-coupled receptors and their ligands, and G-CSF regulate neutrophil retention in the bone marrow and release into the circulation. Binding of SDF-1 (CXCL12) to its receptor CXCR4 mediates neutrophil retention in the bone marrow. Binding of the chemokines MIP-2 or KC to CXCR2 during inflammation induce neutrophil shape changes and release into the circulation. Binding of other inflammatory mediators (such as complement fragments or the bacterial peptide fMLP) to G protein-coupled receptors also induce shape changes and rapid release of neutrophils into the circulation. G-CSF disrupts the CXCR4/SDF-1 signaling axis and enhances release induced by MIP-2 or KC. Crosstalk between CXCR4 and CXCR2 regulates neutrophil release during inflammation through mechanisms that are not yet completely clear

function, both at steady state and in conditions of stress (Panopoulos and Watowich 2008; Nicholson et al. 1994, 1995; Shimoda et al. 1994). Mice that are deficient in G-CSF or G-CSFR have low circulating counts and decreased numbers of neutrophils and their precursors in the bone marrow (Liu et al. 1996; Lieschke et al. 1994; Basu et al. 2002; Richards et al. 2003). However, mice deficient in G-CSF or G-CSFR do have neutrophils, albeit in reduced numbers, suggesting that G-CSF-independent pathways for neutrophil production partially compensate for a lack of G-CSF signaling. G-CSF is also important for neutrophil trafficking into the circulation both at baseline and during pulmonary bacterial infection (Semerad et al. 2002; Gregory et al. 2007).

After release from the bone marrow, neutrophils circulate in the blood. The concentration of neutrophils in the blood varies depending on the site within the vasculature. For example, differences in the neutrophil concentrations in tail, eye, and heart have been identified (Nemzek et al. 2001). In general, concentrations increase

in vascular beds where blood flow and pressure are less and the diameters and branching geometry of the bed favors increased concentration. This increased concentration is often described as a marginated pool, but these pools are unlikely to be stagnant and in disequilibrium with the circulating pool. The mechanisms for the increased concentration in the pulmonary capillaries are discussed in a subsequent section.

In animals without an inflammatory focus, the circulating half-life of neutrophils is 4–14 h (Basu et al. 2002; Dancey et al. 1976; Deubelbeiss et al. 1975; Eash et al. 2009; Gomez et al. 2008; Lord et al. 1991; Price et al. 1996). Neutrophils that do not migrate into inflammatory sites become senescent, in which they are unresponsive to stimuli that would otherwise induce degranulation or respiratory burst, and die by apoptosis. The mechanisms that regulate senescence are not well understood, and may be regulated by microRNAs (Ward et al. 2011). At steady state, apoptotic neutrophils accumulate in the liver, bone marrow, and spleen, suggesting these are the sites of clearance for effete neutrophils (Furze and Rankin 2008a, b; Suratt et al. 2001). Constitutive removal of apoptotic neutrophils requires macrophages in the bone marrow stroma and marginal zone of the spleen (Gordy et al. 2011). The capillaries of the lungs may also be a site of clearance, since intravascular neutrophils that get trapped in narrow capillary segments may undergo apoptosis or other forms of cell death (Bicknell et al. 1994). The recognition systems whereby the reticuloendothelial system recognizes effete neutrophils are becoming clearer, although numerous mechanisms are likely to contribute, including increased expression of CXCR4 (Eash et al. 2009, 2010; Martin et al. 2003; Suratt et al. 2004), an important molecule in the retention of neutrophils within the bone marrow that is discussed below. A recent study reported that expression of the anti-inflammatory phospholipid-binding protein Annexin A1 by mouse bone marrow macrophages is critical for the uptake of apoptotic neutrophils (Dalli et al. 2012).

Constant exposure to the outside world, either through inhalation, ingestion, cutaneous routes, or other means, cannot always be managed by first-line organ-specific host defense mechanisms. An exposure can result in mild inflammatory responses that are unlikely to be experienced by the host as the five cardinal signs of inflammation, rubor (redness), calor (increased heat), tumor (swelling), dolor (pain), and function laesa (loss of function), and thus is not detected clinically. However, these common responses may influence circulating neutrophils by inducing efflux of neutrophils from the blood stream or even in altering production by the bone marrow, as may occur during more chronic noxic exposures. The lungs are an important route of exposure, even though airflow patterns through the nasopharynx, the nasopharyngeal surfaces themselves, and the mucociliary clearance mechanisms in the bronchi and trachea are highly functional first-line defense mechanisms. For example, a reduction in inhaled atmospheric particulates, as occurs upon relocation from an urban level of particular matter to that found in Antarctica, is associated with a reduction in the numbers of mature and immature neutrophils circulating in the blood, and these numbers increase upon return to the urban environment (Sakai et al. 2004). Similarly, circulating neutrophil counts in cigarette smokers are higher

than in age-matched nonsmokers in the same environment (van Eeden and Hogg 2000; Corre et al. 1971).

Thus, in healthy subjects, the number of circulating neutrophils reflects a dynamic balance between production and continual release from the bone marrow, the marginating pools, the clearance of neutrophils from the circulation, and neutrophil migration from the circulation into the tissues in response to a constant barrage of stimuli, where they usually perform any needed function and are cleared by resident macrophages. A clinically relevant injury or infection will alter this equilibrium.

4.2.2 Mechanisms of Neutrophil Release from the Bone Marrow During Inflammation

In response to injury or infection, the number of neutrophils in the circulation can undergo a rapid increase that largely reflects the release of neutrophils from the bone marrow. Marginating pools of mature or nearly mature neutrophils are present both within the stroma of the bone marrow and within the venous sinusoids. Many inflammatory mediators that are chemotactic agents or activators of neutrophils can cause this acute release. For example, infusion of the chemotactic bacterial peptide fMLP (*N*-formyl-L-methionyl-L-leucyl-L-phenylalanine, also abbreviated fMLF), the chemokine interleukin-8 (IL-8), or its murine homolog MIP-2, or complement protein fragments (particularly C5a) induce neutrophil release from the venous sinusoids and/or from the marginating pool of mature neutrophils in the bone marrow stroma into the sinusoids and then the circulation (Jagels et al. 1995; Jagels and Hugli 1992, 1994; Burdon et al. 2008). Neutrophil egress from the stroma into the venous sinusoids usually occurs through the endothelial cells, rather than between them. For example, neutrophils were observed to squeeze through pores in the bone marrow endothelium in response to MIP-2. In perfused rat femurs, the endothelial cells presented a barrier, and neutrophil mobilization by MIP-2 required p38 MAPK activity but was not affected by the presence of a nonspecific matrix metalloprotease inhibitor (Burdon et al. 2008). Thus, neutrophil mobilization is an active process that requires the integration of many signals and pathways involving neutrophils as well as other cells in the bone marrow.

4.2.2.1 The Importance of Biomechanical Properties of Neutrophils in Their Production and Release

Neutrophils appear to undergo changes in their biomechanical properties as they mature (Fig. 4.2). Early studies showed that myelocytes were stiffer than mature neutrophils (Lichtman 1970), and that mature granulocytes were better able to transit through smaller pore diameters and respond to a chemoattractant than less mature cells (Giordano and Lichtman 1973). Studies using magnetic twisting cytometry showed that mature neutrophils isolated from the bone marrow are stiffer than those

isolated from circulating blood (Saito et al. 2002). This increased stiffness was associated with the presence of an f-actin rim beneath the cell membrane, and disruption of the actin cytoskeleton by treatment with cytochalasin D reduced the stiffness of bone marrow neutrophils to the level seen in circulating neutrophils (Saito et al. 2002). The greater stiffness of immature neutrophils may facilitate their retention within the bone marrow, by preventing or slowing the passage of these cells through the stroma or pores in the sinusoidal wall, along with other factors such as CXCR4/SDF-1 signaling and adhesivity (discussed below). Furthermore, it is also possible that the process of neutrophil migration through sinusoidal endothelial cells alters their biomechanical properties to decrease their stiffness.

During an inflammatory stimulus, bone marrow neutrophils deform in order to transit toward and enter into the venous sinusoids. Their biomechanical properties may be an important factor in their retention or release. Chemokines and other mediators that act through serpentine receptors (also called heptahelical receptors or G protein-coupled receptors) share the ability to alter the cytoskeleton of neutrophils, which is often evaluated as changes in f-actin in neutrophils. This rearrangement of actin appears initially to result in f-actin beneath the cell membrane, inducing an increase in stiffness, which further remodels after 1–2 min to a flattening out and cytoskeletal changes that vary within regions of the neutrophils. In fact, the initial stiffening may cause neutrophils to round up and come off the stroma or sinusoidal endothelium (Luscinskas et al. 1992; Hechtman et al. 1991), which could also facilitate release. Studies show that stimulation by fMLP or complement fragments in vitro caused an increase in stiffness in both circulating and bone marrow neutrophils and an increase in f-actin beneath the cell membrane (Saito et al. 2002). Pretreatment with cytochalasin D prevented the stiffening induced by inflammatory mediators, suggesting that f-actin rearrangement was responsible for the increased stiffness. Infusion of intravascular fMLP and complement fragments induced an extremely rapid release (within seven minutes) of mature neutrophils from the bone marrow into the circulation (Saito et al. 2002; Kubo et al. 1998). The fMLP-induced increase in circulating neutrophil counts was not prevented by pretreatment with colchicine, indicating that microtubule rearrangements were not required for this process (Saito et al. 2002). These observations suggest that structural and mechanical changes induced by circulating mediators may facilitate the release of bone marrow neutrophils into the circulation.

4.2.2.2 The Role of CXCR4 and SDF-1 in Bone Marrow Release of Neutrophils

Disruption of G protein signaling by treatment with pertussis toxin led to leukocytosis and mobilization of hematopoietic stem/progenitor cells from the bone marrow (Papayannopoulou et al. 2003), indicating that signaling through pertussis toxin-sensitive G protein-coupled receptors is important in retaining primitive as well as more mature hematopoietic cells within the bone marrow. One of these G protein-coupled receptors, CXCR4, has been studied extensively and is expressed

on a wide variety of hematopoietic cells, including neutrophils (Broxmeyer 2008). Its ligand is SDF-1 (CXCL12), a CXC chemokine produced by stromal cells in the bone marrow and a key signal for retaining and maintaining cells in the bone marrow through its binding to CXCR4 (Broxmeyer 2008). Ma and colleagues showed that mice whose bone marrow was reconstituted with CXCR4-deficient fetal liver cells had reduced levels of granulocytic cells in the bone marrow and elevated numbers of circulating mature and immature granulocytes (Ma et al. 1999). The critical role of CXCR4/SDF-1 signaling in retaining neutrophils is also supported by studies of WHIM syndrome, in which mutations that lead to prolonged signaling or hyperactivation of CXCR4 result in immune abnormalities and failure of bone marrow release of mature neutrophils, resulting in severe neutropenia, myeloid hyperplasia, and apoptosis of mature neutrophils within the bone marrow (myelokathexis) (Kawai and Malech 2009). Other studies interfering with CXCR4 signaling have shown that treatment of mice with a CXCR4 antagonist (Martin et al. 2003) or with CXCR4 blocking antibody (Suratt et al. 2004) mobilized neutrophils from the bone marrow. Selective deletion of CXCR4 in myeloid cells caused increased numbers of circulating neutrophils with no increase in immature forms and elevation in the ratio of circulating to bone marrow neutrophils, indicating a cell-autonomous requirement for CXCR4 in neutrophil retention in the bone marrow (Eash et al. 2009). Thus, many approaches have supported the concept that SDF-1/CXCR4 signaling serves as a retention signal for neutrophils in the bone marrow. However, the molecular mechanisms initiated by SDF-1 binding to CXCR4 that are responsible for this retention are not yet clear.

Disruption of CXCR4/SDF-1 signaling in the bone marrow may be a common feature of neutrophil release induced by some chemokines or G-CSF. Mobilization of bone marrow neutrophils induced by the CXC chemokine KC (CXCL1) was enhanced by treatment with a CXCR4 antagonist (Martin et al. 2003) or blocking antibody (Suratt et al. 2004), whereas *Cxcr2−/−* neutrophils were not mobilized by transiently inhibiting CXCR4 (Eash et al. 2010). Pretreatment of murine neutrophils with KC led to a decrease in the calcium flux in response to SDF-1, suggesting that KC and other chemokines may mobilize neutrophils in the bone marrow by disrupting SDF-1 signaling in the bone marrow (Suratt et al. 2004). Conversely, SDF-1 attenuated neutrophil responses to KC in vitro (Martin et al. 2003). These studies suggest that signaling through CXCR4 and CXCR2 act in opposite ways to regulate neutrophil release.

The interplay between CXCR4/SDF-1 signaling, CXCR chemokines and G-CSF is complex. Neutrophil mobilization in response to pulmonary infection with *P. aeruginosa* was reduced in mice lacking G-CSF receptor, indicating that G-CSF signaling may be critical for neutrophil recruitment in this infection (Gregory et al. 2007). Antibody neutralization of G-CSF resulted in fewer neutrophils within the lungs at 48 h of *S. pneumoniae* pneumonia by less than but had no effect at 24 h (Knapp et al. 2004). G-CSF may induce neutrophil release from the bone marrow either directly by reducing expression of CXCR4 on myeloid cells (Kim et al. 2006) or by disrupting CXCR4 signaling through reducing levels of SDF-1 in the bone marrow (Semerad et al. 2005; Christopher et al. 2009). Giving KC, MIP-2 or G-CSF

intravenously resulted in increased neutrophil numbers in the blood and decreased numbers in the bone marrow of mice, and infusion of G-CSF and KC together mobilized a significantly greater number of neutrophils compared with either mediator alone in a perfused femoral bone marrow system (Wengner et al. 2008). Antibody neutralization of G-CSF, MIP-2, KC, or MIP-2 and KC together reduced neutrophil mobilization from the bone marrow and recruitment to the peritoneum of mice in thioglycollate-induced acute peritonitis, but inhibition of G-CSF did not alter the response to a selective CXCR4 antagonist (Wengner et al. 2008). G-CSF-induced neutrophil mobilization is attenuated in *Cxcr2−/−* mice or in wild type (WT) mice given blocking antibody to CXCR2, and G-CSF treatment induces CXCR2 ligands in the bone marrow (Eash et al. 2010; Kohler et al. 2011). Notably, Kohler and colleagues found that G-CSF did not directly induce CXCR2 ligands, but rather induced thrombopoietin, which induced CXCL1 production from megakaryocytes (Kohler et al. 2011). Taken together, these studies suggest that neutrophil release is regulated by a complex interplay between CXCR4/SDF-1 signaling in the bone marrow and mobilization signals induced by G-CSF and inflammatory mediators.

CXCR4/SDF-1 signaling in the periphery may play a role in neutrophil mobilization and recruitment to tissue during inflammation. In LPS-induced pneumonitis, SDF-1 expression was upregulated in lung epithelium and SDF-1 blockade prevented neutrophil recruitment to the airspace at 24 h after injury (Petty et al. 2007). A recent study reported that SDF-1 blockade prevented the increase in circulating neutrophils and decrease in BM neutrophils induced by sepsis at 12 h (Delano et al. 2011). Taken together these studies suggest that generation of an SDF-1 gradient in the periphery is required for mobilization. Notably, neutrophil mobilization in the murine model of polymicrobial sepsis did not require MyD88, IFNα/βR, TRIF, or TLR4, and was not inhibited by CXCR2 blockade, suggesting that other pathways can modulate CXCR4/SDF-1 signaling to induce mobilization (Delano et al. 2011).

4.2.2.3 The Functions of Rac2

The small GTPase Rac2 is expressed in leukocytes and activated by signaling downstream of many receptors, including G protein-coupled receptors of chemokines/chemoattractants and the β2 integrin, CD11/CD18 expressed on leukocytes. It regulates a wide variety of functions in neutrophils, including cytoskeletal organization and rearrangements, superoxide production, chemotaxis, phagocytosis, transcription, and cell growth and proliferation (Bokoch 2005). *Rac2−/−* mice have circulating neutrophil counts that are several times those seen in wild type animals and a slight increase in marrow granulopoiesis, which persist in otherwise asymptomatic mutant mice (Roberts et al. 1999). *Rac2−/−* mice have higher numbers of circulating HSC/Ps at baseline and after G-CSF treatment compared to WT littermates despite having similar numbers of HSC/Ps in the bone marrow (Yang et al. 2001), suggesting that Rac2 may be important in the retention and mobilization of these

cells from the bone marrow. *Rac2−/−* HSC/P adhered less to bone marrow stromal cells in vitro and exhibited growth defects in stroma-dependent cultures than wild type cells, indicating that Rac2 in hematopoietic cells is required for optimal growth and development (Jansen et al. 2005). Neutrophil kinetic studies suggested that Rac2 modulates the time from the last mitosis to release of neutrophils into the circulation and does not prolong their circulating half-life (Gomez et al. 2008). Lethally irradiated wild type mice reconstituted with a mixture of wild type and *Rac2−/−* stem cells were protected from neutrophilia, and neutrophils constituted a greater percentage of the *Rac2−/−* leukocytes than wild type leukocytes in these mice (Gomez et al. 2008). These findings are consistent with the role of Rac2 in transducing signals downstream of integrin activation (please see next section). However, whereas Rac2 deficiency in hematopoietic cells alone resulted in increased neutrophil production in the bone marrow, Rac2 deficiency in both hematopoietic and nonhematopoietic cells was required for the increase in circulating neutrophil counts, suggesting a role for Rac2 in nonhematopoietic cells in regulating bone marrow release of neutrophils (Gomez et al. 2008).

4.2.2.4 Adhesion Molecules and the Regulation of Neutrophil Production and Release

Adhesion of hematopoietic cells to stromal cells or to the extracellular matrix within the bone marrow plays an important role in retaining hematopoietic cells within the bone marrow and in mediating the return of some hematopoietic cells to the bone marrow from the circulation ("homing"). Conversely, adhesive interactions may be important in mobilizing hematopoietic cells to enter the circulation, by allowing hematopoietic cells to crawl through the bone marrow stroma toward the sinusoids and cross the sinusoidal endothelium. Bone marrow neutrophils express the integrin VLA-4 ($\alpha 4\beta 1$ or CD49d/CD29) which binds to VCAM-1 expressed by bone marrow stromal cells and endothelium (Petty et al. 2009). Blocking antibodies against VLA-4 or VCAM-1 given intravascularly caused neutrophilia at 4 h, in the absence of any inflammatory stimulus (Petty et al. 2009). Evidence of interaction between CXCR4 and VCAM-1 in neutrophil mobilization was observed (Petty et al. 2009). In contrast, baseline neutrophil release in a model of rat perfused femoral bone marrow was not inhibited by blocking CD49d (the alpha subunit of VLA-4), and an anti-CD49d antibody partially prevented neutrophil mobilization induced by MIP-2 (Burdon et al. 2005). The basis for this discrepancy is not yet clear, but these data underline the complexities of VLA-4/VCAM-1, CXCR4/SDF-1, and CD11/CD18/ICAM-1 axes and signaling. Light may be shed on these complexities by observations made in immature bone marrow hematopoietic cells, where VLA-4 plays an important role in mediating homing to and mobilization from the bone marrow by binding to VCAM-1 (Bonig et al. 2006; Priestley et al. 2006; Scott et al. 2003). Interestingly, lacking CXCR4 expression in hematopoietic cells abolished G-CSF-induced HSC/P mobilization in mice, but had no effect on mobilization induced by blocking VLA-4 (Christopher et al. 2009), suggesting

multiple independent or redundant mechanisms of HSC/P release that may also operate in neutrophils.

Integrins of the β2 integrin subfamily (CD11/CD18 complex) are the main integrins expressed on the surface of leukocytes. Patients with heterogeneous mutations in the gene that encodes CD18 suffer from the clinical syndrome Leukocyte Adhesion Deficiency type I (LAD I). LAD I is characterized by extremely high levels of circulating neutrophils, recurrent bacterial infections, impaired wound healing, and functional defects in neutrophils. Mice that are completely deficient in CD18 (*Itgb2-/-*) express no functional leukocyte β2 integrins, including LFA-1 (CD11a/CD18 or αLβ2) and Mac-1 (CD11b/CD18 or αMβ2) and have a phenotype similar to human LAD I patients, including granulocytosis, spontaneous infections, and myeloid hyperplasia (Scharffetter-Kochanek et al. 1998). Mice deficient in CD11a/CD18 (LFA-1) (Ding et al. 1999) or the CD11/CD18 receptor ICAM-1 (Sligh et al. 1993) have modestly elevated circulating neutrophil counts.

The neutrophilia in CD18 deficiency is present within the first few days of life, and occurs even when mice were housed in clean, specific pathogen-free facilities and in the absence of discernible infections. When lethally irradiated wild type (WT) mice are given a 1:1 mixture of WT and CD18-deficient fetal liver cells, neutrophilia is inhibited by greater than 95 % compared with WT mice given CD18-deficient stem cells alone (Horwitz et al. 2001), indicating that the neutrophilia seen in CD18 deficiency can be largely corrected by the presence of WT hematopoietic cells. Weinmann and colleagues demonstrated that circulating neutrophils in CD18-deficient mice show decreased apoptosis, and the delay in apoptosis of CD18-deficient neutrophils is abolished in the presence of WT leukocytes (Weinmann et al. 2003). These studies led to the hypothesis that neutrophilia in CD18 deficiency is mainly due to the inability of mutant leukocytes to defend the host from microbial pathogens, resulting in chronic infection and subsequent chronic stimulation of the bone marrow. The presence of WT neutrophils is thus postulated to remove the stimuli that increase neutrophil production in the bone marrow by conferring protection to the host.

Cell-intrinsic mechanisms that were not corrected by the presence of wild type cells contributed to the granulocytosis observed in CD18 deficiency, because a mild granulocytosis developed in mice that received a mixture of wild type and CD18-deficient stem cells (Horwitz et al. 2001). In chimeric mice with both wild type and CD18-deficient bone marrow, a larger than expected fraction of the circulating neutrophils are CD18-deficient and a much larger proportion of the CD18-deficient circulating leukocytes are neutrophils, even in the presence of normal numbers of wild type neutrophils in the blood and bone marrow (Horwitz et al. 2001). The proportion of apoptotic Gr-1+ cells in both the bone marrow of chimeric animals and in vitro cultures of wild type and CD18-deficient HSCs was lower in CD18-deficient than in wild type cells (Gomez and Doerschuk 2010). These data suggest that CD18 can directly regulate neutrophil production, in part by limiting the survival of neutrophils and their precursors.

A novel mechanism through which the defects of CD18-deficient neutrophils in host defense result in neutrophilia was suggested by Forlow and colleagues, who showed that CD18-deficient mice have increased levels of circulating IL-17 and G-CSF and that blocking IL-17 or G-CSF suppressed the neutrophilia in these mice

(Forlow et al. 2001). The cytokine IL-17 is produced by T cells and induces G-CSF production, and is itself induced by IL-23 produced by macrophages and dendritic cells (von Vietinghoff and Ley 2008). Stark and colleagues demonstrated a novel feedback loop in which phagocytosis of apoptotic neutrophils by tissue-resident macrophages and dendritic cells inhibits the production of IL-23, thus shutting down the IL-17A/G-CSF signaling axis and preventing increased granulopoiesis in the bone marrow (Stark et al. 2005).

Similar concepts may underlie neutrophilia found in mice deficient in signaling through CXCR2. *Cxcr2−/−* mice have increased numbers of neutrophils in the bone marrow and circulation, and *Cxcl5−/−* (LIX-deficient) mice have a similar but milder phenotype (Mei et al. 2012). The authors showed that the phenotype was due to increased IL-17A in the ileum, and the treatment with anti-IL17A or antibiotics resulted marked reduction in neutrophils in the blood and bone marrow (Mei 2012). The authors propose that CXCL5 (LIX) produced by enterocytes attract circulating neutrophils into the gut where they feedback into the IL-17A/G-CSF signaling axis. Thus, the failure of neutrophils to migrate into the tissues may underlie the reactive neutrophilia observed in mice deficient in adhesion molecules or CXCR2 signaling. Interestingly, uptake of apoptotic neutrophils by bone marrow-derived macrophages induces G-CSF production, whereas LPS-induced G-CSF production by peritoneal macrophages is inhibited (Furze and Rankin 2008a), suggesting that neutrophil uptake may induce distinct signaling pathways depending on the macrophage population and the microenvironment, with IL-17 signaling playing a role in neutrophil kinetics during inflammation by inducing G-CSF.

4.3 Neutrophil Margination and Sequestration in Pulmonary Microvasculature and Recruitment into Lung Tissue

4.3.1 Neutrophil Trafficking and Margination in the Normal Pulmonary Microvasculature

Within the capillaries of the pulmonary circulation in healthy lungs, there is an increased concentration of neutrophils relative to other vessels. This increased concentration of neutrophils has been termed the marginated pool, although the implication that this pool is stagnant is unlikely to be true (Hogg 1987). Many studies have shown that this increased concentration is explained by increased transit time of neutrophils through the pulmonary capillary bed, compared to the rapid transit times of erythrocytes. This longer transit time is likely due to constraints set by the diameters of the capillaries and the neutrophils, and the biomechanical properties of the neutrophils (Brumwell et al. 1991; Doerschuk et al. 1993; Hogg et al. 1994; Lien et al. 1987, 1990; Wiggs et al. 1994; Yoder et al. 1990; MacNee et al. 1989; Selby et al. 1991). Capillary segments are often narrower than the diameter of spherical neutrophils, indicating that neutrophils must stop and deform in order to traverse

these narrow segments. Video microscopy demonstrated that in contrast to the continuous movement of the discoid erythrocytes that can fold, leukocytes move in hops, stopping once or more in transiting through the capillary bed from a pulmonary arteriole to a venule (Lien et al. 1990). The prolonged transit time of neutrophils and other leukocytes through the pulmonary capillaries may be important for host defense, by allowing neutrophils to sample their microenvironment for the presence of inflammatory stimuli and to respond appropriately. The observation that transit times are longer has become controversial of late (Summers et al. 2010), but the numerous ways in which neutrophil transit times have been studied (imaging, morphometry, computational modeling), the many species (human, dog, rabbit, mice), the many ways in which neutrophils have been isolated, and the numerous studies showing the effect of epinephrine and respiratory maneuvers on the release of this pool suggest that neutrophil transit times are longer than erythrocyte transits, resulting in an increased concentration of neutrophils in the pulmonary capillary blood that is likely important for host defense in the lungs.

4.3.2 Neutrophil Sequestration in the Lungs During Inflammation

Neutrophils migrate from the circulation to the tissues in response to inflammatory stimuli. The recruitment of neutrophils and other leukocytes to the tissues during inflammation occurs in a sequence of events that includes leukocyte recognition of the inflammatory site, sequestration within the microvasculature, firm adhesion, transmigration through the endothelium, and migration in the tissues (Fig. 4.3).

The mechanisms through which neutrophils are sequestered at a site of inflammation within the alveolar spaces appear quite different in the pulmonary microcirculation compared to the systemic microcirculation. In the systemic microcirculation, the initial steps of leukocyte capture and rolling occur within the post-capillary venules and are mediated by selectins, a family of calcium-dependent lectins that includes L-selectin expressed on leukocytes, P-selectin expressed on platelets and endothelial cells, and E-selectin expressed on endothelial cells. Neutrophils express L-selectin and several ligands that can bind to selectins expressed on leukocytes, platelets, and endothelial cells. Among the best characterized selectin ligands on neutrophils are PSGL-1, ESL-1, and CD44. Neutrophil PSGL-1 can bind to all three selectins, whereas ESL-1 and CD44 can bind to E-selectin expressed on activated endothelial cells. Binding of E-selectin to PSGL-1, ESL-1, or CD44 on neutrophils elicits distinct signals that correlate with their distribution on the cell surface (Hidalgo et al. 2007). The biology of selectin adhesion has been extensively studied in vitro and within the systemic post-capillary venules.

In contrast, the major site of neutrophil sequestration within the pulmonary microvasculature in response to an inflammatory stimulus is the pulmonary capillaries, and this sequestration does not require rolling. Rolling does not occur in the pulmonary capillaries, because of the spatial restraints arising from the diameter of many

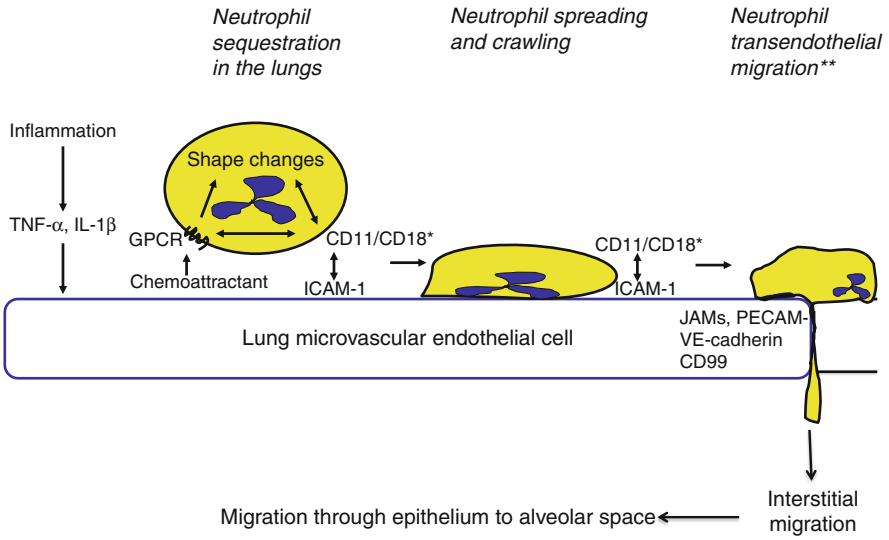

Fig. 4.3 Postulated pathway of neutrophil recruitment during inflammation in the lungs. Complex signaling processes occurring in and between neutrophils and endothelial cells facilitate the early stages of neutrophil recruitment from the circulation. *Asterisk* depending on the stimulus, neutrophils migrate into the airspaces of the lungs using CD11/CD18-dependent or -independent mechanisms. *Double asterisks* most transendothelial migration occurs paracellularly as depicted; a minority of cells migrate through endothelial cells (transcellularly). Please see text for details

pulmonary capillaries being narrower than those of round neutrophils (Doerschuk et al. 1993; Gebb et al. 1995; Lien et al. 1991). Rather, mediator-induced changes in the biomechanical properties of neutrophils appear to underlie their sequestration in the pulmonary capillaries (Doerschuk 2001). The binding of inflammatory mediators present at the inflammatory site to G protein-coupled receptors on leukocytes leads to changes in the biomechanical properties of neutrophils that decrease their ability to deform and change shape. In normal lungs as mentioned above, the transit times of neutrophils are longer than for erythrocytes, allowing them 2–20 s or more to assess their environment. The binding of neutrophil receptors by chemokines or bacterial products induces an f-actin rim to form beneath the cell membrane within seconds, which causes neutrophils to become stiffer and less deformable (Downey et al. 1991; Inano et al. 1992). This increased stiffness appears to prevent them from deforming and passing into the pulmonary venules, resulting in their sequestration at inflammatory sites. During bacterial pneumonias in rats, neutrophils with f-actin rims were preferentially retained in the lungs over those without f-actin rims at a time when sequestration was actively ongoing (Yoshida et al. 2006). CD18 blockade had no effect on this preferential sequestration of f-actin rimmed neutrophils, and L-selectin expression or platelet binding made no difference in which neutrophils were preferentially retained. Furthermore, L-selectin-deficient mice had no defect in neutrophil sequestration within lung capillaries induced by intravascular activated complement or intra-alveolar *S. pneumoniae* (Doyle et al. 1997).

Although selectins appear to play no role in the immediate recruitment of neutrophils in the pulmonary capillaries, this family of adhesion molecules was required for prolonged retention of neutrophils in the lungs in response to intravascular complement fragments (Kubo et al. 1999). During bacterial pneumonia, the requirement for selectins may depend upon the bacterial species. For example, neutrophil sequestration and migration in response to *S. pneumoniae* does not appear to require selectins, whereas L-selectin does contribute to emigration induced by *Escherichia coli* (Doyle et al. 1997; Mizgerd et al. 1996). This requirement may reflect a role of selectins in integrin activation (see below) and other signaling processes in neutrophils and other cells. For example, platelet-derived P-selectin-mediated platelet-neutrophil interactions, and P-selectin blockade abrogated neutrophil recruitment and lung injury in a model of acid-respiration induced lung injury (Zarbock et al. 2006). Thromboxane A2 released by platelet-neutrophil aggregates increased the expression of ICAM-1, the receptor for the major leukocyte integrin, on endothelial cells and adhesion of neutrophils to endothelial cells (Zarbock et al. 2006).

4.3.3 Neutrophil Adhesion to Endothelial Cells and Migration into the Lungs

4.3.3.1 The Biology of Integrin Activation

Firm adhesion of leukocytes is mediated by activated integrins present on the cell surface. Integrins expressed on resting leukocytes generally have low affinity for their ligands. Integrin activation caused by binding of various agonists to receptors on the cell surface induces structural changes in integrins that result in increased affinity for ligands (inside-out signaling) and clustering of integrins on the cell surface. Integrins on leukocytes can be rapidly activated by the binding of chemokines and other inflammatory mediators to cognate G protein-coupled receptors on the leukocyte surface. Binding of agonists to the N terminal extracellular portion of their cognate G protein-coupled receptors results in activation of the associated cytoplasmic G protein, which dissociates into the GTP-bound Gα subunit and Gβγ dimers. The G protein subunits activate downstream effectors, including phospholipase C, the small GTPase Rap, GEFs including CALDAG-GEF1, and the cytoskeletal protein talin, that mediate integrin activation to allow high affinity binding to ligands. Upon binding ligand, integrins transmit signals that control a wide variety of cellular processes, including structural and mechanical changes, adhesion, and migration (outside-in signaling). Integrin structure and signaling are discussed in detail in recent reviews (Abram and Lowell 2009; Luo et al. 2007).

The functional importance of integrin activation is apparent in patients with LAD type 3/type 1 variant (LAD III/1V), who manifest defects in integrin activation in leukocytes and platelets that lead to immune deficiencies and bleeding disorders. The disease is due to mutations in the gene FERMT3 that encodes Kindlin-3 (Malinin et al. 2009; Moser et al. 2009; Svensson et al. 2009; Kuijpers et al. 2009). Kindlin-3 binds to the distal portion of the cytoplasmic tail of β1 and β3 integrins on platelets

and β2 integrins on leukocytes and mediates inside-out activation (Moser et al. 2008, 2009). Kindlin-3-deficient neutrophils do not firmly adhere to and spread on β2 ligands ICAM-1 and iC3b after stimulation, and show defects in adhesion to endothelial cells and extravasation in response to inflammatory mediators (Moser et al. 2009).

In addition to rapid activation by GPCRs, integrins on neutrophils can also be activated by engagement of L-selectin by glycoproteins, or by binding of selectin ligands on neutrophils by E-selectin (expressed by stimulated endothelial cells) or P-selectin (expressed on platelets and endothelial cells). The transition of integrins from low to high affinity states can be bridged by an intermediate affinity state triggered by rolling on selectins, which can be modulated by hemodynamic shear forces (Alon and Ley 2008). Engagement of PSGL-1 on neutrophils by E-selectin on endothelial cells can activate rolling on ICAM-1 by LFA-1, via Syk, the Src family kinase Fgr, and the immunotyrosine activation motifs (ITAM)-containing adaptors FcRγ and DAP12 (Zarbock et al. 2007a, 2008). PSGL-1 binding to P-selectin expressed by platelets and endothelial cells activated LFA-1 and Mac-1 through a pathway that involved phosphorylation of Naf1 by Src family kinases and subsequent activation of PI(3)K (Wang et al. 2007). Yago showed that E-selectin engages CD44 or PSGL-1 to activate slow rolling on LFA-1 through the Src kinases Hck, Fgr, and Lyn, the adaptors FcRγ and DAP12, Syk, Btk, and p38 MAPK (Yago et al. 2010). Thus binding of endothelial selectins to neutrophil ligands induces signaling pathways that result in activation of β2 integrins.

In vivo, GPCR and selectin-dependent pathways likely cooperate to induce integrin activation. Very little information is available about the integration of these signaling pathways to regulate adhesion and migration in the lungs, although there are hints that this is occurring. In venules of cremaster muscles inflamed by TNF-α, both pertussis-toxin inhibitable G protein-coupled receptors including CXCR2 and E-selectin contributed to neutrophil adhesion (Smith et al. 2004). Similarly, during thioglycollate-induced peritonitis, defects in neutrophil recruitment were observed only when E-selectin-deficient mice were treated with pertussis toxin or when CXCR2-deficient mice were given a blocking anti-E-selectin antibody (Smith et al. 2004). Other studies are pursuing an understanding of the complexities of integrin activation during neutrophil recruitment, including roles for Syk, Galphai2, the Src kinase Fgr, and the adaptors FcR gamma and DAP12 (Zarbock et al. 2007b, 2008; Van Ziffle and Lowell 2009). The contributions of G protein-coupled receptors and selectin ligands to integrin activation are very likely to depend on the stimulus and the recruitment site.

4.3.3.2 Integrin Activation in the Lungs and Integrin-Independent Adhesion and Migration

As discussed in detail above, much of the recruitment of neutrophils to the distal airways and the alveoli occurs through the pulmonary capillaries, which are too narrow to allow rolling (Fig. 4.3). Thus, the selectin- and integrin-mediated processes that mediate neutrophil rolling and tethering in the systemic circulation may not be required for the initial processes of neutrophil sequestration. However, integrin

activation by GPCR and/or selectin signaling may be required for neutrophil adhesion and migration along the endothelium, transendothelial cell migration and travel into the tissues, as well as carrying out their effector function. GPCR signaling is required for neutrophil recruitment and lung injury in a number of models of acute lung injury. Deficiency of the GPCR CXCR2 or blockade of CXCR2 ligand interactions resulted in reduction in neutrophil recruitment and lung injury in a mouse model of ventilation-induced lung injury (Belperio et al. 2002). Signaling through CXCR2 expressed on neutrophils and nonhematopoietic cells mediated neutrophil recruitment and lung injury induced by inhaled LPS (Reutershan et al. 2006). Gαi2 in neutrophils was required for KC-induced neutrophil arrest and for neutrophil recruitment into the lung induced by LPS inhalation (Zarbock et al. 2007b). Engagement of ESL-1 on neutrophils by E-selectin led to activation of the integrin CD11b/CD18 (αMβ2) on microdomains in neutrophils, capture of platelets and release of reactive oxygen species (ROS) in cremasteric venules in a model of transfusion-related acute lung injury (Hidalgo et al. 2009). Blocking E-selectin or CD11b/CD18 but not P-selectin prevented lung injury in this model, suggesting that heterotypic interactions may be occurring in the lung as well.

In the post-capillary venules of the systemic circulation, neutrophil adhesion usually requires the CD11/CD18 adhesion complex. However, neutrophil migration from the pulmonary capillaries to inflammatory sites in the airspaces of the lung utilizes either CD11/CD18-dependent or CD11/CD18-independent mechanisms. Even in CD11/CD18 dependent emigration, inhibition of this adhesion complex blocks only 70–80 % of neutrophils from migrating, and 20–30 % of neutrophils still migrate. The adhesion pathway depends on the stimulus. For example, in the lungs, stimuli that induce primarily CD18-dependent emigration include *E. coli*, *E. coli* lipopolysaccharide, *P. aeruginosa*, IgG immune complexes, IL-1, and phorbol myristate acetate (PMA). Those inducing CD11/CD18-independent neutrophil emigration include *S. pneumoniae*, group B *Streptococcus*, *Staphylococcus aureus*, hydrochloric acid, hyperoxia, pulmonary PMN sequestration early in the course of ventilator-induced lung injury, C5a, and the chemokine KC (Doerschuk 2000; Mackarel et al. 2000; Ridger et al. 2001; Doerschuk et al. 1990; Choudhury et al. 2004). In vitro, fMLP-induced CD18-dependent migration of neutrophils across either pulmonary arterial endothelial cells or HUVECs, whereas IL-8, LTB4, and sputum from patients with purulent bronchiectasis induced CD18-independent neutrophil transendothelial migration (Doerschuk 2000; Mackarel et al. 2000; Ridger et al. 2001; Doerschuk et al. 1990; Morland et al. 2000).

The mechanisms underlying CD18-independent neutrophil recruitment into the lung are poorly understood. CD11/CD18-independent leukocyte migration has also been reported in liver sinusoids, a site where selectin-dependent rolling and tethering also do not occur (reviewed in (Lee and Kubes 2008)) and where binding of CD44 to hyaluronan contributes to neutrophil sequestration in inflamed liver sinusoids (McDonald et al. 2008). However, in the lungs, CD44 had no role in neutrophil migration into the airspaces of the lungs when induced by *S. pneumoniae*, a stimulus that induces neutrophil recruitment through predominantly CD18-independent mechanisms (Wang et al. 2002; van der Windt et al. 2011). However, curiously, in pneumonias induced by E. coli and Klebsiella pneumoniae, stimuli

which induce CD18-dependent neutrophil emigration, CD44 deficiency enhanced neutrophil recruitment (Wang et al. 2002; van der Windt et al. 2010). Studies addressing the adhesion molecules and signaling pathways mediating CD18-independent migration of neutrophils in the lungs have suggested a partial and sometimes small contribution of VLA-4 (CD29d) in mediating neutrophil recruitment during *S. pneumoniae* pneumonia (Tasaka et al. 2002) and for both CD29b and CD29d during KC-induced pulmonary inflammation (Ridger et al. 2001). The mechanisms mediating CD18-independent adhesion in the lungs remain unclear, despite their obvious importance. The remainder of this section will focus on CD18-dependent neutrophil recruitment and signaling into the endothelium.

4.3.3.3 Neutrophil Trafficking from the Capillaries to the Alveoli

Thus, during an acute inflammatory response in the lung, neutrophils rapidly sequester in the pulmonary capillary bed (Fig. 4.3). Endogenous or exogenous chemoattractants alter the biomechanical properties of circulating neutrophils, resulting in a prolonged transit time for a neutrophil to cross the pulmonary capillary bed and neutrophil sequestration. As an inflammatory response progresses, cytokines are produced to activate lung parenchymal cells including the endothelium. Chemokines are induced, which are necessary to activate the integrins on neutrophils and to further signal the neutrophils to stop in the lung. In addition, adhesion molecules such as ICAM-1 are expressed and activated on endothelial cells, and these molecules mediate firm adhesion of neutrophils to the endothelium. Neutrophils then spread and crawl on the endothelial surface until they reach the site for transmigration. Intravascular crawling is mediated by CD11b/CD18 on the neutrophils and ICAM-1 on endothelial cells. This spreading and crawling implies that the adhesive bonds between integrins and their ligands must be displaced while new bonds are formed. This requires coordinated signaling from chemokines, integrins, and ICAM-1. Besides regulating neutrophil adhesion and locomotion on endothelial cells, signaling during adhesion also modulates endothelial cell junctions, and induces expression of inflammatory genes. These responses likely play important roles in the progression of an inflammatory response by modulating endothelial cell permeability, neutrophil transmigration and production of inflammatory mediators. Neutrophil transmigration through the endothelium can occur at endothelial cell borders (paracellular transmigration) or through endothelial cells (transcellular migration) in vitro and in vivo. During paracellular migration another set of adhesion molecules are engaged that include platelet endothelial cell adhesion molecule (PECAM)-1, VE-cadherin, junctional adhesion molecules and CD99 (Muller 2009).

In vivo studies suggest that most neutrophils sequestered within the capillaries of inflamed lungs reenter the circulation, while only a small fraction transmigrate to reach the extravascular space (Doerschuk et al. 1994). Whether this is a random process or whether there are localized responses that guide neutrophils to reach the site of transmigration and how these responses occur remain important questions.

The path neutrophils take from the capillary to the alveoli has been described elsewhere (Burns et al. 2003). In other tissues and often in vitro, whether a sequestered

neutrophil will take a paracellular or transcellular route to migrate through the endothelium appears to depend on the nature of the inflammatory stimulus, the tissue microenvironment, the level of ICAM-1 expression, and the localization of ICAM-1 (Yang et al. 2005; Carman and Springer 2004; Feng et al. 1998; Cinamon et al. 2004; Millan et al. 2006; Nieminen et al. 2006; Woodfin et al. 2010). However, in the pulmonary microvasculature, the majority of the neutrophils (and perhaps nearly all) take the paracellular route between endothelial cells (Burns et al. 2003; Walker et al. 1995; Behzad et al. 1996). Fibroblasts in the interstitium may guide the migrating neutrophils from the endothelial cell junctions to discontinuities at the basal aspect of the epithelial borders. Neutrophils then migrate into the alveolar space through tricellular corners at the junctions of type I and type II epithelial cells (Burns et al. 2003; Walker et al. 1995; Behzad et al. 1996). These sites may be preferred due to the discontinuous nature of the tight junctions. A role for both the leukocyte adhesion complex CD11/CD18 and the β1 integrin CD29 are important in neutrophil migration through the pulmonary interstitium toward the alveolar space (Ridger et al. 2001; Ong et al. 2003).

Once in the alveolar space, neutrophils adhere to the apical surface of epithelial cells, possibly through ICAM-1 interactions. Interestingly, a soluble form of ICAM-1 (sICAM-1) is present in the plasma and in the lung lining fluid. sICAM may be generated through alternative splicing or by proteolytic cleavage of the membrane-bound form (mICAM-1). The mechanisms of sICAM-1 production are different in type I alveolar epithelial cells (AEC) and pulmonary microvascular endothelial cells (PVEC). High baseline release of sICAM-1 in AEC and presence in the alveolar epithelial lining fluid were observed. TNFα or LPS had little effect on sICAM-1 expression in AEC, but greatly increased sICAM-1 from PVEC (Mendez et al. 2008). AEC sICAM-1 shedding was inhibited by a serine protease inhibitor; whereas protease inhibitors had no effect on PVEC sICAM-1 expression. These differences may reflect the roles of sICAM-1 in the vasculature versus the air spaces. Overexpression of sICAM-1 in the alveolar space distal lung resulted in decreased survival after intranasal infection with *K. pneumoniae* (Mendez et al. 2011). At 24 h, a greater percentage of the transgenic mice (SPC-sICAM-1) had bacteria in the spleen compared with WT mice, while bacterial burden in the lungs was similar, and the number of neutrophils in the BALF was threefold greater in the SPC-sICAM-1 mice. These data suggest that sICAM-1 modulates host defense toward pathogens in the lung.

4.4 Neutrophil Functions in the Lung

4.4.1 Bactericidal Functions

Upon arrival at the site of infection, neutrophils contribute to microbial killing by binding and phagocytosing pathogens, and subsequently releasing highly toxic granule contents and radicals into the phagosome (Fig. 4.4). Oxidant production and other effector functions is enhanced by priming neutrophils with inflammatory

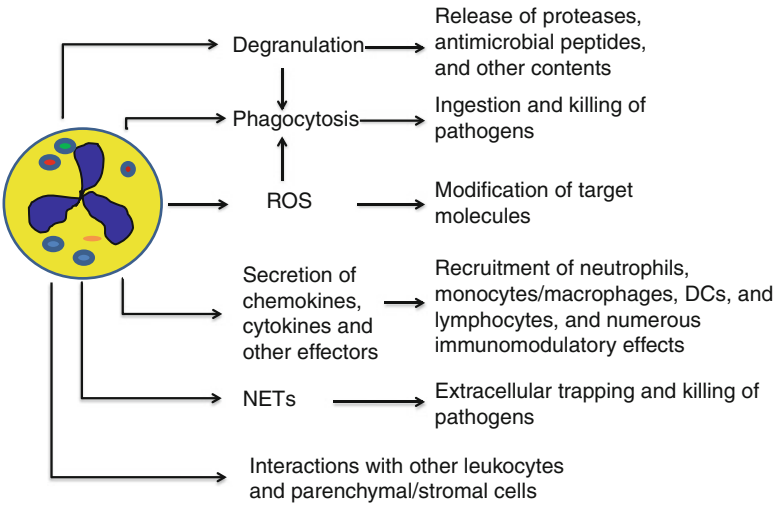

Fig. 4.4 Neutrophil effector functions during inflammation. Release of granule contents and reactive oxygen species into phagosomes or into the surrounding environment occurs upon stimulation. Granule contents and ROS have direct antimicrobial effects, as well as modulating the inflammatory response. Other neutrophils functions include secretion of soluble signaling molecules, extracellular pathogen killing through NETs, and regulating host defense through direct interactions with cells present in the inflammatory milieu, including other leukocytes

mediators or chemokines and cytokines, including TNF-α, IL-8, or IFN-γ, or by the processes of adhesion and migration. The recognition of microbes or microbial products may be mediated by pattern recognition receptors, including toll-like receptors, C-type lectin receptors, NLRs, and RIG-I helicase receptors. Human neutrophils have been demonstrated to express all the TLRs except TLR3, and also express CLEC7A (dectin 1), CLEC2 (CLEC1B), RIG-I, MDA5, NOD1, and FPR1 (reviewed in (Mantovani et al. 2011)).

Uptake of bacteria and other particles by phagocytes (neutrophils, macrophages, and to a lesser extent dendritic cells) is markedly enhanced by coating the particle surface with opsonins, which include antibodies, complement fragments, pentraxins, and collectins such as mannan- binding lectin and in the lung, SP-A and SP-D (Greenberg and Grinstein 2002). Targets coated with antibodies or complement fragments bind phagocytic receptors expressed on the surface of phagocytes (Lee et al. 2003; Flannagan et al. 2009). The main phagocytic receptors in neutrophils are Fcγ receptors that bind particles coated with IgG, and complement receptors that recognize particles coated with complement fragments (Greenberg and Grinstein 2002; Lee et al. 2003). The low affinity Fc receptors FcγRIIIB and FcγRIIA are constitutively expressed in human neutrophils, whereas the high affinity receptor FcγRI is induced by the treatment with G-CSF or IFN-γ (Repp et al. 1991; Cassatella et al. 1990; Nimmerjahn and Ravetch 2006). FcγRIIA has ITAM in its cytoplasmic domain which become phosphorylated upon ligand binding. FcγRIIIB is GPI-anchored to the cell membrane and may signal intracellularly by acting in concert with FcγRIIa in lipid microdomains (Chuang et al. 2000; Marois et al. 2011).

FcγRI has a short cytoplasmic tail and requires an associated gamma-chain to induce signaling. Binding of ligands to Fc receptors leads to receptor clustering and recruitment of Src family kinases, which phosphorylate tyrosine residues in the ITAM domains, leading to recruitment of the tyrosine kinase Syk and activation of small GTPases, and triggering a signaling cascade that mediates target engulfment (Lee et al. 2003; Flannagan et al. 2009).

Complement receptor 3 (CR3) is the leukocyte integrin CD11b/CD18 (Mac-1), and it binds particles coated with C3bi, a cleavage product of C3b which is in turn produced by proteolysis of C3 during complement activation. Whereas phagocytosis of IgG-coated particles is characterized by the extension of pseudopods that surround and engulf the target, complement-coated targets are observed to sink into the cell, indicating distinct mechanisms of engulfment (Lee et al. 2003). Crosstalk between the Fc receptors and complement receptors modulate their respective activities. For example, in human monocytes, CR3-mediated phagocytosis is inhibited by binding FcγRI and enhanced by binding FcγRII (Huang et al. 2011).

The nascent phagosome undergoes a complex process of maturation, whereby it acquires the machinery and materials for microbial killing and degradation (reviewed in (Lee et al. 2003; Flannagan et al. 2009; Nordenfelt and Tapper 2011)). The process culminates in the release of granule contents into the phagosome and the assembly and activation of NADPH oxidase on the membrane. In contrast to macrophages, phagocytosis in neutrophils occurs in seconds rather than minutes, maturation involves the fusion of preformed granules with the phagosome rather than the endocytic maturation pathway in macrophages, large amounts of oxidants are produced through NADPH oxidase activity, and the phagosomal pH is neutral rather than acidic (Nordenfelt and Tapper 2011; Bianchi et al. 2009).

Neutrophil granule contents are synthesized and packaged during neutrophil development in the bone marrow (Borregaard and Cowland 1997; Borregaard et al. 2007; Faurschou and Borregaard 2003). Neutrophil granules are generally classified based on the timing of their synthesis and their major contents. Primary (azurophil) granules are made earliest and contain myeloperoxidase, as well as serine proteases, defensins, and bactericidal permeability-increasing protein. Specific or secondary granules are peroxidase-negative and contain lactoferrin, as well as the cathelicidin hCAP-18 (precursor for the antimicrobial peptide LL-37), whereas tertiary granules contain gelatinase. The membrane of specific granules contains the cytochrome b558 moiety of NADPH oxidase which is incorporated into the phagosome and cell membrane upon neutrophil activation. Secretory vesicles are made during the terminal stages of maturation and enriched in receptors including the complement receptors CR1 (CD35) and CR3 (CD11b/CD18), and albumin. The membranes of secretory vesicles are incorporated into the plasma membrane in response to chemotactic factors and early during leukocyte recruitment, thus supplying membrane proteins including adhesion receptors that are critical for neutrophil function. Using a calcium ionophore or the chemotactic peptide fMLF to induce degranulation, the contents of granules are released in the opposite order to their synthesis: secretory vesicles are mobilized most readily, the threshold for exocytosis of gelatinase granules, and specific granules are progressively higher (Sengelov et al. 1993).

Non-oxidative killing of microbes by neutrophils is effected by antimicrobial peptides and proteases stored in granules and released into the phagosome, including serine proteases, matrix metalloproteinases, and various antimicrobial peptides. In addition to their role in killing pathogens in the phagosome, neutrophil granule contents may also kill pathogens extracellularly in neutrophil extracellular traps (NETs, described in the following section).

The neutrophil serine proteases neutrophil elastase (NE), cathepsin G (CG), and proteinase-3 are structurally related, abundantly expressed enzymes stored in the azurophilic granules (Pham 2006, 2008). Before being packaged into primary granules, the proenzyme form of these serine proteases are processed by dipeptidyl peptidase (DPPI, or cathepsin C) to yield the active forms. Serine proteases can cleave a large variety of substrates, including bacterial constituents, components of the extracellular matrix, plasma proteins, cytokines, and growth factors (Pham 2006, 2008). The diversity of substrates indicates that neutrophil serine proteases may play many roles in the inflammatory response.

Neutrophil serine proteases have been shown to kill a variety of microbial pathogens in vitro. For example, purified NE or CG kill *S. pneumoniae* in vitro, and inhibitors of serine proteases abrogate this microbicidal activity (Standish and Weiser 2009). NE degrades virulence factors of Gram-negative enterobacteria *Shigella*, *Salmonella*, and *Yersinia* (Weinrauch et al. 2002). Serine proteases can regulate host defense by proteolytic modification of cytokines and chemokines which results in either enhanced activity or inactivation of their targets, and by activation of specific cellular receptors (Pham 2006, 2008). For example, serine proteases process IL-8 to more active truncated forms (Padrines et al. 1994), NE induces apoptosis of lung epithelial cells through PAR-1 activation (Suzuki et al. 2005, 2009), and CG and NE cleave IL-33 into active mature forms (Lefrancais et al. 2012).

The role of serine proteases in infection models have been tested in vivo using mice that are deficient in one or a combination of these enzymes, or mice deficient in DPPI. DPPI-deficient mice have no defect in clearance of the fungus *Aspergillus fumigatus* from the lung, and studies of bacterial clearance remain to be pursued (Vethanayagam et al. 2011). Surprisingly, DPPI deficiency was protective in a murine model of sepsis due to increased levels of IL-6 (Mallen-St Clair et al. 2004). Humans with Papillon–Lefevre syndrome due to DPPI deficiency have pyogenic liver abscesses, but no clear defect in neutrophil killing of *S. aureus* or *E. coli* (Pham et al. 2004; Almuneef et al. 2003). NE-deficient mice exhibit impaired host defense following intraperitoneal infection with *K. pneumoniae* and *E. coli* but not *S. aureus* (Belaaouaj et al. 1998). NE null mice have increased susceptibility and impaired bacterial killing in *P. aeruginosa* pneumonia (Hirche et al. 2008). However, NE is not required for neutrophil recruitment into the lungs or peritoneum in response to *P. aeruginosa* or LPS (Hirche et al. 2004). Purified CG does not inhibit the growth of *S. aureus*, *K. pneumoniae*, or *E. coli*, and no defect in clearance or survival upon challenge in vivo with any of these organisms (MacIvor et al. 1999). In a model of pneumonia induced by *S. pneumoniae*, mice deficient in CG or in both CG and NE had reduced survival and increased bacterial load (Hahn et al. 2011). These studies indicate that the serine proteases have site- and organism-specific and often nonredundant roles in host defense against microbes.

The matrix metalloproteinases are a family of zinc-dependent endopeptidases expressed in many cell types, including neutrophils, that degrade extracellular matrix components and are thus implicated in tissue remodeling, but also regulate host defense by targeting cell adhesion molecules, cytokines, and growth factors (Greenlee et al. 2007). MMP-8 (neutrophil collagenase) and MMP9 (gelatinase B) are stored in secondary granules and secreted or expressed on the surface upon activation. The levels of MMP-8 and MMP-9 were elevated in lung lavage fluid and plasma from patients with hospital-acquired pneumonia (Hartog et al. 2003), suggesting that these MMPs may play a role during pulmonary infections. Membrane-expressed MMP8 has recently been shown to cleave MIP-1α and attenuate injury in LPS-induced ALI (Quintero et al. 2010). IL-8 binding to CXCR2 stimulates the release of MMP9 (gelatinase B) which processes IL-8 to increase its activity (Van den Steen et al. 2000; Chakrabarti and Patel 2005). A recent study demonstrated that MMP9 (gelatinase B) was critical for efficient phagocytosis and superoxide production by neutrophils, and cleaved IL-17A in vitro (Hong et al. 2011). MMP2/9 double-deficient mice have more neutrophils in the lungs, greater bacterial load, and are more susceptible to *S. pneumoniae*-induced acute pneumonia (Hong et al. 2011). Clearly MMP-8 and MMP-9 can modulate the host response by processing some cytokines directly, but their effect can also be indirect, for example collagen digestion by MMP-8, MMP-9, and prolyl endopeptidase generates proline-glycine-proline fragments which are potent neutrophil chemoattractants (Gaggar et al. 2008; Weathington et al. 2006).

The antimicrobial peptides defensins and cathelicidins are small cationic peptides with antimicrobial and immune regulatory properties (Yang et al. 2004). There are four human neutrophil α-defensins, human neutrophil peptides 1–4, which are stored in primary granules and released upon neutrophil activation (Lehrer and Lu 2012). The human cathelicidin hCAP18 is cleaved by serine proteases to yield the antimicrobial fragment LL-37. Defensins and cathelicidins can kill a broad range of pathogens through permeabilization of target membranes. In addition to their antimicrobial effects, these antimicrobial peptides can modulate immune responses by serving as alarmins to recruit and activate immune cells (Chertov et al. 1996; Grigat et al. 2007).

In resting neutrophils, the NADPH oxidase complex is separated into cytoplasmic and membrane-bound components. When neutrophils are activated by a variety of stimuli, including inflammatory mediators, adhesion via integrins or binding of opsonized particles, the cytoplasmic components of the phagocyte NADPH oxidase (p40, p47, p67, and the small GTPase Rac) associate with the membrane bound cytochrome b558 heterodimer of gp91 and p22 to assemble the functional enzyme that catalyzes the production of superoxide from molecular oxygen (Quinn and Gauss 2004; Nauseef 2004; Babior 2004). Superoxide anion is converted spontaneously or enzymatically into hydrogen peroxide, which is converted by myeloperoxidase into the potent microbicide hypochlorous acid. Superoxide and other ROS formed downstream can interact with a large variety of molecules and alter their target structure and function. Fusion and release of granule contents into the phagosome is coincident with NADPH oxidase activity, so that the engulfed bacterium is exposed to high ROS levels, proteases, and a plethora of antimicrobial proteins. NADPH oxidase-generated oxidants are generally thought to be major effectors of

bacterial killing. An alternative hypothesis has implicated protease activation in the phagosome by ion fluxes generated during NADPH oxidase activation, but this remains controversial (Segal 2005; Nauseef 2007). It seems clear, however, that oxidase activity is required for host defense caused by some organisms. For example, in mice with pulmonary *A. fumigatus* or systemic *Burkholderia cepacia* infection, NADPH oxidase activity rather than serine proteases was required for protection (Vethanayagam et al. 2011). Interestingly, NADPH oxidase appears to downmodulate inflammation in certain conditions (Marriott et al. 2008; Morgenstern et al. 1997; Segal et al. 2010), most clearly through ROS-mediated inactivation of chemokines, cytokines, and other inflammatory mediators. Consistent with both the microbicidal functions and the inflammatory downmodulatory effects, patients with chronic granulomatous disease characterized by having defects in NADPH oxidase function often have *B. cepacia* and *A. fumigatus* infections and over-exuberant sterile inflammation.

ROS can modify the function of many signaling molecules by targeting thiols on cysteine residues. Signaling molecules that are targeted by ROS leading to changes in their signaling pathways include the protein tyrosine phosphatases, the small GTPase Rho, and Src kinases. Notably, a recent paper showed that NADPH oxidase deficiency or inhibiting NADPH oxidase led to defects in chemotaxis in human neutrophils, implicating NADPH oxidase-generated oxidants as regulators of neutrophil chemotaxis (Hattori et al. 2010). NADPH oxidase is also required for the production of IFN-γ by neutrophils during *S. pneumoniae* pneumonia (Yamada et al. 2011). NADPH oxidase is required for elaboration of NETs, as discussed in the following section.

4.4.2 NET Formation

NETs were first described as extracellular structures composed of decondensed chromatin with histones and granular contents that are able to bind and kill bacteria (Brinkmann et al. 2004). Inducers of NET formation include bacteria and their components, fungi, PMA, cytokines, and chemokines including IL-8 and IFN-γ (von Kockritz-Blickwede and Nizet 2009). The formation of NETs is initially marked by chromatin decondensation, perhaps through posttranslational histone modification (Neeli et al. 2008, 2009; Li et al. 2010) or through cleavage by neutrophil elastase translocated to the nucleus (Papayannopoulos et al. 2010). This is followed by breakdown of the nuclear and granular membranes and contact between granular contents and nuclear material, and subsequently the cell ruptures and NETs are released extracellularly (Fuchs et al. 2007). NET formation requires NADPH oxidase (Bianchi et al. 2009; Fuchs et al. 2007) and is regulated by neutrophil elastase and myeloperoxidase (Papayannopoulos et al. 2010; Metzler et al. 2011). This pathway of NET formation usually occurs over a span of several hours and is likely a form of cell death (Fuchs et al. 2007; Remijsen et al. 2011a). Rapid NET formation has also been induced by platelet binding to neutrophils after TLR4 stimulation (Clark et al. 2007).

Other pathways of NET formation occur more rapidly and may not require lytic cell death. Live GM-CSF-primed neutrophils form extracellular nets in response to TLR4 or C5a signaling by extruding their mitochondrial DNA (Yousefi et al. 2009). Release of chromatin in vesicles from intact neutrophils induced by *S. aureus*, distinct from later lytic release of DNA, has been reported as an oxidant-independent pathway of NET production (Pilsczek et al. 2010). The Raf-MEK-ERK pathway was identified by a chemical inhibitor screen as a critical step for NET formation, likely upstream of NADPH oxidase (Hakkim et al. 2011). Notably, PKC inhibition by staurosporine led to a marked decrease in NET induction by PMA and PAF but not by *Helicobacter pylori*, indicating some redundancy in NET signaling pathways depending on the stimulus.

NETs have been reported to kill bacteria, fungi, and protozoa in vitro (Brinkmann et al. 2004; Fuchs et al. 2007; Guimaraes-Costa et al. 2009; Parker et al. 2012; Urban et al. 2006, 2009; Young et al. 2011). The antimicrobial functions of NETs are due to killing by histones, oxygen radicals, and microbicidal granular contents and to trapping of pathogens in the chromatin mesh (Brinkmann et al. 2004; Clark et al. 2007; Parker et al. 2012; Urban et al. 2006, 2009; Papayannopoulos and Zychlinsky 2009). NETs from human neutrophils stimulated with PMA contained molecules with known antimicrobial properties: granular proteins (leukocyte elastase, lactotransferrin, azurocidin, cathepsin G, myeloperoxidase, leukocyte proteinase-3, lysozyme C, and neutrophil defensins 1 and 3), nuclear components (histones), and the cytoplasmic calprotectin complex (Urban et al. 2009).

Determining the specific contribution of NETs in host defense in vivo is complicated because many of the neutrophil constituents present in NETs and the processes required in NET formation are involved in other antimicrobial processes (Fig. 4.4). Supporting an antimicrobial role for NETs in vivo is the observation that bacterial pathogens appear to have evolved mechanisms to counter them. Pathogenic group A streptococci possess endonucleases that allow them to escape from NETs (Buchanan et al. 2006; Walker et al. 2007). Pneumococcal strains express an endonuclease that allows them to escape from NETs, and mutant pneumococci lacking endonuclease have impaired ability to spread to the lung and blood in mice infected intranasally (Beiter et al. 2006). Pneumococci also modify their surface charge to repulse antimicrobial peptides present in NETs and synthesize a capsule that enables them to evade entrapment (Wartha et al. 2007).

NETs may have deleterious effects by entangling or activating host immune cells, or by directly damaging tissues through contact with NET contents. NETs have been implicated in the pathogenesis of autoimmune diseases (Hakkim et al. 2010; Kessenbrock et al. 2009; Leffler et al. 2012; Garcia-Romo et al. 2011; Lande et al. 2011), gout (Mitroulis et al. 2011), sepsis (Clark et al. 2007), and venous thrombosis (von Bruhl et al. 2012; Brill et al. 2012; Fuchs et al. 2010). In vitro, NETs damage endothelial cells and the lung epithelial cancer cell line (A549) likely through the activities of proteases and histones (Clark et al. 2007; Saffarzadeh et al. 2012; Gupta et al. 2010).

NETs may play a role in host defense in the lung during bacterial pneumonias. NETs were induced in a murine model of acute pneumonia induced by *K. pneumoniae*

(Papayannopoulos et al. 2010) and *S. pneumoniae* (Yamada et al. 2011; Beiter et al. 2006; Wartha et al. 2007). Notably fewer NETs were observed when pneumonia was induced by *E. coli* (Yamada et al. 2011). IFN-γ production by neutrophils was required for NET formation and decreased bacterial load (Yamada et al. 2011).

4.4.3 Recruitment of More Neutrophils from the Bone Marrow and Blood

Inflammatory mediators emanating from the inflammatory stimulus itself (bacterial constituents such as fMLP, for example), or produced by resident cells and cells recruited to the lung during the infection can recruit neutrophils into the lungs from the blood and bone marrow. Alveolar macrophages, epithelial cells, and endothelial cells produce mediators that recruit neutrophils. Among these mediators are complement fragments, lipid mediators such as LTB4, cytokines such as IL-1β and TNF-α, and chemokines such as IL-8. Neutrophils contribute to this process in several ways, primarily by producing mediators themselves. For example, human neutrophils make lipid mediators and IL-8, and are thus able to attract more neutrophils into the site of inflammation. LL-37, a cathelicidin released upon degranulation, is chemotactic for neutrophils, monocytes, and T lymphocytes (De et al. 2000). Neutrophil proteases may also contribute to this process by modifying chemokines and their receptors, or cleaving collagen to generate the chemoattractant PGP (discussed previously).

4.4.4 Immunoregulatory Functions

The complex and critical role of neutrophils in regulating innate and adaptive immunity is increasingly being appreciated (Mantovani et al. 2011). In addition to their well-described roles in pathogen killing and clearance, neutrophils regulate the immune response by producing and releasing molecules with immunoregulatory activities (Fig. 4.4). Proteases, antimicrobial peptides, and radicals that are released upon neutrophil activation can regulate the functions of surrounding cells, recruit immune cells, or act on signaling molecules in the surrounding milieu, as described in the previous sections. Neutrophils can also produce soluble cell signaling molecules, including potent cytokines such as IFN-γ and TNF-α, chemokines such as IL-8, growth factors such as G-CSF, pattern recognition molecules that enhance pathogen recognition by neutrophils and other cells, and lipid-derived mediators (Mantovani et al. 2011). For example, neutrophils make IFN-γ in pneumonias induced by *S. pneumoniae* and *S. aureus*, but not by *E. coli* or *P. aeruginosa* (Yamada et al. 2011). Production of IFN-γ by neutrophils requires NADPH oxidase. IFN-γ modulates NET production and improves bacterial clearance (Yamada et al. 2011).

Direct contact between neutrophils and other cells can modulate their respective functions. For example, engulfment of apoptotic neutrophils modifies macrophage function, as described below. Crosstalk through direct interactions between

neutrophils and monocytes, dendritic cells, or T cells, leading to modulation of the subsequent immune response, has also been demonstrated. For example, CD49d-expressing neutrophils induce FcεRI expression on lung dendritic cells, facilitating the recruitment of Th2 cells in a murine model of post-viral asthma (Cheung et al. 2010). Finally, neutrophils are critical for resolving inflammation or setting the stage for the immune response, as described in the next section.

4.4.5 Resolution of Inflammatory Response or Transition from Inflammatory to Innate Immune Response

Resolution is not merely the passive cessation of pro-inflammatory responses, but rather requires an active response from the host, resulting in tissue repair and return to homeostasis. Ideally, neutrophilic inflammation is self-limiting and resolves with the proper removal of apoptotic neutrophils by scavenging macrophages and tissue repair. Several classes of lipid-derived mediators of resolution have been identified including lipoxins, D- and E-series resolvins, protectins, and maresins. Resolvins bind G protein-coupled receptors, decreasing neutrophil recruitment and downregulating production of inflammatory mediators and ROS, while promoting apoptotic neutrophil uptake by macrophages (Uddin and Levy 2011). For example, in a model of acid aspiration-induced lung injury and bacterial challenge with *E. coli*, prophylactic treatment with RvE1 decreased neutrophil accumulation in the lungs and bacterial load, and improved survival (Seki et al. 2010). These were associated with decreased levels of pro-inflammatory mediators IL-1beta, IL-6, HMGB-1, MIP-1alpha, MIP-1beta, keratinocyte-derived chemokine, and MCP-1 (Seki et al. 2010). RvD1 and RvD2 target leukocytes and reduce neutrophil recruitment (Spite et al. 2009).

A role for CD44 has also recently been described in the process of resolution. Interestingly, deficiency of CD44 results in improved clearance of either *S. pneumoniae* or *K. pneumoniae* (van der Windt et al. 2010, 2011), suggesting that CD44 facilitates bacterial growth and dissemination. However, CD44-deficient mice also show delayed resolution of the inflammatory process (van der Windt et al. 2010, 2011) suggesting that CD44 contributes to resolution through its interactions with hyaluronan or other ligands. Studies investigating bleomycin-induced lung inflammation and fibrosis also show that CD44 is important in resolving the inflammatory process (Teder et al. 2002).

In addition to their immunoregulatory and pro-resolution functions described previously, neutrophils can modulate the transition from inflammation to innate immune response by activating antigen presenting cells through alarmins. Lactoferrin, α-defensins, cathelicidin, and HMGB-1 are considered alarmins for their ability to serve as endogenous danger signals that alert the immune system (Yang and Oppenheim 2009). Neutrophils can also modulate monocyte recruitment by releasing granule proteins that activate endothelial cells or modify chemokines by proteolysis, and neutrophils and monocytes/macrophages interact and regulate each other's functions (Soehnlein and Lindbom 2010; Soehnlein et al. 2009). For example, uptake of apoptotic neutrophils modulates macrophage function, as described below.

4.5 Clearance of Neutrophils

4.5.1 Apoptosis

The fate of emigrated neutrophils can be one of the several death pathways with varying inflammatory and immunogenic consequences for the host; apoptosis, NETosis and necrosis (Fig. 4.1) (Bratton and Henson 2011). Neutrophil apoptosis occurs via the intrinsic and extrinsic pathways resulting in death without release of cellular contents that may otherwise be released into the surrounding tissue and amplify inflammation (Bratton and Henson 2011; Witko-Sarsat et al. 2011; Fox et al. 2010). As described in several recent reviews (Witko-Sarsat et al. 2011; Fox et al. 2010), the distinct features of neutrophil apoptosis include the key role of the prosurvival factor Mcl-1, the dual role of TNFα which can induce apoptosis or prolong the lifespan of neutrophils, the proapoptotic role of ROS and NADPH oxidase activation, and the role of the granule protein cathepsin D which can activate caspase-8 (Conus et al. 2008, 2012) or process the pro-apoptotic Bcl-2 protein Bid to promote apoptosis (Blomgran et al. 2007). These and other pathways may be modified during the inflammatory response to prolong neutrophil lifespan. For example, cathepsin D release in the cytosol is blocked during inflammation, inhibiting its activation of caspase 8 (Conus et al. 2008). These reviews and the studies described therein highlight the complex nature of apoptosis programs in neutrophils, and the differences between spontaneous versus inflammation-induced apoptosis, indicating that regulation of neutrophil life span may be a therapeutic target.

Apoptotic neutrophils are removed by scavenging macrophages (efferocytosis) that are attracted to the site by "find me" signals and recognize the so-called "eat me" signals on the surface of apoptotic neutrophils (Bratton and Henson 2011). Diffusible find me signals include extracellular nucleotides (ATP and UTP), while the most studied "eat me" signal is phosphatidylserine, which can directly engage receptors on macrophages or bind soluble pattern recognition molecules that are recognized by macrophage receptors (Bratton and Henson 2011). In the inflammatory microenvironment, pattern recognition molecules that coat apoptotic target cells facilitate efferocytosis (Litvack and Palaniyar 2010). For example, the lung collectins SP-A and SP-D regulate inflammatory mediator production and efferocytosis in contrasting ways, depending on the signaling complexes bound and the phagocyte subset (Janssen et al. 2008; Gardai et al. 2003).

During inflammation, the induction of neutrophil apoptosis and clearance of apoptotic neutrophils limit tissue injury and promote resolution (Fox et al. 2010; El Kebir and Filep 2010; Elliott and Ravichandran 2010). Apoptotic neutrophils are generally functionally spent, and uptake of apoptotic neutrophils by macrophages prevents the release of toxic neutrophil contents into the tissues. Phagocytosis of apoptotic cells by macrophages suppresses macrophage release of pro-inflammatory mediators such as TNFα, and increases macrophage release of anti-inflammatory mediators such as IL-10 and TGFβ (Fox et al. 2010; El Kebir and Filep 2010; Elliott and Ravichandran 2010). Neutrophil apoptosis as well as clearance of apoptotic

neutrophils is regulated by pro-inflammatory cytokines and toll-like receptor agonists. Pro-inflammatory mediators such as TNF-α, high mobility group protein-1 and toll-like receptor agonists such as LPS, peptidoglycans, and CpG DNA can rescue neutrophils from apoptosis and inhibit apoptotic cell clearance in the lung (Borges et al. 2009; Liu et al. 2008; Michlewska et al. 2009; Banerjee et al. 2011).

4.5.2 NETosis

NETosis was coined to describe the death of neutrophils associated with the production of NETs, described above (Steinberg and Grinstein 2007). Morphologically, NETosis proceeds in the following stages: initially, the nuclei lose their lobules and chromatin begins to decondense; then, the nuclear envelope vesiculates and chromatin is decondensed, subsequently the granules disappear, and the chromatin mixes with cytoplasmic and granular contents, as evidenced by colocalization of elastase with chromatin (Fuchs et al. 2007). Compared with apoptosis and necrosis, the distinguishing morphological features of NETosis are the mixing of nuclear and cytoplasmic constituents after the nuclear membrane disintegrates and the disappearance of internal membranes and cytoplasmic organelles (Fuchs et al. 2007). In contrast to apoptosis, neutrophils undergoing NETosis do not exhibit chromatin condensation or DNA fragmentation, do not express PS on the outer surface of the cell membrane prior to cell rupture, and are resistant to pan-caspase inhibitor (Fuchs et al. 2007; Remijsen et al. 2011a, b). Notably, NETs may be produced without undergoing NETosis, as in the case of neutrophils that extrude mitochondrial DNA (Yousefi et al. 2009) or neutrophils that release chromatin in vesicles (Pilsczek et al. 2010).

4.5.3 Necrosis

Primary necrosis occurs when emigrated neutrophils simply disintegrate at the site of inflammation, releasing their contents into the surrounding environment, with potentially deleterious effects due to the cytotoxic properties of their contents and to increased inflammation. Secondary necrosis occurs when apoptotic neutrophils are not taken up by scavenging phagocytes because the scavenging capacity is exceeded by a large number of apoptotic cells or the macrophages themselves are damaged or targeted by pathogens (Silva 2010a, b). Secondary necrosis may be particularly relevant in the pneumonia, where accumulation of apoptotic neutrophils leads to increased inflammation without increased bacterial load (Silva 2010b; Haslett 1999). A pathway of programmed necrosis or necroptosis occurring via death receptor signaling and requiring the kinases RIP1 or RIP3 has also been described (Vandenabeele et al. 2010; Han et al. 2011). Neutrophils exposed to *Shigella flexneri* underwent death by this pathway rather than apoptosis (Francois et al. 2000).

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Chapter 5
Toll-Like Receptors in the Airway Epithelium

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The immune response to infection is initiated by the detection of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) distributed on the cell surface, within endosomal structures, and in the cytoplasm (Kawai and Akira 2011; Kumar et al. 2011). The mammalian network of PRRs includes toll-like receptors (TLRs), which signal responses to a broad range of microbial ligands (Kawai and Akira 2011); C-type lectin receptors such as dectin-1, which recognizes fungal beta-glucan (Kawai and Akira 2011; Osorio and Reis e Sousa 2011); the large family of nucleotide-binding oligomerization domain-like receptors that respond to diverse microbial components (Broz and Monack 2011; Elinav et al. 2011); the RIG-I-like helicases that detect viral RNA (Loo and Gale 2011); and incompletely characterized cytosomal DNA sensors (Barbalat et al. 2011; Hornung and Latz 2010). Recognition of specific microbial ligands by these receptors triggers signaling events that activate components of the innate immune response, including the induction of pro-inflammatory cytokines, the release of antimicrobial peptides, and phagocyte activation. TLRs are the best characterized PRRs for activation of immune responses to bacteria in the lungs, and are the focus of this chapter.

5.1 Toll-Like Receptors

TLRs are members of the Toll-IL-1 receptor superfamily. They are type-1 transmembrane glycoproteins with an extracellular domain composed of leucine-rich repeats that confer ligand specificity, a transmembrane component, and a cytoplasmic

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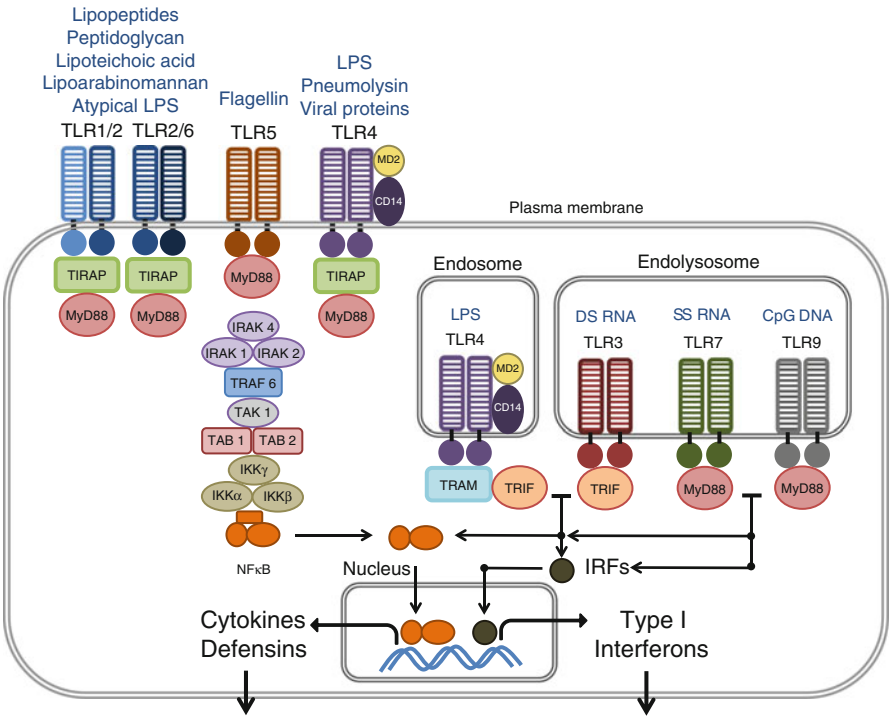

Fig. 5.1 Activation of innate immunity by Toll-like receptor (TLR) mediated recognition of pathogen-associated molecular patterns (PAMPs) at the cell surface and within endosomal structures

Toll-IL-1 receptor domain that is required for downstream signaling (Kumar et al. 2011). Ten human and 12 mouse TLRs have been described that vary in pattern recognition, the use of co-receptors, subcellular location, and tissue distribution (Kumar et al. 2011). Humans and mice share TLRs 1–9, whereas human TLR 10 has no known murine analog, and mouse TLRs 11–13 are unknown in humans.

TLRs recognize diverse PAMPs (Fig. 5.1). TLR2 senses the broadest range of microbial components, including lipopeptides that are found in the cell walls of all bacteria (Kawai and Akira 2010, 2011; Kumar et al. 2011). TLR2 forms heterodimers with TLR1 and TLR6 that create distinct recognition domains. Triacyl-lipopeptides are recognized by TLR1/TLR2 heterodimers, whereas diacyl-lipopeptides are detected by the TLR2/TLR6 heterodimer. TLR2 also senses peptidoglycan and lipoteichoic acid found in the cell walls of Gram-positive bacteria, lipoarabinomannan from mycobacteria, and certain atypical forms of lipopolysaccharide (LPS) (Kawai and Akira 2010, 2011; Kumar et al. 2011). In addition, TLR2 detects fungal zymosan, protozoal glycolipids, and components of measles and vaccinia viruses (Kawai and Akira 2010, 2011). TLR4 is the primary LPS receptor, acting in concert with a secreted protein, MD-2, and facilitated by surface bound CD14 and serum LPS-binding protein (Kawai and Akira 2010). TLR4 also detects pneumolysin made by *Streptococcus pneumoniae* (Malley et al. 2003; Dessing et al. 2009) and

several viral envelope proteins (Kawai and Akira 2010, 2011). Flagellin, the major structural protein of flagella, is the only known ligand for TLR5 (Kawai and Akira 2010). TLR3 recognizes double-stranded RNA, including genomic RNA of rheoviruses and double-stranded RNA produced during the replication of single-stranded RNA viruses such as RSV and influenza (Kawai and Akira 2011). TLR7 and 8 detect single-stranded RNA, as found in influenza, HIV, and some bacteria (Kawai and Akira 2011). TLR9 detects unmethylated cytidine phosphate guanosine (CpG) DNA motifs that are common in bacteria and some viruses, but rare in mammalian cells (Kawai and Akira 2010, 2011; Kumar et al. 2011). Thus, the TLRs that are involved in recognition of bacterial ligands are TLR 2 (along with its co-receptors TLR1 and TLR6), TLR4, TLR5, and TLR9.

TLR pattern recognition is compartmentalized within the cell, which facilitates stratification of downstream signaling and protection from autoimmunity (Kawai and Akira 2010, 2011; Barton and Kagan 2009; Blasius and Beutler 2010). TLRs 1, 2, 4, 5, and 6 are found on the plasma membrane, where they trigger rapid, NFκB-dependent responses to extracellular microbial components (Fig. 5.1). Surface binding of LPS by TLR4 triggers dynamin-mediated endocytosis of the receptor complex that results in secondary signaling, using alternate adaptors, leading to delayed NFκB activation and type I interferon production (Kawai and Akira 2010; Blasius and Beutler 2010) (Fig. 5.1). TLR2 also can be internalized after ligand binding, with resultant type I interferon production (Kawai and Akira 2011). All nucleic acid recognition occurs within endosomal compartments, a key adaptation to prevent receptor activation to self. TLRs 7, 8, and 9 are localized to endoplasmic reticulum in unstimulated cells, but rapidly traffic to endolysosomes by a highly regulated process (Kawai and Akira 2010, 2011; Barton and Kagan 2009). This is a fluid model, as study of the cellular localization of TLRs is a rapidly advancing area.

TLR adaptors control downstream signaling (Kawai and Akira 2011; Kumar et al. 2011). TLRs utilize four sorting adaptors: myeloid differentiation primary response gene 88 (MyD88), Toll-IL1 receptor domain- containing adaptor protein (TIRAP), Toll-IL-1 receptor domain-containing adaptor inducing interferon-beta (TRIF), and TRIF-related adaptor molecule (TRAM) (Fig. 5.1). The adaptors engaged by each receptor determine which signaling pathways will be activated. TLR2 and TLR4 require TIRAP to recruit MyD88 to the receptor complex, whereas TLR5 does not. TLR3 is the only TLR that does not use MyD88. TLR4 is the only TLR to use all four adaptors: TIRAP and MyD88 mediate signaling triggered by surface ligation of TLR4, whereas TRAM and TRIF are required for signaling by endosomal TLR4. NFkB activation can follow ligation of either surface or endosomal TLRs, whereas activation of interferon releasing factor occurs only after intracellular recognition by TLRs (Kawai and Akira 2011; Kumar et al. 2011). The signaling pathways triggered by TLR ligation are further discussed in the Chap. 7.

TLRs recognize damage-associated molecular patterns (DAMPs) as well as PAMPs (Xiang and Fan 2010; Kovach and Standiford 2011). DAMPs are endogenous molecules released in the setting of tissue injury and cell death, whether caused by infection, trauma, malignancy, or other insult. Examples of DAMPs that are recognized by TLRs include high mobility group protein 1, heat shock proteins, oxidized lipoproteins and phospholipids, beta-defensins, and extracellular matrix

proteins. Both PAMPs and DAMPs can be involved in the response to infection. For example, influenza RNA is recognized by TLR3 and TLR7 (Lund et al. 2004; Le Goffic et al. 2007), but most of the acute lung injury associated with influenza infection is caused by TLR4-mediated recognition of oxidized phospholipids released by damaged cells (Imai et al. 2008). In a broad sense, TLRs comprise an early warning system for danger, either in the form of microbial invasion, or cellular injury. Thus, TLRs are involved in activating host defenses against infection, and in triggering inflammation and repair processes in response to cellular injury. The remainder of this chapter focuses on the role of TLRs in host defense against bacterial infections of the lungs.

5.2 TLRs in the Airway Epithelium

TLRs are expressed by many cell populations in the lungs, including airway epithelial cells, alveolar macrophages, dendritic cells, fibroblasts, and endothelial cells (Xiang and Fan 2010; Kovach and Standiford 2011; Parker and Prince 2011). Primary human airway epithelial cells have been found to functionally express all of the TLRs known to be involved in bacterial recognition (TLR1, TLR2, TLR4, TLR5, TLR6, and TLR9) (Armstrong et al. 2004; Becker et al. 2000; Hertz et al. 2003; Jia et al. 2004; Koff et al. 2008; Melkamu et al. 2009; Platz et al. 2004; Ritter et al. 2005; Sha et al. 2004; Zhang et al. 2005), as have immortalized cell lines derived from human airway epithelial cells (Sha et al. 2004; Zhang et al. 2005; Adamo et al. 2004; Greene et al. 2005; Guillot et al. 2004; Homma et al. 2004; John et al. 2010; March et al. 2010; Mayer et al. 2007; Monick et al. 2003; Muir et al. 2004; Regueiro et al. 2009; Soong et al. 2004). Many TLRs are expressed at relatively low levels by resting airway epithelial cells, but TLR gene and protein expression can be upregulated in response to cytokines and PAMPs, and TLRs can be rapidly trafficked to the cell surface upon infectious challenge (Armstrong et al. 2004; Melkamu et al. 2009; Ritter et al. 2005; Adamo et al. 2004; Homma et al. 2004; Regueiro et al. 2009). In some cases the function of TLRs on airway epithelial cells is dampened by the absence of necessary co-receptors and accessory proteins (Lee et al. 2012). For example, the relative insensitivity of airway epithelial cells in comparison with mononuclear phagocytes to the presence of LPS can be attributed to low expression of the TLR4 cofactors MD2 or CD14 (Becker et al. 2000; Jia et al. 2004; March et al. 2010). Similarly, weak TLR2-mediated responses by bronchial epithelial cells to some bacterial ligands may result from low levels of CD36 (Mayer et al. 2007). TLR-mediated recognition of bacterial ligands by airway epithelial cells leads to the production of pro-inflammatory cytokines and chemokines, and the release of antimicrobial peptides (Parker and Prince 2011). Regulation of TLR and accessory molecule expression in airway epithelial cells provides a means for managing respiratory mucosal sensitivity to the presence of microorganisms, and a pathway for amplification of innate immune responses.

5.3 TLRs in Models of Acute Bacterial Pneumonia

Studies in mouse models have yielded insights into the roles of TLRs in host defense against acute bacterial infection of the lower respiratory tract. In general, these studies have shown that the functions of individual TLRs are pathogen-specific, cooperative, and partially redundant.

Consistent with its role in recognizing multiple cell wall components of Gram-positive bacteria, TLR2 has been found to contribute to intrapulmonary cytokine and inflammatory responses after intranasal challenge with *S. pneumoniae*, but without influencing bacterial clearance or survival from primary infection (Knapp et al. 2004; Dessing et al. 2008). The absence of TLR1 or TLR6 (co-receptors for TLR2) also did not affect survival from murine pneumococcal pneumonia (Albiger et al. 2007). In contrast, TLR2 did not have a measurable role in lung inflammation from secondary pneumococcal pneumonia induced 14 days after influenza infection (Dessing et al. 2007). However, mice treated with antibiotics for secondary pneumococcal pneumonia 7 days following influenza infection exhibited increased survival in the absence of TLR2, suggesting that TLR2 mediates an injurious response in this particular setting (Karlstrom et al. 2011). TLR2 also recognizes lipoproteins present in *Mycoplasma pneumoniae* that are involved in triggering innate immune responses to *M. pneumoniae* infection of the lower respiratory tract (Chu et al. 2005; Shimizu et al. 2005, 2007, 2008). TLR2-mediated signaling contributes to clearance of *Mycoplasma* from the lungs (Wu et al. 2007; Love et al. 2010).

The individual role of TLR2 in the pulmonary host response to extracellular Gram-negative bacterial infections is pathogen-dependent. After intratracheal challenge with the oral anaerobe *Porphyromonas gingivalis*, TLR2-deficient mice exhibited impaired bacterial clearance in association with blunted cytokine responses and reduced bacterial killing by neutrophils and alveolar macrophages (Hajishengallis et al. 2008). In contrast, lung inflammation and bacterial clearance after intranasal infection with *Acinetobacter baumannii* was augmented in TLR2-deficient mice in comparison with wild type controls (Knapp et al. 2006). Pulmonary cytokine responses to infection were differentially influenced by the absence of TLR2 in this study: intrapulmonary levels of macrophage inflammatory protein-2 and monocyte chemotactic protein-1 were increased, whereas concentrations of the CXC chemokine KC were decreased in TLR2-deficient mice. Pulmonary inflammation after *Klebsiella pneumoniae* infection also was increased in the absence of TLR2, though bacterial clearance was mildly delayed in this model (Wieland et al. 2011). After challenge of TLR2-deficient mice with *Haemophilus influenzae,* lung inflammation was augmented in one study (Lorenz et al. 2005) and unimpaired in another (Wieland et al. 2005), without influencing early bacterial clearance. Similarly, pulmonary inflammatory responses to *Pseudomonas aeruginosa* (Skerrett et al. 2007; Lorenz et al. 2004) and *Burkholderia thailandensis* (West et al. 2009) were unimpaired in the absence of TLR2. The intrapulmonary production of KC modestly reduced in TLR2-null mice in several of these studies, but most cytokine responses were normal or increased in the absence of TLR2. Collectively, these

investigations suggest that the TLR2-mediated signaling serves to modulate host responses to Gram-negative bacteria, in a manner that may be suppressive overall.

TLR4 is the principal LPS receptor, and serves an important role in the innate immune response to Gram-negative bacteria in the lungs. Microarray analysis of mouse lungs 4 h after intratracheal infection with *K. pneumoniae* revealed that the upregulation of more than 60 genes was dependent on functional TLR4 (Schurr et al. 2005). Not surprisingly, TLR4-deficient mice have been reported to have impaired pulmonary cytokine and inflammatory responses to numerous Gram-negative pathogens, including *K. pneumoniae* (Wieland et al. 2011; Schurr et al. 2005; Happel et al. 2003), *H. influenzae* (Wieland et al. 2005; Lorenz et al. 2004; Wang et al. 2002), *E. coli* (Lee et al. 2005; Jeyaseelan et al. 2007), *A. baumannii* (Knapp et al. 2006), *Bordetella bronchiseptica* (Mann et al. 2005), and *P. aeruginosa* (Skerrett et al. 2007; Faure et al. 2004; Power et al. 2004; Ramphal et al. 2005). TLR4-mediated signaling influenced the outcome of many Gram-negative infections, but all. Pulmonary clearance of *K. pneumoniae* (Wieland et al. 2011; Schurr et al. 2005), *H. influenzae* (Wieland et al. 2005; Lorenz et al. 2004; Wang et al. 2002), *A. baumannii* (Knapp et al. 2004), *B. thailandensis* (West et al. 2009), and the cytotoxic, unflagellated PA103 strain of *P. aeruginosa* (Faure et al. 2004) was at least partially dependent on TLR4. Furthermore, survival from respiratory tract infection with *K. pneumoniae* (Schurr et al. 2005; Bhan et al. 2010; Branger et al. 2004), *B. bronchiseptica* (Mann et al. 2004a, b, 2005), and PA103 *P. aeruginosa* (Faure et al. 2004) was reduced in the absence of TLR4 (Schurr et al. 2005; Mann et al. 2004a, b, 2005; Bhan et al. 2010; Branger et al. 2004). On the other hand, pulmonary clearance of *E. coli* (Lee et al. 2005) and the PAK strain of *P. aeruginosa* (both flagellated and unflagellated isotypes) (Skerrett et al. 2007; Ramphal et al. 2005) did not require TLR4.

TLR4 also recognizes pneumolysin, a virulence factor produced by *S. pneumoniae* (Malley et al. 2003; Dessing et al. 2009). However, lung inflammation after intranasal inoculation with pneumolysin is mediated by TLR2 as well as TLR4 (Dessing et al. 2009). Early pulmonary cytokine and inflammatory responses after intranasal infection with *S. pneumoniae* were not impaired in the absence of functional TLR4 alone (Branger et al. 2004), but bacterial clearance and survival from pneumococcal pneumonia were reduced in TLR4-deficient mice (Malley et al. 2003; Karlstrom et al. 2011; Branger et al. 2004).

TLR5 recognizes a conserved element of flagellin (Smith et al. 2003), the structural protein of bacterial flagella, and is required for pulmonary inflammation after intranasal inoculation with flagellin (Feuillet et al. 2006). TLR5-knockout mice exhibited mildly impaired cytokine responses, neutrophilic inflammation, and bacterial clearance after acute infection with *P. aeruginosa* (Morris et al. 2009). Survival from high-dose *P. aeruginosa* infection also was impaired in TLR5-null mice (Feuillet et al. 2006).

TLR9 detects unmethylated CpG motifs that are common in bacterial DNA but rare in mammalian cells (Kawai and Akira 2011; Kumar et al. 2011; Hornung and Latz 2010). An independent role for TLR9 in innate immunity to bacterial pneumonia is supported by studies that have shown impaired bacterial clearance and reduced

survival after infection with either *S. pneumoniae* or *K. pneumoniae* in TLR9 null mice (Albiger et al. 2007; Bhan et al. 2010). Although the pulmonary inflammatory responses to these pathogens were independent of TLR9, the antimicrobial functions of alveolar macrophages were reduced in TLR9-deficient animals (Albiger et al. 2007; Bhan et al. 2010).

Bacterial infection triggers signaling via multiple TLRs, hence it stands to reason that the absence of more than one TLR would be more deleterious to innate immunity than individual deficiencies. Indeed, mice lacking both TLR2 and TLR4 or both TLR4 and TLR9 were more susceptible to *K. pneumoniae* than animals deficient for the individual receptors (Wieland et al. 2011; Bhan et al. 2010). In the case of *P. aeruginosa* infection of the lower respiratory tract, TLR4 and TLR5 seem to be the key PRRs for host defense. The presence of either TLR4 or TLR5 is sufficient to signal cytokine responses to *P. aeruginosa* by mouse airway epithelial cells and alveolar macrophages (Raoust et al. 2009). The absence of TLR4 or both TLR2 and TLR4 did not impair resistance to pulmonary infection with wild type *P. aeruginosa*, but resulted in markedly blunted cytokine and inflammatory responses and reduced survival after infection with a strain of *P. aeruginosa* lacking the TLR5 ligand flagellin (Skerrett et al. 2007; Ramphal et al. 2005, 2008). Similarly, mice null for both TLR4 and TLR5 were more susceptible to fatal *P. aeruginosa* pneumonia than wild type animals or mice lacking TLR5 alone (Feuillet et al. 2006).

Mouse studies also have informed our understanding of the role of TLR adaptors in host defense against bacterial pneumonia. MyD88 is required for rapid downstream signaling triggered by all TLRs involved in recognition of bacterial ligands (Kawai and Akira 2011). Accordingly, MyD88-null mice are effectively pan-Toll knockouts for early innate immune responses and have profoundly impaired initial pulmonary cytokine and inflammatory responses to all bacterial pathogens that have been tested (Wieland et al. 2005; Jeyaseelan et al. 2006, 2007; Power et al. 2004, 2006; Ramphal et al. 2005; Albiger et al. 2005; Cai et al. 2009; Skerrett et al. 2004a; Wiersinga et al. 2008). MyD88 is required for pulmonary clearance of *P. aeruginosa* (Power et al. 2004; Ramphal et al. 2005; Skerrett et al. 2004a), *K. pneumoniae* (Cai et al. 2009), *H. influenzae* (Wieland et al. 2011), *B. pseudomallei* (Wiersinga et al. 2008), and *S. pneumoniae* (Albiger et al. 2005), but not *S. aureus* (Skerrett et al. 2004a). Murine resistance to staphylococcal pneumonia appears to be uniquely independent of the TLRs and the IL-1 receptor family, which also depends on MyD88 for downstream signaling, even though resistance to systemic infection with *S. aureus* requires MyD88 (Takeuchi et al. 2000). MyD88-deficient mice have been demonstrated to exhibit impaired survival after pulmonary infection with *P. aeruginosa* (Ramphal et al. 2005; Skerrett et al. 2004a), *S. pneumoniae* (Albiger et al. 2005), and *K. pneumoniae* (Cai et al. 2009).

TIRAP is required for early signaling via TLR2 and TLR4 (Kawai and Akira 2011). Mice lacking this adaptor resemble TLR2/TLR4 double null mice in exhibiting impaired cytokine and inflammatory responses, diminished bacterial clearance, and reduced survival after intratracheal infection with *E. coli* or *K. pneumoniae*, but not *P. aeruginosa* (Jeyaseelan et al. 2005, 2006), which can trigger responses via TIRAP-independent TLR5. TRIF mediates signaling from endosomal TLR4, which

recognizes LPS with delayed kinetics in comparison with MyD88-mediated responses (Kawai and Akira 2011). TRIF contributes to innate immune responses and bacterial clearance after pulmonary infection with *P. aeruginosa* (Power et al. 2007), *E. coli* (Jeyaseelan et al. 2007), and *K. pneumoniae* (Cai et al. 2009), but not *H. influenzae* or *B. pseudomallei* (Wieland et al. 2005; Wiersinga et al. 2008).

5.4 The Airway Epithelium in TLR-Mediated Resistance to Acute Bacterial Pneumonia

The construction of bone marrow chimeras, in which lethally irradiated wild type or TLR-deficient mice are reconstituted with marrow harvested from wild type or TLR-deficient donors have helped define the relative contributions to innate immunity of TLR signaling by radiosensitive marrow-derived cells, such as macrophages and leukocytes, and radioresistant structural cells, such airway epithelial cells. For example, the pulmonary inflammatory response to inhaled LPS is absent in mice lacking TLR4 or MyD88 (Hollingsworth et al. 2005; Noulin et al. 2005). Chimera studies indicated that TLR4 expression by marrow-derived cells was sufficient to reconstitute this response (Hollingsworth et al. 2005), but that MyD88 expression by both marrow-derived and structural cells was required (Noulin et al. 2005). Similarly, lung inflammation after airway stimulation with flagellin requires both TLR5 and MyD88 (Feuillet et al. 2006; Noulin et al. 2005). Experiments with bone marrow chimeras have shown that the early TNF-α response to airway stimulation with flagellin required TLR5 expression by marrow-derived cells (Noulin et al. 2005), but neutrophilic lung inflammation was mainly dependent on TLR5 expression by structural cells (Feuillet et al. 2006; Noulin et al. 2005).

Innate immunity to acute *P. aeruginosa* pneumonia requires MyD88-dependent signaling (Power et al. 2004; Ramphal et al. 2005; Skerrett et al. 2004a), and chimera studies have demonstrated distinct, complementary roles for MyD88 in bone marrow-derived and radioresistant structural cells in pulmonary host defense (Hajjar et al. 2005). Early production of pro-inflammatory cytokines such as TNF-α and IL-1β in response to pulmonary challenge with *P. aeruginosa* requires MyD88 expression by marrow-derived cells, whereas the release of CXC chemokines and granulocyte macrophage-colony stimulating factor (GM-CSF) is dependent on MyD88 expression by structural cells (Hajjar et al. 2005). Furthermore, neutrophil recruitment and containment of bacterial replication in the first 4 h after infection requires MyD88 expression by structural cells (Hajjar et al. 2005). By 24 h after infection, MyD88 expression by either marrow-derived or structural cells is sufficient to support lung inflammation and bacterial clearance (Hajjar et al. 2005). That airway epithelial cells are the key non-marrow-derived cell population in mediating innate immunity to *P. aeruginosa* in the lungs is supported by studies with transgenic mice in which selective reconstitution of MyD88 in the airway epithelium restored early neutrophil recruitment and bacterial clearance (Mijares et al. 2011). These studies complement earlier investigations of the role that NFκB activation in airway

epithelial cells plays in pulmonary innate immunity. Targeted inhibition of NFκB activation in airway epithelial cells was observed to blunt the inflammatory response to inhaled LPS (Poynter et al. 2003; Skerrett et al. 2004b), and to reduce resistance to *P. aeruginosa* (Sadikot et al. 2006; Chen et al. 2008). Conversely, specific upregulation of NFκB activation in airway epithelium augmented resistance to *P. aeruginosa* pneumonia (Sadikot et al. 2006; Chen et al. 2008).

Recognition of the roles of TLRs in stimulating innate immune responses has suggested opportunities for TLR-based immunotherapy. Intratracheal treatment with a lipopeptide TLR2 agonist augmented resistance to *S. pneumoniae* (Reppe et al. 2009). Similarly, airway stimulation with the TLR5 ligand flagellin conferred protection to *S. pneumoniae* and *P. aeruginosa* by promoting neutrophil recruitment and stimulating antimicrobial peptide release from airway epithelium (Munoz et al. 2010; Yu et al. 2010). Intranasal treatment with CpG oligodeoxynucleotides that stimulate TLR9 enhanced resistance to infection with *K. pneumoniae* and *B. pseudomallei* (Deng et al. 2004; Wongratanacheewin et al. 2004). Knowing that multiple TLRs are involved in activating innate immunity to bacterial infection, it follows that combinations of TLR agonists may have synergistic therapeutic effects. Indeed, intranasal treatment with a combination of TLR2 and TLR9 agonists that stimulates microbial killing by airway epithelial cells was synergistic in protecting against respiratory tract infection with *S. pneumoniae* or *P. aeruginosa* (Duggan et al. 2011).

5.5 Conclusions

Pathogen recognition by TLRs in the airway epithelium contributes to the innate immune by triggering the release of antimicrobial peptides with direct antimicrobial activity and by stimulating the production of mediators that recruit leukocytes to the site of infection. Regulation of TLR and accessory molecule expression modulates the sensitivity of airway epithelial cells to the presence of microorganisms. The roles of individual TLRs and associated signaling adaptors in the mucosal immune response are pathogen-specific, cooperative, and partially redundant. Deeper understanding of how TLRs and other PRRs contribute to coordinated immune responses will yield new opportunities for therapeutic modulation of mucosal immunity.

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Chapter 6
Type I Interferon Responses to Airway Pathogens

Dane Parker

The type I interferons (IFNs) have been studied extensively in the context of viral infections, and have now been recognized as influencing the outcome of bacterial infections. The innate immune system possesses numerous receptors, including TLRs, RIG-like and cytosolic receptors to recognize specific molecular patterns of invading pathogens, be they viral or bacterial. Several of these receptors result in the activation of the type I IFN signaling pathway via intracellular recognition of products such as: DNA, RNA, LPS, and peptidoglycan. Type I IFN signaling leads to the activation of inteferons alpha and beta that via the IFNAR receptor and JAK/STAT signaling influence the transcription of hundreds of genes. The receptors that lead to type I IFN signaling can be found on numerous cell types in the airways: epithelial cells, macrophages, and dendritic cells, which all respond to pathogens in unique ways to produce type I IFNs. The effect of type I IFNs on the infection outcome is variable. Type I IFNs can lead to protective as well as sensitizing effects depending upon the bacterial pathogen and the site of infection. Type I IFNs are able to exert their effects through both direct activation of antimicrobial gene products as well as immunomodulation of cell activation and chemotaxis. Bacterial pathogens, both intracellular and extracellular are able to activate the type I IFN response in the airway. Their ability to activate this pathway and the host outcome will be discussed.

6.1 The Type I IFN Signaling Cascade

Type I IFNs (the main types produced are IFN-α (multiple genes) and IFN-β) are produced in response to a variety of different ligands via intracellular receptors (Table 6.1) (Decker et al. 2005). The activation of type I IFNs produces specific

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Table 6.1 Host receptors that lead to type I IFN production

Receptor	Target	References
TLR3	dsRNA	Alexopoulou et al. (2001)
TLR4	LPS	Poltorak et al. (1998)
TLR7/8	ssRNA	Diebold et al. (2004), Heil et al. (2004)
TLR9	CpG DNA	Hemmi et al. (2000)
DAI	Cytosolic DNA	Takaoka et al. (2007)
STING	Cyclic di-GMP	Burdette et al. (2011)
IFI16	Cytosolic DNA	Unterholzner et al. (2010)
RNA polymerase III-RIG-I	Poly(dA:dT)-5′PPP RNA	Ablasser et al. (2009), Chiu et al. (2009)
LRRFIP1	dsRNA and dsDNA	Yang et al. (2010)
NOD2	Muramyl dipeptide, 5′PPP RNA	Leber et al. (2008), Pandey et al. (2009), Sabbah et al. (2009)
DDX41	DNA	Zhang et al. (2011)
DHX9, DHX36	DNA	Kim et al. (2010)

antiviral and bacterial factors as well as inducing responses that influence cell recruitment (Debes et al. 2006; Der et al. 1998; Kelly-Scumpia et al. 2010; Loetscher et al. 2001; Manicone et al. 2008; Parker et al. 2011, 2012; Qian et al. 2007; Sanda et al. 2006; Satoh et al. 2006; Watanabe et al. 2010; Wenzel et al. 2006; Yarovinsky et al. 2008), especially critical to host defense in the lung. Several TLRs are involved in type I IFN activation (Table 6.1). TLR4 senses LPS, along with lipid binding protein, CD14, and MD2, and activates signaling via MyD88 at the surface. However, once LPS has been endocytosed by the cell it is brought to TIR-domain-containing adapter-inducing interferon-β (TRIF) via TRIF-related adaptor molecule (TRAM) that then leads to the activation of TANK binding kinase 1 (TBK1) and interferon regulatory factor 3 (IRF3) and IRF7 before IFN-β production (Fitzgerald et al. 2003; Hacker et al. 2006; Kagan et al. 2008). The endosomally located TLRs 7/8 (TLR7 murine, TLR8 human) and 9 utilize the myeloid differentiation primary response 88 (MyD88) adaptor molecule. Initiation of these receptors recruits IL-1 receptor-associated kinase 4 (IRAK-1) and IRAK-4, which activate IRF5 and IRF7. IRAK-1 also interacts with TRAF-6 that leads to NF-κB production (Blasius and Beutler 2010; Parker and Prince 2011; Takeuchi and Akira 2010) (Fig. 6.1). NF-κB is able to further stimulate production of type I IFNs (Lenardo et al. 1989).

Conserved receptors that respond to viral nucleic acids are also stimulated by bacteria whose components gain access to these receptors. Receptors can be endosomal, appropriate for recognition of phagocytosed microbes, both viruses and bacteria and cytosolic, which may require processing of the endocytosed bacteria and release from the endosome (Barbalat et al. 2011; Barton and Kagan 2009; Ishikawa et al. 2009; Keating et al. 2011; Takaoka et al. 2007). Viral RNA is detected by the retinoic acid inducible gene (RIG)-like receptors (RLR) RIG-I and melanoma differentiation-associated protein (MDA5) (Fig. 6.1). Bacterial DNA can also be sensed via these receptors through RNA polymerase III activity that can convert

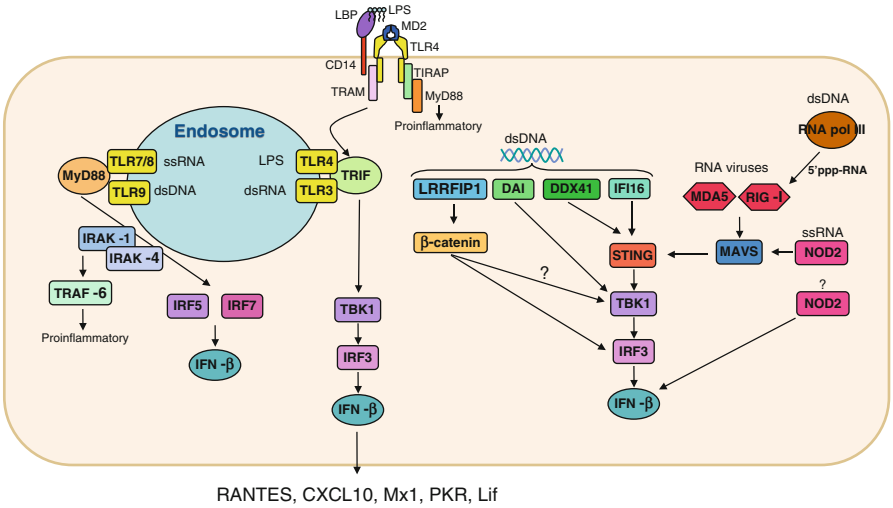

Fig. 6.1 Mechanisms involved for activation of type I IFN signaling. A number of receptors are able to activate type I IFN signaling. The majority of type I IFN activating receptors are located inside cells, the exception being TLR4, which is internalized to signal with its adaptor TRIF. The MDA5 and RIG-I receptors that sense viral RNA can also sense bacterial DNA converted to RNA by RNA polymerase III. All signaling pathways lead to activation of an interferon regulatory factor (IRF) before producing IFN-β

DNA to 5′triphosphate RNA that is then detectable by RIG-I. RIG-I and MDA5 both then signal to IPS-1 (IFN-β promoter stimulator 1) (also called mitochondrial antiviral signaling protein (MAVS)) before TBK1 and IRF3 (Parker and Prince 2011) (Fig. 6.1). Viral RNA and presumably bacteria RNA is also sensed by TLR3.

A number of cytosolic receptors that activate type I IFN signaling have been identified recently. DNA-dependent activator of IFN-regulatory factors (DAI) and IFI16 (Unterholzner et al. 2010) through stimulator of interferon genes (STING) (Ishikawa et al. 2009) both lead to activation of type I IFN signaling through TBK1 and IRF3 (Fig. 6.1). STING has since been found to act as a receptor for the bacterial signaling molecules, cyclic dinucleotides (Burdette et al. 2011; Sauer et al. 2011; Woodward et al. 2010). STING also couples with the helicase DDX41 to induce type I IFN production (Zhang et al. 2011). The nucleic acid binding protein LRRFIP1 is so far unique in the sense that it recognizes both RNA and DNA to activate type I IFNs (Yang et al. 2010) (Fig. 6.1).

The type I IFNs bind to the interferon alpha/beta receptor (IFNAR) that activates the JAK/STAT signaling cascade. JAK/STAT signaling leads to the phosphorylation of Janus kinase (JAK) members Jak1 and Tyk2 that recruit and phosphorylate STAT1 and STAT2 that then bind to IFN-stimulated response elements (ISRE), which ultimately leads to the induction of hundreds of genes (Benveniste and Qin 2007; Der et al. 1998; Kawai et al. 2001; Minisini et al. 2011; Sanda et al. 2006; Schoggins et al. 2011).

6.2 Mediators of Type I IFN Signaling in the Airway

Numerous cells types in the airway contribute to production of type I interferons including epithelial cells, macrophages, and denritic cells (DC). The airway epithelium expresses the full complement of TLR and NOD receptors; their distribution and the availability of adaptor proteins influences the ability for the epithelium to sense specific pathogen-associated molecular patterns (PAMPS) (Berube et al. 2009; Mayer et al. 2007; Muir et al. 2004; Platz et al. 2004) (reviewed in chapter 5). As acute pneumonia is associated with excessive inflammation the induction of proinflammatory signaling needs to be tightly controlled. A major component of immunity in the airway epithelium is the activation of regulatory proteins such as NF-κB, activator protein-I (AP-I), interferon regulatory factors (IRFs), and the mitogen-activated protein kinases (MAPK) (Parker and Prince 2011; Sha et al. 2004; Yoshikawa et al. 2010). The airway epithelium also secretes several antimicrobial peptides and produces chemokines and cytokines that act to recruit neutrophils and professional phagocytes to the site of infection (Parker and Prince 2011). Exactly how the participation of type I IFN signaling is coordinated with the many proinflammatory cascades and their regulatory components is an area of active investigation. It is well established that the airway epithelium is able to sense viral infection via type I IFN receptors (Parker and Prince 2011; Ritter et al. 2005; Wang et al. 2009; Xing et al. 2010) and that these airway cells alone confer protection from viral infection. This was demonstrated by comparing the susceptibility of STAT1 null mice reconstituted with WT or STAT1 null bone marrow prior to viral infection. STAT1 null mice reconstituted with WT bone marrow were still susceptible to infection (Shornick et al. 2008), indicating the ability to prevent viral infection lies within the epithelium. Less clear is exactly what innate immune mechanisms are directly responsible for this protection.

The role of the airway epithelium in host defenses from bacterial infection has been studied in the context of *S. aureus*, *S. pneumoniae*, and *P. aeruginosa* (Martin et al. 2009; Parker et al. 2011, 2012). Even though activation of type I IFN signaling is mediated by intracellular receptors, shed components from extracellular organisms can be endocytosed or specific bacterial products delivered intracellularly through pore-forming toxins. As an example *S. pneumoniae* undergoes autolysis that releases peptidoglycan and DNA (Chetty and Kreger 1981; Kadioglu et al. 2008), which can act as a ligand for type I IFN activation (Parker et al. 2011). Pneumococcal production of the pore-forming toxin pneumolysin facilitates entry of these ligands, including DNA, into the epithelial cell (Parker et al. 2011), where they activate intracellular receptors. Similarly, protein A is shed from the surface of actively growing *S. aureus*, released into the airways and recognized by a number of receptors that stimulate multiple immunostimulatory cascades (Gomez et al. 2004, 2005, 2007; Movitz 1976). Endocytosis of bound protein A could presumably participate in the activation of type I IFN signaling that has been observed in response to S. aureus protein A (Mayer et al. 2007). This is likely the pathway involved in the induction of type I IFNs by shed LPS from Gram-negative organisms

such as *P. aeruginosa*, which is taken up into endosomal compartments and stimulates TLR4/TRIF signaling resulting in type I IFN production (Kagan et al. 2008; Zanoni et al. 2011).

For actively phagocytic cells such as macrophages and DCs, the internalization of bacteria also results in production of type I IFNs. Plasmacytoid DCs (pDCs) are especially responsive to nucleic acids (via TLR7 and TLR9), producing large quantities of type I IFNs before maturation (Liu 2005). Type I IFN induction can be expedited by bacteria that express toxins that facilitate their escape from the endosome. Cytosolic bacteria or their components then induce type I IFN through ligation with cytosolic receptors (Burdette et al. 2011; Leber et al. 2008; McCaffrey et al. 2004; Woodward et al. 2010). It is difficult to sort out how important type I IFN signaling is in the overall function of macrophages and DCs in host defenses against these common pathogens. In depletion studies, both macrophages and DCs have been shown to be important in protection against *S. aureus* and *S. pneumoniae* (Martin et al. 2011; Zhang et al. 2009), while their roles against infection with *P. aeruginosa* are less consistent in different models (Cheung et al. 2000; Kooguchi et al. 1998). DCs may be especially relevant in the acquisition of infection by these organisms that first colonize the upper airways before causing lower respiratory tract infection. DCs are concentrated in the upper respiratory tract and progressively decrease in density in the lower respiratory tract (Condon et al. 2011). The combined production of type I IFNs by both the mucosal epithelium and the abundant DCs in the upper airways may be especially relevant in the susceptibility to pneumonia caused by these pathogens. Cross signaling between the epithelium and immune cells is well illustrated with DCs. Cytokines and chemokines produced by epithelial cells (e.g., GM-CSF, IL-1β, thymic stromal lymphopoietin) are effective in changing the activation state of DCs and their ability to uptake antigen and traffic to local lymph nodes (Hammad and Lambrecht 2008; Rate et al. 2009).

6.3 Type I IFNs in Host Defenses

Type I IFNs have multiple effects in host defense, many of which appear to be pathogen-associated. Best studied are the antiviral effects (Schoggins et al. 2011) and in response to typical respiratory viruses the induction of type I IFN signaling leads to the expression of hundreds of gene products (Benveniste and Qin 2007; Der et al. 1998; Kawai et al. 2001; Minisini et al. 2011; Sanda et al. 2006; Schoggins et al. 2011). Proteins such as Mx1, PKR, 2′-5′-oligoadenylate synthases, and adenosine deaminases, exert direct antiviral effects (Haller et al. 2007; Pfaller et al. 2011; Quinton et al. 2008; Salomon et al. 2007). Mx1, a GTPase, inhibits viral replication while PKR is able to bind dsRNA, destabilizing RNA duplex structures, and altering protein synthesis (Decker et al. 2005; Haller et al. 2007; Yang et al. 1995). While there are no reports to date of similarly direct interactions of type I IFN-induced gene products on specific bacterial structures, these interactions also seem likely. Despite the intracellular localization of the receptors involved in type I

IFN signaling, consequences of IFN-α and β induction cannot be readily ascribed to infection by intracellular vs. extracellular bacterial pathogens. Murine models indicate that type I IFNs prevent infection due to *P. aeruginosa*, *S. pneumoniae*, *Bacillus anthracis*, *Legionella pneumophila*, and polymicrobial sepsis (Freudenberg et al. 2002; Kelly-Scumpia et al. 2010; Lippmann et al. 2011; Parker et al. 2011, 2012; Walberg et al. 2008), while enhancing susceptibility to others (*S. aureus*, *Listeria monocytogenes*, *Chlamydia muridarum*, and *Mycobacterium tuberculosis*) (Martin et al. 2009; Nagarajan et al. 2008; O'Connell et al. 2004; Qiu et al. 2008; Stanley et al. 2007). The variable consequences of type I IFN signaling are no more evident when *L. monocytogenes* and *L. pneumophila* are compared. Both organisms are intracellular and are known to activate many of the same receptors (Burdette et al. 2011; Chiu et al. 2009; Ishikawa et al. 2009; Lippmann et al. 2011; Monroe et al. 2009; Sauer et al. 2011) yet have opposing effects on host outcome (O'Connell et al. 2004; Opitz et al. 2006; Sauer et al. 2011). Thus, the precise factors that orchestrate these protective and sensitizing effects in response to the type I IFNs are still largely unknown. It seems likely, given the multiple signaling events that must be orchestrated in response to a given pathogen that the predominance of specific responses, perhaps inflammasome activation or induction of proinflammatory mechanisms of cell death, may predominate overall in a setting of invasive infection whereas the type I IFN cascade may be especially critical in the early stages of pathogenesis.

6.4 Type I IFNs and Innate Immune Signaling

Type I IFNs affect many diverse cellular processes that influence the outcome of bacterial infection. An important consequence of type I IFN production is their effect on chemokine and cytokine expression that results in bacterial killing. IFN-β along with TLR stimulation leads to expression iNOS, a major effector of bacterial killing. Although iNOS is an effective antibacterial, its activation can be beneficial or detrimental to the final outcome of the infection (Utaisincharoen et al. 2003; Yao et al. 2001; Zwaferink et al. 2008). The CXCR3 chemokines, CXCL9 (MIG), CXCL10 (IP-10), and CXCL11 (I-TAC) are also directly induced by type I IFN signaling. The CXCR3 chemokines have properties similar to the antibacterial defensin peptides with direct antibacterial effects against a number of Gram-positive and Gram-negative organisms (Cole et al. 2001; Yang et al. 2003). These chemokines, produced in a variety of cells including airway epithelial cells (Kelsen et al. 2004; Spurrell et al. 2005) perform a variety of functions, and are best known for their recruitment of T cells (Debes et al. 2006; Manicone et al. 2008; Qian et al. 2007). In the context of polymicrobial sepsis, regulation of CXCL10 by type I IFNs is important in neutrophil recruitment (Kelly-Scumpia et al. 2010). In an inhalation model of anthrax, neutralization of these chemokines increased severity of infection. Type I IFNs also induce production of IL-27 that suppresses IL-17, an important cytokine for clearance of extracellular organisms through neutrophil recruitment

(Henry et al. 2010) (Chapter 3). This is consistent with other observations that type I IFNs impair neutrophil chemokine production (Shahangian et al. 2009).

Type I IFNs also participate in pathogen eradication through activation of cell death cascades. An important mechanism for the resolution of intracellular infection, either viral or bacterial, is through induction of host cell death, through either apoptosis or proinflammatory mechanisms. Interferon-associated apoptosis has been observed in the context of *L. monocytogenes* infection, as IFN-β acts to sensitize cells to the effects of listeriolysin (Carrero et al. 2004), and in *Chlamydia muridarum* pulmonary infection (Carrero et al. 2004; Qiu et al. 2008). However, in other cells types and with different toxins the converse is observed, indicating that effects of type I IFNs on apoptosis are not universal (Yarovinsky et al. 2008). Type I IFNs may also serve to down regulate the exaggerated proinflammatory effects and cell death initiated through inflammasome activation. Type I IFNs inhibit the NLRP1 and NLRP3 inflammasomes preventing production of caspase-1-dependent IL-1β maturation (Guarda et al. 2011). As there is a crucial balance between pro and anti-inflammatory signaling, especially in the lung, it is perhaps not surprising that diverse effects can be elicited by the same pathways depending upon the nature and extent of the infection. Additional beneficial effects of type I IFN expression in the setting of bacterial infection include: activation of DCs, macrophages, NK cells and B cells, enhanced neutrophil survival, protection from septic shock, enhanced cell migration, priming of CD8 cells, and polarization of effector T cells (Bukholm et al. 1984; Dalod et al. 2002; Decker et al. 2005; Karaghiosoff et al. 2003; Le Bon et al. 2003; Longhi et al. 2009; Martinez et al. 2008; Parker et al. 2012; Sakamoto et al. 2005; Swanson et al. 2010).

The role type I IFNs play is thus dependent on the nature of the infection, be it protective or sensitizing the host to infection. Below the role of type I IFNs in the setting of several respiratory bacterial pathogens, intracellular and extracellular, is discussed and their influence of the ability of the host to efficiently clear the infection.

6.4.1 Legionella pneumophila

L. pneumophila, an important intracellular pathogen is especially important in health care associated infections, and in immunocompromised hosts. This organism readily infects macrophages, and has an elaborate set of *dot/icm* genes devoted to its adaptation to the intracellular milieu. Not surprisingly, the activation of type I interferons is an important host response to these organisms that are adapted to grow intracellularly. The activation of type I IFN signaling is dependent on the *Legionella* type IV secretion system (Opitz et al. 2006; Stetson and Medzhitov 2006) that facilitates exposure of *Legionella* nucleic acids to cytosolic sensors. The activation of type I IFN signaling is likely a major component of the host-pathogen interaction as *L. pneumophila* has possibly the only known bacterial protein that can suppress type I IFN production. Expression of SdhA has been shown to actively repress the type I IFN response

resulting from RIG-I and MDA5 (Monroe et al. 2009). The *L. pneumophila* secretion system is required for induction of type I IFN signaling in both macrophages and epithelial cells. The protein MAVS/IPS-1 plays a role in the ability of *L. pneumophila* to activate type I IFN in epithelial cells, which leads to IRF3 activation and *Ifnb* induction (Opitz et al. 2006). The mechanisms involved in *L. pneumophila* activating type I IFNs is complex. Many ligands and receptors have been identified that participate in sensing *L. pneumophila*. Both DNA and RNA via RNA polymerase III, RIG-I, MDA5, and STING are involved (Chiu et al. 2009; Lippmann et al. 2011; Monroe et al. 2009; Sauer et al. 2011). The end result of type I IFN activation is the prevention invasion and replication (Coers et al. 2007; Plumlee et al. 2009; Schiavoni et al. 2004). In vivo pulmonary models demonstrate the importance of type I IFNs as *Ifnar*$^{-/-}$ mice have increased bacterial loads of *L. pneumophila* compared to WT mice (Lippmann et al. 2011). Thus, the innate immune defenses against one of the bacterial pathogens most highly adapted to the intracellular niche relies substantially upon the participation of the type I IFN cascade.

6.4.2 *Mycobacterium tuberculosis*

M. tuberculosis is another intracellular pathogen of major global significance that activates type I IFN signaling in macrophages as well as in the murine lung (Pandey et al. 2009; Stanley et al. 2007). In contrast to *Legionella*, more varied roles for the type I IFNs in *M. tuberculosis* infection have been reported, with the predominant effect being they appear to enhance infection. Activation of type I IFNs by *M. tuberculosis* is dependent on the ESX-1 secretion system and appears to be initiated by Nod2 via the unusual peptidoglycan contained in the mycobacterial cell wall (Pandey et al. 2009; Stanley et al. 2007). In an attempt to mimic chronic infection, instillation of purified IFN-β leads to increased mycobacterial loads in the lung (Antonelli et al. 2010; Manca et al. 2001). Type I IFNs appear to suppress protective immune responses, including the production of IL-1β, important in mycobacterial immunity (Novikov et al. 2011) and foster the generation of a permissive population of macrophages (Manca et al. 2001). Prolonged exposure to type I IFNs also leads to increased lung necrosis and an impaired Th1 response to these organisms (Antonelli et al. 2010; Manca et al. 2001). The participation of type I IFNs in the process of acute Mycobacterial infection is much less definitive and requires further study (Ordway et al. 2007; Stanley et al. 2007).

6.4.3 *Streptococcus pneumoniae*

S. pneumoniae (Chapter 9) the most common cause of acute bacterial pneumonia has been shown to activate type I IFN in multiple sites in the respiratory tract. In addition to the intracellular bacterial pathogens which would be expected to

stimulate receptors involved in type I IFN responses, there is accumulating evidence that PAMPs from primarily extracellular pathogens activate type I IFN signaling in both immune and epithelial cells. As discussed above, multiple cell types can participate in type I IFN responses. In the upper airway nasal-associated lymphoid tissue, lung tissue, airway epithelial cells as well as macrophages and dendritic cells, are able to activate *Ifnb* transcription and STAT signaling, as well as type I-dependent genes such as *Mx1* and *PKR* (Joyce et al. 2009; Parker et al. 2011). Several investigators have examined the mechanisms through which *S. pneumoniae* activate type I IFN signaling. Pneumococcal expression of the pore-forming, cholesterol-dependent cytolysin pneumolysin (Ply) is required for the induction of type I IFN responses in nasal epithelial cells and lymphoid tissue, lung tissue and immune cells (Joyce et al. 2009; Parker et al. 2011). Ply does not act directly to activate type I IFN production via a typical ligand-receptor interaction. Instead Ply pore formation facilitates the introduction of pneumococcal DNA into the cytoplasm (Parker et al. 2011). *S. pneumoniae* with mutations in the autolysin gene *lytA* is unable to activate type I IFN signaling. Less DNA, as detected by 16S rRNA levels, is observed inside epithelial cells stimulated with a *ply* strain of *S. pneumoniae* and treatment of pneumococcal lysates with DNase also reduces type I IFN induction. Pneumococcal DNA in bone marrow derived immune cells such as DCs and macrophages is sensed via cytosolic receptors, such as STING and DAI, which are upregulated in the lung in response to *S. pneumoniae* and the downstream components TBK1 and IRF3 (Koppe et al. 2012; Parker et al. 2011). The mechanism for induction of type I IFN in immune cells is likely to deviate from the model in epithelial cells where the organism remains extracellular. In epithelial cells Ply is likely to act as a pore for DNA to enter in to cells. In immune cells it may aid in escape from endosomes allowing ligands to be exposed to cytosolic receptors.

Type I IFN signaling plays a protective role against *S. pneumoniae* infection. Early studies in the field identified a role for IFN-β in preventing infection as mice treated with anti-IFN-β antibody succumbed rapidly to infection (Weigent et al. 1986). In the usual disease process, *S. pneumoniae* colonizes the nasopharynx before descending into the lower respiratory tract. In the absence of type I interferon signaling, *S. pneumoniae* has an increased propensity to colonize the nasopharynx (Parker et al. 2011). This observation is also consistent with the density of DC throughout the respiratory tract. In the upper respiratory tract, where the colonization phenotype was observed, DC are present in high density (Condon et al. 2011). The density of DC diminishes further down the respiratory tract.

The reduced density of DC in the lower respiratory tract may explain the lack of a phenotype in pulmonary infection of *Ifnar*$^{-/-}$ mice with *S. pneumoniae*. Only in the context of superinfection post influenza did *Ifnar*$^{-/-}$ mice display increased susceptibility to pneumococcal infection, instilled directly into the lungs (Shahangian et al. 2009). In the upper airways, the increased colonization in the absence of type I IFN signaling is strain-dependent (Nakamura et al. 2011; Parker et al. 2011). Superinfection with influenza increased colonization of *S. pneumoniae* in the nasopharynx, most likely as a result of decreased CCL2 production (Nakamura et al. 2011).

6.4.3.1 Other Streptococci

Group A and B streptococci (GAS and GBS; *S. pyogenes* and *S. agalactiae*) also activate type I IFNs. The mechanism of type I IFN induction by GAS varies by host cell type. Macrophages sense GAS DNA and utilize STING, TBK1, and IRF3, with partial MyD88 involvement, while DCs are able to detect RNA and require MyD88 and IRF5 (Gratz et al. 2008, 2011). Induction of type I IFNs by GAS does not require production of its cytolysin, streptolysin O (SLO). The involvement of type I IFNs in protection against GAS has been well demonstrated in a subcutaneous models of cellulitis (Gratz et al. 2011) in which *Ifnar*$^{-/-}$ mice were less able to resolve the infection and recruit neutrophils.

Similar to *S. pneumoniae*, the hemolysin of GBS is required to induce *Ifnb* (Charrel-Dennis et al. 2008). The mechanism of type I IFN activation by GBS also varies depending upon the cell type studied. In macrophages nucleic acids are sensed. DNA via TBK1 and IRF3 activates type I IFNs (Charrel-Dennis et al. 2008) as well as ssRNA via a MyD88-dependent mechanism (Deshmukh et al. 2011). In DCs, phagosomally associated organisms signal type I IFN production via TLR7, MyD88, and IRF1 (Mancuso et al. 2009). Type I IFNs are important for protection against GBS in animal models. Both subcutaneous and intraperitoneal infection of *Ifnar*$^{-/-}$ mice leads to significant increases in mortality (Mancuso et al. 2007). The role that type I IFNs play in clearance of GAS and GBS from the airway and their ability to activate signaling in epithelial cells remains to be determined.

6.4.4 Staphylococcus aureus

S. aureus is a major cause of pneumonia in both health care associated and community settings. The initiation of the type I IFN response is likely to originate in the respiratory epithelium. Airway epithelial cells initiate production of *Ifnb* and activation of the STAT pathway in response to *S. aureus* (Martin et al. 2009). A mechanism for this has been proposed to involve the virulence factor protein A. Lung sections from *S. aureus* infected mice show activation of STAT3, which can be induced by IL-6 (Martin et al. 2009). Protein A is released from the surface of *S. aureus* during growth (Movitz 1976) and is taken up by airway epithelial cells. The ability of protein A to induce type I IFN production has been demonstrated in both mouse and human lymphocytes (Smith et al. 1983). Protein A can signal via the receptors TNFR1 and EGFR to induce TNF production (Gomez et al. 2004, 2007). TNF itself can induce production of IRF1 and type I IFNs (Fujita et al. 1989; Schmitz et al. 2007). The role of IRF1 in type I IFNs has been linked to signaling via TLR9 (Schmitz et al. 2007). In bone marrow derived dendritic cells, *S. aureus* is able to activate type I IFN production through TLR9 recognition of DNA (Parker et al. 2012). This is a potentially important mechanism in the upper respiratory tract where dendritic cells are concentrated (Condon et al. 2011).

Infection with *S. aureus* is a major complication of influenza and initiates significant production of type I IFNs. Staphylococcal infection following influenza

insult results in increased lung damage and reduced ability to clear the infection (Lee et al. 2010) *S. aureus* superinfection following influenza in *Ifnar*$^{-/-}$ mice results in improved outcomes compared to the WT (Kudva et al. 2011). It is not surprising then that production of type I IFNs in response to *S. aureus* is detrimental in murine models of infection (Martin et al. 2009). Intranasal infection with high doses of *S. aureus* results in mortality while *Ifnar*$^{-/-}$ mice are protected. Consistent with the negative impact of type I IFNs on *S. aureus* infection, *Tlr9*$^{-/-}$ mice fare better against infection (Parker et al. 2012).

The fact that *Ifnar*$^{-/-}$ mice are protected from *S. aureus*-induced mortality is likely due to the reduced host inflammatory response. At early time points *Ifnar*$^{-/-}$ mice have reduced levels of TNF and IL-6, important proinflammatory cytokines (Denis et al. 1991; Maillet et al. 2011; Martin et al. 2009; Pedroza et al. 2011). It has also been shown that type I IFNs can stimulate TNF (Huys et al. 2009). Additional mechanisms that could explain the poorer outcome are the ability of type I IFNs to suppress the Th17 response (Kudva et al. 2011) and the induction of apoptosis in granulocytes (Navarini et al. 2006).

6.4.5 *Pseudomonas aeruginosa*

P. aeruginosa is a common opportunistic pathogen that is a cause of significant morbidity and mortality for individuals with cystic fibrosis (CF) and a major cause of pneumonia in hospital-associated infections. As the cell wall of *P. aeruginosa* contains LPS, it is not surprising that the TLR4-LPS signaling cascade is involved in type I IFN production. All of the receptor components (TLR4, TRIF, MD2 and CD14), and downstream proteins (TBK1, IRF3) involved in LPS signaling are involved in type I IFN production (Parker et al. 2012). Type I IFNs appear to play an important role in protection from *P. aeruginosa* infection based on a series of in vivo studies. Mice lacking various components of the TLR4-LPS signaling pathway, TLR4, TRIF, and IRF3 all show reduced abilities to clear *P. aeruginosa* and have impaired cytokine production and PMN recruitment (Carrigan et al. 2010; Faure et al. 2004; Power et al. 2007; Ramphal et al. 2008). *P. aeruginosa* has been shown to induce *Ifnb* transcription in lung tissue and this was entirely dependent upon IRF3. In agreement with the knockout mouse studies, mice with enhanced type I IFN signaling display improved clearance of *P. aeruginosa* (Parker et al. 2012). Type I IFNs were stimulated in the airway via the TLR3 ligand poly(I:C), which increased expression of the macrophage and DC activation marker CD86. The enhanced expression of CD86, although not investigated, might have led to improved phagocytosis and presentation of ligands by antigen presenting cells leading to the improved clearance of *P. aeruginosa*.

A difference in the ability to induce type I IFNs is present in epithelial cells with mutations in the cystic fibrosis transmembrane regulator (CFTR). Cell lines with mutations in CFTR induce significantly less type I IFNs and type I IFN-dependent gene products than WT lines in response to LPS and *P. aeruginosa*

(Parker et al. 2012). This signaling defect appears to be limited to type I IFN signaling as NF-κB-dependent gene transcription in CF lines is normal. This difference in type I IFN signaling is also limited to TLR4 as the TLR3 ligand, poly(I:C), induced *Ifnb* and phosphorylation of STAT1 and IRF3 comparable to WT cell lines. The TLR4 specificity of the defect in type I IFN signaling is interesting in the context of infection with CF patients. *P. aeruginosa* over time develops altered LPS structures that have reduced abilities to active proinflammatory signaling (Cigana et al. 2009), a mechanism presumably to maintain a chronic state and avoid the host immune system. It remains to be determined what effects these LPS modifications have on the activation of type I IFN signaling and if this contributes to chronic infection.

6.5 Conclusions

The airway is able to utilize a range of cell types to sense and respond to invading pathogens. The role of type I IFN signaling in response to bacterial pathogens varies greatly: ranging from protective to sensitizing the host to infection. The overall outcome is likely to be dependent upon the site of infection, simultaneous activation of various signaling cascades as well as the density of infection. The mechanisms underlying host outcome due to activation of type I IFN signaling are still being defined but can be related to activation of professional phagocytes in cases of protection vs. creating permissive phagocytes hyper-susceptible to apoptosis in sensitizing cases. The ability to activate this signaling cascade occurs regardless of the cellular lifestyle of the organism, as extracellular organisms' PAMPS gain access to intracellular receptors via surface proteins and introduction of ligands via secretion systems. The varying host outcomes associated with type I IFN signaling in the airway indicate the complexity in this signaling cascade and much is yet to be delineated to define the host mechanisms involved.

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Chapter 7
Transcriptional Signaling Hubs in Epithelial Cells During Pneumonia

Lee J. Quinton and Joseph P. Mizgerd

7.1 Introduction

The respiratory surface is composed of heterogeneous epithelial cells with diverse cell-specific functions (Franks et al. 2008). The epithelium constitutes a barrier between the ambient environment and the internal milieu, and as such the epithelial cells help protect the body from bacteria and other materials that are inhaled or aspirated into the air spaces of the lung. Epithelial cells have constitutive activities that are clearly essential to host defense, including the elaboration of a surface lining fluid with chemical and physical properties that enhance antibacterial defense. In the airways, the mucociliary escalator traps inhaled materials and propels them out of the lungs, transporting them up the airways and past the glottis until they become swallowed and eliminated. This defense is a product of the airway epithelium, including coordinated efforts of glandular, secretory, and ciliated cells. The common and severe lung infections in patients with genetic defects influencing mucociliary escalator function, such as primary ciliary dyskinesia (Bush et al. 2007) or cystic fibrosis (Boucher 2007), highlight the critical role of this constitutive host defense system. In the alveoli, the type II cells basally secrete the lipid and protein components of the surfactant lining fluid. The surfactant proteins A and D are collectins with diverse host defense roles against bacteria, and the significance of these constitutive defenses are highlighted by the susceptibility to lung infection of mice with genetic deficiencies of surfactant proteins A or D (Wright 2005). Thus, the epithelial cells of the lung constitutively function to protect the lung from bacterial infection. When bacteria are too numerous or too virulent to be eliminated by constitutive defenses, recruited innate immune defenses are needed. These innate immune responses involve the integrated functions of structural

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elements such as lung epithelial cells and immune cells such as macrophages and neutrophils. This chapter will focus on the innate immune responses of lung epithelial cells, including their molecular regulation and contributions to mucosal immunology and tissue homeostasis during acute bacterial pneumonia. Specifically, we will highlight knowledge about two transcription factor signaling hubs which appear to be especially important to epithelial responses during pneumonia, NF-κB, and STAT3.

7.2 Balancing Host Defense and Lung Injury During Pneumonia by Regulation of NF-κB

NF-κB was first discovered (Sen and Baltimore 1986) as a nuclear factor (NF) that bound the enhancer of the kappa light chain of B cells (κB), but it is now recognized to be activated by a diverse set of signals related to inflammation or cell stress and to influence the expression of hundreds of genes involved in myriad physiological and pathological processes (Baltimore 2011). NF-κB transcription factors are homodimers or heterodimers from a pool of five different proteins, RelA (also known as p65), p50, c-Rel, RelB, and p52. NF-κB proteins are present in the cytoplasm of perhaps all nucleated cells, but upon activation NF-κB accumulates in the nucleus where it binds DNA and regulates gene transcription. To our knowledge, only 2 NF-κB proteins have been implicated in the mucosal immunology of acute bacterial infection, RelA and p50. Both translocate to the nucleus in response to bacterial stimuli in the lungs (Mizgerd et al. 2002), but they appear to have very distinct functions. The mutation of RelA in all cells results in spontaneous bacterial infections, including pneumonia (Alcamo et al. 2001). RelA deficiency severely compromises expression of innate immunity products elicited by bacterial products in the lungs including chemokines, cytokines, adhesion molecules, and colony stimulating factors, resulting in defective neutrophil recruitment and impaired host defense (Alcamo et al. 2001; Quinton et al. 2007). Thus, RelA is essential to inducing innate immunity genes that recruit and activate neutrophils to eliminate bacteria in the lung. In contrast, p50 does not appear essential to the expression innate immunity genes during pneumonia (Mizgerd et al. 2003). Rather, mutation of the gene encoding p50 (*Nfkb1*) results in excessive inflammatory gene expression (Mizgerd et al. 2003), consistent with data suggesting that p50 often limits rather than promotes transcriptional responses (Hayden and Ghosh 2008). The overabundance of inflammatory mediators due to this NF-κB dysregulation is disastrous during infection. While bacteria can be eliminated from the lungs as efficiently in the p50-deficient mice as their wild type counterparts, acute lung injury is exacerbated with lethal consequences (Mizgerd et al. 2003). Thus, p50 is essential to limiting expression of RelA-driven innate immunity genes in order to attenuate inflammatory injury. RelA and p50 play opposing roles in the regulation of innate immunity gene expression, and their balance is critical to the outcome of infection. These studies helped establish and provide strong support for the fundamental concept that NF-κB governs the expression of inflammatory genes during pneumonia. Sufficient expression of NF-κB-mediated innate immunity genes

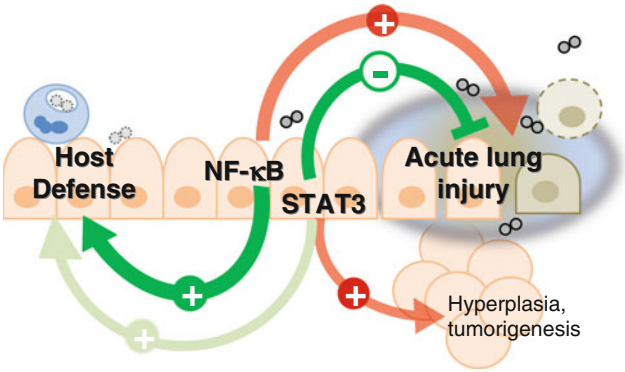

Fig. 7.1 Overview of the roles played by NF-κB and STAT3 in epithelial cells during acute bacterial pneumonia

is essential to neutrophil-mediated host defense against bacteria in the air spaces, but too much causes lung injury (Fig. 7.1).

Humans with polymorphisms or mutations in NF-κB pathways support this fundamental concept. We have not seen reports of humans with a complete deficiency of RelA, but deficiencies of upstream signaling molecules have been identified, such as mutations in the genes for MyD88 or IRAK-1, are responsible for rare genetic diseases. MyD88- or IRAK-1-deficient cells from these patients cannot activate NF-κB in response to diverse Toll-like receptor agonists or IL-1, although NF-κB can be activated by other pathways such as TNF-α (Ku et al. 2007; von Bernuth et al. 2008). These patients with narrowed but not eliminated abilities to activate NF-κB are highly susceptible to pyogenic bacteria, especially pneumococcus (Ku et al. 2007; von Bernuth et al. 2008). Pneumonia is rarely diagnosed, perhaps because the alveolar flooding essential to this diagnosis depends on the inflammatory responses that are dampened by mutation, but the failure of NF-κB-driven host defense results in severe and life-threatening invasive infections from these respiratory pathogens (Ku et al. 2007; von Bernuth et al. 2008). A clear example of the need to limit these same responses during infection comes from patients with TLR1 polymorphisms that increase NF-κB activation after stimulation of this pattern recognition receptor (Wurfel et al. 2008). Among sepsis patients, those with TLR1 polymorphisms which exaggerate NF-κB activation have increased acute lung injury and increased death (Wurfel et al. 2008). These compelling human patient data support the concept that NF-κB governs the expression of inflammatory genes which are essential determinants of pneumonia outcome.

7.3 NF-κB in Epithelial Cells During Acute Bacterial Pneumonia

NF-κB has distinct and important roles in many cell types during pneumonia, including epithelial cells, endothelial cells, macrophages, neutrophils, hepatocytes, and others (Mizgerd 2008; Quinton and Mizgerd 2011). Several lines of evidence

suggest that the general concept for how NF-κB applies to mucosal immunology during acute bacterial pneumonia applies specifically to NF-κB in lung epithelial cells, thanks in large part to genetically engineered mouse studies in which NF-κB pathways were targeted selectively in lung epithelial cells.

The CC10 promoter can be used to target Clara cells of the conducting airway. When NF-κB in Clara cells is interrupted by the CC10-driven overexpression of a dominant-negative inhibitor IκB-α (dnIκBα) protein, in which the serine normally phosphorylated by IκB Kinase-β (IKK-β) is replaced by alanine, acute pulmonary inflammation elicited by intranasal LPS is blunted. The CC10-driven dnIκBα transgene nearly eliminates neutrophil recruitment and the net expression of CXCL1 and CXCL2 in the lungs (Poynter et al. 2003). These data suggest a remarkably profound role of airway epithelial cell NF-κB in this setting. The results may be characteristic of intranasal delivery of a nonliving and non-replicative stimulus more than a bacterial pneumonia, but the study clearly indicates that NF-κB in epithelial cells of the conducting airway can be absolutely vital for responses to some bacterial products in the air spaces. The CC10 system has also been used to mutate the gene for IKK-β selectively in Clara cells. During pneumonia caused by intranasal delivery of group B Streptococcus, the CC10-driven mutation of IKK-β leads to a modest (threefold) but significant increase in bacterial burdens (Fong et al. 2008). Although not targeting NF-κB specifically, these data suggest that NF-κB activation in Clara cells of the conducting airway is necessary for maximal host defense against bacteria in the lungs.

A complementary set of studies targeting MyD88 (upstream of NF-κB in both the TLR and IL-1R signaling pathways) is also revealing about host defense roles of these pathways in airway epithelial cells. As in humans (von Bernuth et al. 2008), MyD88 deficiency renders mice extremely susceptible to *Pseudomonas aeruginosa*, with multi-log increases in bacterial burden during pneumonia (Skerrett et al. 2004a). Reciprocal bone marrow chimera experiments demonstrate that having MyD88 in the non-hematopoietic compartment (with leukocytes remaining MyD88-deficient) is sufficient to maintain fully effective host defenses, comparable to wild type mice (Hajjar et al. 2005). Taking this one step further, the CC10 system was used to restore MyD88 to conducting airway epithelial cells selectively, and this dramatically decreases *P. aeruginosa* burdens in the lungs of MyD88-deficient mice (Mijares et al. 2011). Therefore, MyD88-mediated activation of NF-κB in just the epithelial cells of the airway is enough to amply rescue local host defense in mice with global deficiencies in this signaling pathway.

The surfactant protein-C (SPC) promoter can be used to target type II alveolar epithelial cells in the parenchyma, and NF-κB has been interrupted with this approach by overexpression of the dnIκBα protein (Skerrett et al. 2004b). NF-κB interruption in type II cells using this approach modestly but significantly reduced responses to the aerosolized delivery of LPS, including neutrophil recruitment and the net expression of CXCL1, CXCL2, TNF-α, and IL-1β in the lungs (Skerrett et al. 2004b). These data suggest a limited but measurable role for NF-κB in type II cells in acute pulmonary inflammatory responses to aerosolized LPS, which is

relevant to but not a direct model of bacterial pneumonia. When these mice were infected with *Streptococcus pneumoniae* by intratracheal instillation, there was a significant defect in bacterial clearance leading to an approximately 30-fold increase in bacterial burdens (Quinton et al. 2007). This defect is less severe than the effects of system-wide mutations in NF-κB (Quinton et al. 2007) or NF-κB-activating pathways (Jones et al. 2005), but these data demonstrate that NF-κB is required in type II cells of the alveoli for maximal host defense during pneumonia.

Genetic engineering in mice has also been used to increase NF-κB activity specifically in lung epithelial cells. Driving a constitutively active IKK-β from the CC10 promoter activates NF-κB in Clara cells (Cheng et al. 2007), and this is sufficient with continued activation to cause lung inflammation (as measured by neutrophil accumulation and expression of many pro-inflammatory cytokines) resulting in acute lung injury (including pulmonary edema, arterial hypoxemia, and death). Therefore, excessive NF-κB activity in lung epithelial cells alone is sufficient to cause lethal inflammatory lung injury.

These cell-targeting studies support the concept that NF-κB has critical roles in airway epithelial cells, but major questions remain unanswered. Regarding epithelial cell specificity, NF-κB has been demonstrated to have roles in both Clara cells and type II alveolar epithelial cells, but other epithelial cells such as ciliated cells or type I cells have yet to be specifically targeted for the NF-κB pathway. Furthermore, the net role of NF-κB in the respiratory epithelium cannot be appreciated from the approaches that have been applied so far, as each has targeted only subsets lung epithelial cells. Regarding NF-κB protein specificity, approaches attempted to target the upstream signaling pathways by interrupting IKK interactions with IκB proteins, but no studies yet have examined roles of individual NF-κB proteins such as RelA or p50 selectively in epithelial cells. Finally, while studies consistently demonstrate that epithelial cell NF-κB is antimicrobial but injurious, which pathways activate NF-κB in these cells and which NF-κB target genes are induced in epithelial cells specifically to protect the host or damage the lungs remains poorly understood, although ideas are emerging and considered below.

7.4 Activation of NF-κB in Epithelial Cells During Pneumonia

NF-κB can be activated by a wide range of stimuli present in infected lungs (Fig. 7.2). The activation of epithelial cells by pattern recognition receptors leading to NF-κB activation is important but will not be discussed here as it is the focus of an additional chapter by Dr. Skerrett in this volume. Materials released by dead and dying cells (sometimes referred to as damage-associated molecular patterms or DAMPs) and other stress-related molecules such as reactive oxygen species are also capable of activating NF-κB, but to our knowledge there is little direct evidence for whether or when DAMPs or other environmental stresses specifically impact NF-κB in the epithelial cells of infected lungs. Host-derived cytokines can activate NF-κB

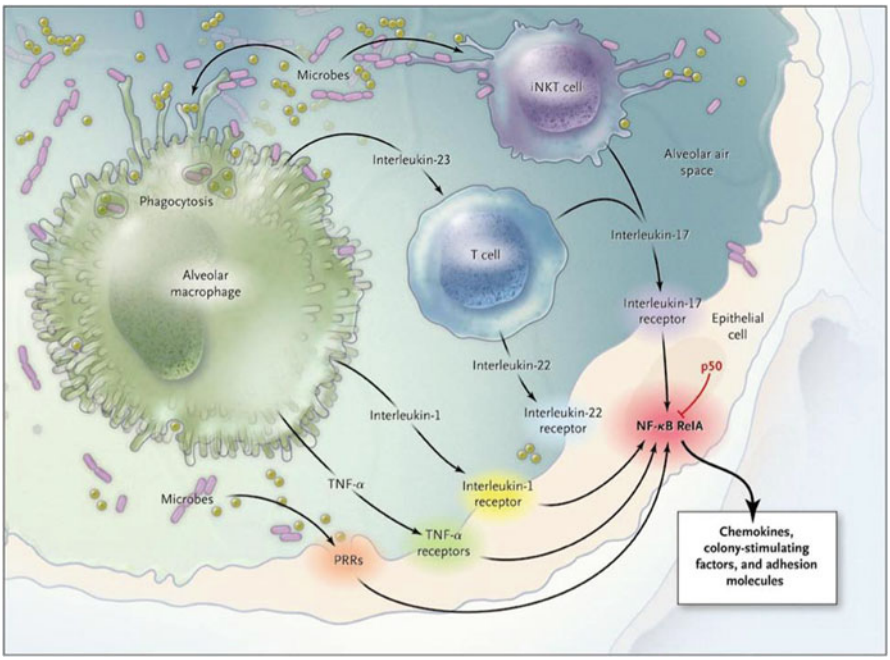

Fig. 7.2 NF-κB activation in epithelial cells during acute bacterial pneumonia. Figure is reprinted from Mizgerd (2008), with permission from the *New England Journal of Medicine*

signaling, some of which are direct sources of epithelial NF-κB activation during pneumonia (Fig. 7.2).

The early response cytokines TNF-α, IL-1α, and IL-1β contribute to host defense against a wide variety of bacteria in the lungs (Mizgerd 2008). For both Gram-negative bacteria and pneumococcal pneumonias, the simultaneous interruption of both TNF and IL-1 pathways has more pronounced effects on host defense than the interruption of either pathway alone (Jones et al. 2005; Mizgerd et al. 2004), suggesting overlapping signaling pathways. The combined interruption of both TNF and IL-1 compromises host defenses against pneumococcus more severely than during Gram-negative bacterial pneumonia, indicating that TNF or IL-1 signaling (but not signaling by both) is especially important for host defense against pneumococcus in the lungs (Jones et al. 2005; Mizgerd et al. 2004). Paralleling these basic studies using mouse models, a clinical trial in rheumatoid arthritis patients testing the combined inhibition of TNF-α (using a soluble receptor) plus IL-1α and IL-1β (using a receptor antagonist) had to be halted early due to increased incidence of severe infectious complications including pneumonia compared to patients receiving either inhibitor alone (Genovese et al. 2004). Together, these mouse and human studies reveal that signaling from TNF or IL-1 is critical to antibacterial defense, especially pneumococcus in the lungs.

While alveolar epithelial cell NF-κB activation is essential to anti-pneumococcal defense, pneumococcus does not directly activate NF-κB in alveolar epithelial cells

(Quinton et al. 2007). During pneumococcal pneumonia, the first recognition of bacteria in the lungs is by alveolar macrophages, which respond to pneumococcus by expressing many cytokines including TNF-α, IL-1α, and IL-1β (Pittet et al. 2011). The combined blockade of both TNF and IL-1, but neither alone, is sufficient to prevent NF-κB activation in alveolar epithelial cells and throughout the lung during pneumococcal pneumonia (Quinton et al. 2007; Jones et al. 2005). We propose that the inability of epithelial cells to immediately recognize and respond to pneumococcus is a major reason that TNF or IL-1 signaling is so much more prominent a component of effective host defense for pneumococcal pneumonia compared to other infections where bacteria can activate NF-κB directly, such as Gram-negative bacterial pneumonias. The interactions of lung epithelial cells with pneumococcus in an infected lung are dynamic (Hammerschmidt et al. 2005), and it is possible that while TNF or IL-1 are essential to the earliest activation of epithelial cells, other pathways may be involved later. Future studies will need to determine whether, over time, changes in either the microbe or the epithelial cells of the infected lung result in stimulation of epithelial NF-κB by pathways other than TNF or IL-1.

While other host-derived factors likely activate NF-κB in epithelial cells of infected lungs, direct evidence is sparse. IL-17 signaling enhances neutrophil-mediated host defense in the lung (Ye et al. 2001; Aujla et al. 2008), and IL-17 activates NF-κB-mediated expression of defense proteins from lung epithelial cells (Fujisawa et al. 2009; Kao et al. 2005; Kao et al. 2008). However, IL-17 also activates other pathways and cells (Bulek et al. 2011; McAleer and Kolls 2011), and the degree to which NF-κB or epithelial cells mediate the protective roles of IL-17 during pneumonia remains speculative.

7.5 Roles of Innate Immunity Genes Induced by NF-κB in Epithelial Cells During Pneumonia

Although it is clear that NF-κB mediates induction of many genes during pneumonia and epithelial cells are a critical site of NF-κB activation during pneumonia, it remains unclear exactly which products induced during pneumonia and essential to the outcome of infection depend upon NF-κB in epithelial cells specifically. Several NF-κB-driven products from epithelial cells may be involved, although data for each are largely circumstantial at present.

Epithelial cells secrete multiple products that recruit and activate neutrophils, such as chemokines, colony stimulating factors, and adhesion molecules (Fig. 7.2). In response to LPS in the lungs, alveolar epithelial type II cells are the predominant source of the neutrophil chemokine CXCL5 (Jeyaseelan et al. 2005). CXCL5 induction is eliminated by the genetic deficiency of RelA during pneumococcal pneumonia (Quinton et al. 2007), and CXCL5 is required for maximal neutrophil recruitment elicited by bacterial LPS in the lungs (Jeyaseelan et al. 2004; Mei et al. 2010). Altogether, these findings strongly suggest that CXCL5 is a critical

pathway through which NF-κB in lung epithelial cells enhances host defense against bacteria in the lungs. Other glutamine–leucine–arginine (ELR)-containing CXC chemokines, which bind the same receptor(s) as CXCL5 and which are also dependent upon NF-κB, are similarly essential for maximal neutrophil recruitment in the infected lung (Mizgerd 2002). Because they can be expressed by epithelial cells (e.g., (Elizur et al. 2008; Liu et al. 2011; Marriott et al. 2012)), it is conceivable that epithelial production of neutrophil chemokines other than CXCL5 further contributes to neutrophil recruitment during pneumonia. In addition to chemokines, granulocyte colony-stimulating factor (G-CSF) and granulocyte macrophage colony-stimulating factor (GM-CSF) can be induced in lung epithelial cells, are mediated by NF-κB in at least some settings, and contribute to antibacterial host defenses during pneumonia (Bozinovski et al. 2002; Laan et al. 2003; Bozinovski et al. 2004; Wu et al. 2011a; Quinton et al. 2002; Knapp et al. 2004; Gregory et al. 2007), suggesting that these may be additional innate immunity products of epithelial NF-κB during pneumonia. Epithelial NF-κB may further enhance host defense against bacteria via the expression of adhesion molecules which direct and activate phagocytes. For example, LPS-induced ICAM-1 induction in the lungs is dependent upon NF-κB (Alcamo et al. 2001), and epithelial ICAM-1 induction enhances macrophage abilities to find and ingest bacteria (O'Brien et al. 1999; Paine et al. 2002). These multiple lines of evidence argue that NF-κB activation in epithelial cells helps recruit and activate phagocytes to kill bacteria in the air spaces.

Beyond their influence on phagocytes, NF-κB in epithelial cells may lead to direct antibacterial functions as well. Many proteins induced in epithelial cells by NF-κB have well-characterized antimicrobial activities, including β-defensins, cathelicidins, and chemokines which are directly bactericidal (Kao et al. 2005; Kao et al. 2008; Kao et al. 2004; Yang et al. 2003; Kolls et al. 2008; Starner et al. 2003; Park et al. 2011; Yu et al. 2010). Mucin proteins are induced by NF-κB in airway epithelial cells, which may enhance mucociliary defense of the airways (Fujisawa et al. 2009; Fujisawa et al. 2011; Thornton et al. 2008). Epithelial cells use oxidative enzymes to generate antibacterial products such as hypothiocyanate (Moskwa et al. 2007), and these systems are inducible (Gattas et al. 2009), but roles of NF-κB have not been tested to our knowledge. While biochemical and cell culture systems implicate these as epithelial host defense pathways, there is remarkably little evidence for how important these are during pneumonia. Perhaps the most compelling argument for direct antimicrobial activity of epithelial cells comes from studies in which the lungs are pharmacologically exposed to nonliving bacterial stimuli shortly before infection, which enhances subsequent defenses against bacteria in the air spaces (Yu et al. 2010; Clement et al. 2008; Duggan et al. 2011; Evans et al. 2010a). The response seems consistently to involve ramping up of the epithelial antimicrobial defenses, independent of phagocytes or other immune cells (Evans et al. 2010a). Protection is stimulated by signaling through MyD88 and likely NF-κB (Duggan et al. 2011; Evans et al. 2010b). These data suggest that NF-κB may enhance direct antibacterial activities of epithelial cells during pneumonia.

7.6 The Enigma of STAT3 and Lung Infection

Nearly 20 years ago, a factor required for IL-6-induced acute phase protein expression was discovered and termed "acute phase response factor" (Wegenka et al. 1993). Shortly thereafter, this factor was cloned in the laboratories of Tadamitsu Kishimoto and James Darnell, and has since come to be known as STAT3 (Akira et al. 1994; Zhong et al. 1994). Two decades later, the enormous scope of STAT3 biology is now well appreciated, albeit poorly understood. STAT3 has been linked to many functions, including but not limited to oncogenesis, inflammation, and cell death (Schindler et al. 2007). In the lungs, STAT3 has emerged as a transcription factor exhibiting both host defense and tissue protective properties, which is somewhat unique given the contradictory nature of these two processes. Like NF-κB (Beg et al. 1995), however, efforts to elucidate the biology of STAT3 in integrated in vivo systems have been hindered by embryonic lethality in STAT3-deficient mice (Takeda et al. 1997). With the advent of site-directed gene deletion, investigators are now better positioned to causally link STAT3 activation with biological outcomes. Combining these efforts with other approaches both directly and indirectly related to STAT3 signaling has revealed STAT3 as a critical yet poorly understood hub controlling inflammation, host defense, and other processes integral to mucosal immunity.

In response to appropriate stimuli, tyrosine-phosphorylated STAT3 forms homodimers or heterodimers with other STAT proteins, ultimately driving nuclear accumulation, DNA binding, and the expression of STAT3-dependent gene programs (Schindler et al. 2007). In the lungs, STAT3 activation occurs in response to a variety of stimuli including pneumonia induced by bacteria (Jones et al. 2006; Quinton et al. 2008) or viruses (Matsuzaki et al. 2006), LPS (Severgnini et al. 2004; Ikegami et al. 2008), immune complexes (Gao et al. 2004), naphthalene (Kida et al. 2008), hyperoxia (Hokuto et al. 2004), and ozone exposure (Lang et al. 2008). While these settings are not all directly relevant to immunobiology, insights gained from these mouse models have shed light regarding STAT3's diverse roles. Generally, STAT3 activity correlates with pulmonary inflammation as does the presence of STAT3-inducing cytokines. As described in further detail below, STAT3 activity appears to be mostly favorable towards lung infection outcomes, attributable in part to its roles in limiting injury, improving barrier integrity, and promoting antimicrobial activity (Quinton and Mizgerd 2011). However, STAT3 has also been linked to hyperplasia and carcinogenesis, demanding an improved understanding as to how these signals are balanced to favor tissue homeostasis (Fig. 7.1).

7.7 STAT3 Deficiency and Human Disease

A rare genetic disease known as hyper-IgE syndrome (HIES, also referred to as Job's syndrome) has provided compelling evidence that STAT3 is a critical determinant of outcome during pneumonia. HIES is directly caused by dominant-negative mutations in the STAT3 gene (Holland et al. 2007; Minegishi et al. 2007). Cells collected from

Table 7.1 Potential cell types influencing HIES-related lung disease

Cell-type	Role of STAT3 in that cell which could prevent pulmonary aspects of the HIES phenotype	Relevant references
Epithelial cells	↓ Inflammatory injury, ↑ repair of the injured lung, ↑ epithelial integrity, ↑ lung host defense	Quinton et al. (2008), Matsuzaki et al. (2006), Ikegami et al. (2008), Kida et al. (2008), Hokuto et al. (2004)
Th17 cells	↑ Local cytokines (IL-17, IL-22) which activate lung epithelial cells to improve defense and limit injury	Milner et al. (2008), Minegishi et al. (2009)
Dendritic cells	↑ Tregs, ↑ IL-10, ↓ inflammatory injury	Saito et al. (2011)
Macrophages	↑ Responsiveness to IL-10, ↓ elaboration of injurious products, ↓ inflammatory injury	Gong et al. (2006), Jin et al. (2012), Takeda et al. (1999), Matsukawa et al. (2005)
Hepatocytes	↑ Acute phase proteins, ↑ host defense, ↓ inflammatory injury	Minegishi et al. (2007), Quinton et al. (2009)

HIES patients, who are heterozygous for the mutant allele, exhibit as little as 25% of normal STAT3 activity. Importantly, these patients have a high incidence of lung infection starting in early childhood, caused by community-acquired pathogens such as *S. aureus*, *S. pneumoniae*, and *H. influenzae* (Freeman and Holland 2009). Pneumatocoeles commonly develop, likely due to abnormal healing of the injured lung, which subsequently become sites for infection by Gram-negative bacteria and fungi (Freeman and Holland 2009). Ultimately, pneumonia is the cause of death for these patients (Freeman et al. 2007). These observations demonstrate that STAT3 is required for host defense and tissue repair during pneumonia.

HIES patients have reduced STAT3 function in all cells of the body, and the severe phenotype likely results from multiple roles of STAT3 in diverse cells which together function to enhance host defense, limit injury, and promote repair (Table 7.1) (Quinton et al. 2008; Matsuzaki et al. 2006; Kida et al. 2008; Hokuto et al. 2004; Minegishi et al. 2007; Milner et al. 2008; Minegishi et al. 2009; Saito et al. 2011; Gong et al. 2006; Jin et al. 2012; Takeda et al. 1999; Quinton et al. 2009; Matsukawa et al. 2005). Epithelial cells are one such site of STAT3 activity, but others are also important. For example, because the development and expansion of Th17 cells is highly dependent upon STAT3 activation downstream of IL-6 and IL-23 (Mathur et al. 2007), Th17 cell differentiation is significantly impaired in HIES patients (Milner et al. 2008; Minegishi et al. 2009; Ma et al. 2008; Woellner et al. 2010). Th17 products, namely IL-17A, IL-17F, and IL-22, are required for mucosal immunity (Ye et al. 2001; Aujla et al. 2008), suggesting that a paucity of Th17 cells may perhaps contribute to pulmonary infections in HIES patients. Lung epithelial cells can themselves be dependent on Th17-derived cytokines for mounting immune responses. A Th17 cytokine cocktail

stimulates the synthesis of select β-defensins and ELR+ CXC chemokines in cultured bronchial epithelial cells and keratinocytes, both representing sites of recurrent infection in HIES patients (Minegishi et al. 2009), more than in other cell types (Minegishi et al. 2009). Therefore, increased dependence of these cell types on Th17 cytokines may predispose lungs and skin to infection during HIES. While this finding does not address the importance of STAT3 in epithelial cells, it provides an axis whereby STAT3 is indirectly required for epithelial-mediated host defense. The facts that bone marrow transplantation fails to restore immune responses in HIES patients (Gennery et al. 2000) and that mice devoid of STAT3 in CD4+ T cells (Harris et al. 2007) or even all T cells (Takeda et al. 1998) fail to reproduce a phenotype consistent with HIES strongly argue that STAT3 in other cells must also contribute to preventing the pulmonary pathophysiology of HIES (Table 7.1). STAT3 in lung epithelial cells is understudied, yet multiple lines of evidence implicate it as a critical determinant of mucosal responses during bacterial pneumonia.

7.8 STAT3 Activating Cytokines: A Window into STAT3 Function in the Lungs

While HIES provides the most direct evidence of STAT3 function in patients with lung infections, associative studies have linked the presence of STAT3-activating cytokines to outcome. For instance, IL-6 and IL-10, both of which signal primarily through STAT3, are elevated in the circulation of patients with pneumonia, and positively associate with sepsis severity and mortality (Kellum et al. 2007; Glynn et al. 1999). IL-6 is enriched in the lungs of patients with pneumonia (Dehoux et al. 1994; Kolsuz et al. 2003), and a common loss-of-function polymorphism in the IL-6 promoter (174G/C) has been positively correlated with invasive pneumococcal disease (Schaaf et al. 2005). Although these associative studies do not provide sufficient evidence to conclude that STAT3 activation is beneficial to patients with lung infections, they strongly support the likelihood that the cytokine-STAT3 axis is involved.

By manipulating local levels of STAT3-activating cytokines or STAT3 itself, investigators have more directly linked this pathway to lung biology, including in some cases epithelial cells specifically. These data uniformly support the involvement of STAT3 signaling in pulmonary inflammation and/or host defense. However, inconsistent and in some cases contradictory findings indicate that the roles of this pathway are undoubtedly context-specific. For instance, introduction of excess IL-6 in the lungs by intratracheal instillation or by epithelial-specific overexpression significantly reduces inflammatory injury in response to either LPS (Ulich et al. 1991) or hyperoxia (Ward et al. 2000), respectively. Anti-inflammatory functions of IL-6 are also supported in at least one loss-of-function study, during which neutrophil recruitment and cytokine expression was increased in IL-6-deficient mice (Xing et al. 1998). These results stand in contrast the conventional perception of IL-6 being a classic pro-inflammatory cytokine, instead suggesting that under certain circumstances, lung IL-6 may serve as more of a tissue protective factor. In the context of infection, however, IL-6 is required

for maximal innate immunity and host defense. During pneumococcal pneumonia, bacterial clearance and lung neutrophil accumulation are compromised in mice lacking a functional IL-6 gene (van der Poll et al. 1997). Similar results occur in IL-6-deficient mice challenged intratracheally with *E. coli*, which have impaired bacterial clearance correlating with reduced neutrophil emigration and decreased STAT3 activity (Jones et al. 2006). Thus, IL-6 contributes to host defense and STAT3 activation in the lungs during bacterial pneumonia.

Evidence of STAT3-activating factors affecting lung inflammation is not limited to IL-6. Lung-specific overexpression of IL-10, an anti-inflammatory cytokine, decreases innate immune responses to LPS (Spight et al. 2005) or *P. aeruginosa* (Sun et al. 2009), resulting in increased mortality in the latter. Consistent with these findings, pharmacological neutralization of IL-10 amplifies acute pulmonary inflammation in response to *S. pneumoniae* and *K. pneumoniae* lung infections (Greenberger et al. 1995; van der Poll et al. 1996). Granulocyte colony-stimulating factor is another STAT3-signaling cytokine produced in the lungs during pneumonia, and it is required for antibacterial host defense (Quinton et al. 2002; Gregory et al. 2007). Other examples that are less well-understood but potentially relevant to pulmonary immune function are the IL-6 family cytokines leukemia inhibitory factor (LIF) and IL-11, both of which are expressed in the lungs during pneumonia (Quinton et al. 2008). Epithelial-specific overexpression of either cytokine is protective in lungs challenged with hyperoxia (Wang et al. 2003; Waxman et al. 1998), and intratracheal LIF administration reduces neutrophil recruitment and cytokine expression in rats exposed to LPS (Ulich et al. 1994). However, the influence of either cytokine on mucosal immunity is unknown. The IL-17 cytokine axis has a well-appreciated role in pulmonary host defense against extracellular bacteria (Ye et al. 2001), and two key components of this pathway, IL-22 and IL-23, have now emerged as key STAT3 inducers. Kolls and colleagues have demonstrated that genetic deletion of either cytokine impairs host defense against Gram-negative bacteria in the lungs (Aujla et al. 2008; Happel et al. 2005; Dubin and Kolls 2007; Dubin et al. 2012). Tissue protective functions have been reported for IL-22, with inhaled IL-22 causing increased STAT3 activity in association with reduced ventilator-induced lung injury (Hoegl et al. 2011), a finding that may extend to lung injury during pneumonia. Other factors, such as leptin, oncostatin M, and IL-27, may also activate STAT3 and contribute to innate immunity in the airspaces (Quinton et al. 2008; Mancuso et al. 2002, 2011; Pearl et al. 2004; Kim et al. 2011; Holscher et al. 2005). These examples constitute a representation of some but not all STAT3-activating cytokines exhibiting relevant immunomodulatory roles, the entirety of which would be beyond the scope of this chapter.

7.9 Lung Epithelium as a Focal Point for STAT3 Activity

Studies focusing on cytokines upstream of STAT3 provide compelling insights regarding potential roles of STAT3 in the lungs. Yet, the degree to which STAT3 itself is responsible for the effects of these (or other) cytokines is only beginning to

be elucidated. Lung epithelial cells represent a key locus of STAT3 activity. Genetic mouse models, and in particular the Cre-*LoxP* system, have enabled site-specific manipulations of STAT3 in airway and alveolar epithelial cells. Together, these studies unequivocally reveal epithelial STAT3 activity as a key determinant of tissue protection, barrier function, and host defense.

One powerful tool for elucidating STAT3 function was generated by Bromberg et al., who showed that substitutions of two cysteine residues in the SH2 domain of STAT3 results in spontaneous dimerization and consequently a constitutively active form of STAT3 (STAT3-C) (Bromberg et al. 1999). Lian and colleagues targeted tetracycline-inducible STAT-C expression to mouse respiratory epithelial cells by combining this strategy with a CCSP-rtTA system (Lian et al. 2005). Following 2 months of doxycycline treatment, mutant mice were protected against hyperoxia-induced lung injury, with increased survival and reductions in several indices of lung injury (Lian et al. 2005). Using the same mouse model, however, it was discovered that long-term overexpression (9 months) of STAT3-C results in inflammatory lung injury and adenocarcinoma formation (Li et al. 2007). These STAT3-C epithelial mutant mice also contain an expanded population of CD11b+ Gr-1+ cells characteristic of myeloid-derived suppressor cells (MDSCs), concordant with decrements in the number and function of CD4+ T lymphocytes (Wu et al. 2011b). Thus, lung tumor abundance may result from a combination of increased tumorigenesis and decreased immunosurveillance. Together, these results indicate that, while protective in some contexts, over-exuberant STAT3 activity in lung epithelial cells can have harmful consequences. Therefore, in response to lung infections and other respiratory challenges, the kinetics of STAT3-signaling cytokines must be balanced to maximize the beneficial effects of STAT3 activation.

While STAT3-C studies show that STAT3 in epithelial cells is sufficient to modulate lung phenotypes, engineering of mice with floxed STAT3 alleles has enabled site-specific investigation of endogenous STAT3 in alveolar epithelial cells. To generate this tool, floxed STAT3 mice (Takeda et al. 1998) were crossed onto a genetic background containing tetracycline-inducible Cre recombinase and rtTA under control of an SP-C promoter (Hokuto et al. 2004). This system results in gene targeting, and in this case STAT3 deficiency, across virtually all alveolar epithelial cells in mice exposed to doxycycline throughout gestation (Hokuto et al. 2004; Perl et al. 2002). Complementing the results observed after STAT3-C overexpression, alveolar epithelial STAT3-deficient mice had significantly increased lung injury in response to hyperoxia compared to littermate controls (Hokuto et al. 2004). Studies from our own laboratory were designed to interrogate the influence of STAT3 during Gram-negative pneumonia. In response to intratracheal *E. coli*, mice lacking STAT3 showed a modest but significant decrease in lung bacterial clearance. However, mutant mice had excessive lung injury compared to controls (Quinton et al. 2008). Interestingly, neutrophil recruitment did not correlate with injury in STAT3-deficient mice, suggesting the STAT3's role(s) in tissue protection may be directly related to epithelial integrity. Overall, these data indicate that alveolar epithelial STAT3 serves to both promote host defense and limit tissue injury during pneumonia, two outcomes that are often contradictory. The role of STAT3 in alveolar epithelial cells has also

been studied in mice challenged with adenovirus (Matsuzaki et al. 2006), LPS (Ikegami et al. 2008), and naphthalene (Kida et al. 2008), all of which caused more robust lung injury in the absence of STAT3. Significant loss of epithelial cells was consistent amongst all of these models, such that injury was targeted to the site of STAT3 deletion. These results during pneumonia and other relevant contexts suggest that STAT3 activity is a critical prerequisite to epithelial barrier integrity, thereby influencing both lung injury and host defense.

7.10 Mechanisms of STAT3 Activation in Respiratory Epithelium

Unlike NF-κB, STAT3 activation does not get triggered by pattern recognition receptors interacting with bacterial products. There is an expansive range of cytokines capable of activating STAT3, with an equally vast array of known physiological functions. As discussed above, many have been linked to lung injury and/or infection, including but not limited to IL-6 (Jones et al. 2006; Quinton et al. 2008; Schaaf et al. 2005; Ward et al. 2000; Xing et al. 1998; van der Poll et al. 1997), OSM (Quinton et al. 2008), IL-11 (Quinton et al. 2008; Waxman et al. 1998), LIF (Quinton et al. 2008; Wang et al. 2003; Ulich et al. 1994), G-CSF (Quinton et al. 2002; Gregory et al. 2007), IL-10 (Greenberger et al. 1995; van der Poll et al. 1996), IL-22 (Aujla et al. 2008; Hoegl et al. 2011), IL-23 (Happel et al. 2005; Dubin and Kolls 2007; Dubin et al. 2012), IL-27 (Pearl et al. 2004; Kim et al. 2011; Holscher et al. 2005), vasoactive intestinal peptide (Ao et al. 2011), and leptin (Mancuso et al. 2002; Mancuso et al. 2011). Based on the heterogeneity of these cytokines, it is clear that the consequences of STAT3 signaling can vastly differ from one situation to another, such that all STAT3 signaling cytokines are not equal. Many of these, namely the IL-6 family, even share common receptor components (gp130), yet they exhibit both overlapping and completely distinct roles. The precise mechanisms whereby so many "similar" factors coordinate distinct functions remain unclear, although receptor distribution is almost certainly a major determinant. In response to lung infection, very little information exists regarding the identity of specific STAT3-activating cytokines required for epithelial STAT3 induction. LIF, IL-6, and IL-22 stand out as three key possibilities.

Although there are at least 10 different IL-6 family cytokine members, only four appear to be induced in lungs during Gram-negative bacterial pneumonia: IL-6, OSM, LIF, and IL-11 (Quinton et al. 2008). To determine the relative contribution of each cytokine to STAT3 activation in epithelial cells, we designed a bioassay in which an alveolar epithelial cell line, MLE-15, was stimulated with bronchoalveolar lavage fluid (BALF) from pneumonic mice in the presence of neutralizing cytokine antibodies. Ex vivo studies of mouse lung epithelial cells has long been a challenge for investigators in this field due to the major experimental limitations posed by cell lines and cultured primary cells, neither of which accurately resemble the alveolar surface of living mouse lungs. MLE-15 cells, however, exhibit many

characteristics and markers exclusive to type 2 alveolar epithelial cells (Cao et al. 2010; Wikenheiser et al. 1993), making them an attractive compromise for experiments that would not otherwise be feasible. Using this ex vivo bioassay, we surprisingly found that the only single cytokine for which neutralization substantially impaired BALF-induced STAT3 activation was LIF (Quinton et al. 2008). The combined neutralization of both IL-6 and LIF reduced STAT3 activation even further, implicating both cytokines as important upstream mediators of this process during pneumonia (Quinton et al. 2008). Although LIF protein is detectable in pleural effusions (Heymann et al. 1996) and is increased in BALF from patients with ARDS (Jorens et al. 1996), its functional relevance in these settings is unknown, making it an important avenue of future investigation. The roles of endogenous IL-6 are more appreciated in response to lung infection, during which it appears to promote mucosal immunity (Jones et al. 2006; van der Poll et al. 1997). Whether these cytokines and/or others integrate to mediate immunity and tissue protection through STAT3 in epithelial cells or other targets is poorly understood. An autocrine loop involving excess LIF-induced STAT3 is associated with lung epithelial tumorigenesis in cultured cells lacking the tumor suppressor gene *Gprc5a* (Chen et al. 2010), again linking excessive STAT3 activity to tumor development, this time in the context of LIF and lung epithelial cells. LIF-induced STAT3 signaling in epithelial cells may be beneficial in the acute phase of infection and injury, but its regulation, like with NF-κB, is equally important for health and homeostasis.

IL-22 is a Th17-derived cytokine that is important during pneumonia, and may be an additional important means through which STAT3 is activated in lung epithelial cells. In vitro, IL-22 induces STAT3 phosphorylation in A549 lung epithelial cells, implicating this cell type as a potential target in response to infection (Hoegl et al. 2011). IL-22 is essential for optimal host defense against *K. pneumoniae* in the lungs, as IL-22 neutralization limits bacterial clearance and survival during this infection (Aujla et al. 2008). IL-22 treatment of cultured lung epithelial cells increases the ex vivo killing of *K. pneumoniae*, dependent upon the antimicrobial protein lipocalin 2 (Aujla et al. 2008). Furthermore, IL-22 enhanced the growth and transepithelial resistance of cultured lung epithelial cells (Aujla et al. 2008). Altogether, these studies suggest that epithelial cells can directly respond to this cytokine in a functionally relevant capacity, likely via STAT3 (Aujla et al. 2008).

7.11 What Does Epithelial STAT3 Do?

STAT3 is important in epithelial cells for the reasons stated above, as are STAT3-signaling cytokines. Yet, the molecular mechanisms through which STAT3 governs biological responses are poorly understood. Overexpression of STAT3-C in A549 lung epithelial cells has been shown to result in numerous gene changes (Dauer et al. 2005). Some of the key gene categories identified by bioinformatic analyses in that study include “apoptosis,” “coagulation,” “invasion,” and “inflammation/immunity” (Dauer et al. 2005). The degree to which genes related to

these processes dictate the biology of STAT3-signaling cytokines during lung infections remains to be determined. Perhaps more relevant to the understanding of endogenous STAT3, Xu et al. transcriptionally profiled primary type 2 alveolar epithelial cells isolated from control mice and mutants lacking functional STAT3 (Xu et al. 2007). Deletion of STAT3 in alveolar epithelial cells dramatically altered the transcriptome, resulting in 1,105 differentially expressed genes (Xu et al. 2007). Gene ontology analysis of differentially expressed transcripts again implicated gene programs controlling apoptosis, along with other interesting biological processes including cell migration, chemotaxis, and lipid biosynthesis. In support of the latter, alveolar surfactant phospholipids are decreased in alveolar epithelial STAT3 mutant mice during hyperoxia, in association with exaggerated lung injury (Hokuto et al. 2004). In fact, mRNA expression is significantly decreased for SP-A, SP-B, and SP-C in these mice, and the effects of STAT3-deficiency are reversed by exogenous SP-B administration (Hokuto et al. 2004). Therefore, it is feasible that surfactant protein synthesis is one possible liaison connecting epithelial STAT3 activation to tissue protection.

The effects of epithelial STAT3 deficiency on lung injury are rather consistent, suggesting that loss of barrier function and perhaps loss of the epithelium itself is at fault. Anti-apoptotic signaling has emerged as perhaps the most likely consequence of STAT3 activity with regards to its effects on epithelial integrity. Indeed, epithelial apoptosis is an important cause of acute lung injury (Martin et al. 2003). As discussed above, gene expression profiles in epithelial cells over-expressing (Dauer et al. 2005) or lacking (Xu et al. 2007) functional STAT3 reveal many differentially expressed transcripts associated with apoptosis, most of which are anti-apoptotic. In support of this possibility, apoptosis was shown to be more prevalent in mice lacking alveolar epithelial STAT3 during viral pneumonia (Matsuzaki et al. 2006). Multiple anti-apoptotic gene products are reduced by STAT3 deletion, one of which, Bcl-x_L, reverses lung injury when administered exogenously to mutant mice (Matsuzaki et al. 2006). Although it is currently unknown whether cell survival is uniformly controlled by STAT3 in different settings of lung infection, it is possible that STAT3-mediated cell survival helps maintain barrier function during severe pulmonary inflammation such as bacterial pneumonia.

Once injury has occurred, such as in the acute phase of lung infection, the functional integrity of the epithelium relies on regeneration, migration, and ultimately, repair (Crosby et al. 2011). STAT3 activation is likely involved at this level as well (Gao and Bromberg 2006). Deletion of alveolar STAT3 in SP-C+ alveolar epithelial cells impairs the recovery of ciliated and non-ciliated epithelium following 2 days of naphthalene-induced lung injury (Kida et al. 2008), implying that STAT3 is required for maximal proliferation and/or differentiation of new epithelium. This is particularly interesting given that CCSP+ (Clara cells) and FoxJ1+ (ciliated epithelial cells) were not targeted for STAT3 deletion. In the same study, the authors determined the influence of STAT3 on cell migration using human bronchial epithelial cells (HBECs) in vitro (Kida et al. 2008). To do this, an HBEC monolayer was disrupted after being transduced with an adenovirus expressing GFP (control) or a dominant-negative version of STAT3, which was sufficient to block

STAT3 activity (Kida et al. 2008). In the absence of STAT3 activity, HBECs do not migrate as efficiently within the injured zone, suggesting that STAT3 may be required for wound repair in vivo, consistent with its presumed role in tissue protection (Kida et al. 2008).

Finally, it is conceivable that STAT3 has functions in epithelial cells beyond its role as a transcription factor. Most notably, it was recently shown that STAT3 in mitochondria is required for optimal function of the electron transport chain, which may influence the homeostasis of epithelial cells in response to stress (Wegrzyn et al. 2009). This process has also been shown to mediate Ras-dependent oncogenesis (Gough et al. 2009), in a manner that is independent of STAT3 nuclear translocation. These discoveries reinforce the concept that STAT3 has cytoprotective yet potentially oncogenic (if uncontrolled) capacity, but they broaden the scope of STAT3 biology outside of transcriptional regulation. While it is certain that STAT3 in epithelial cells is essential to mucosal responses during bacterial pneumonia, non-transcriptional roles for STAT3 in this setting are presently speculative.

7.12 Conclusion

Signaling from NF-κB and STAT3 in epithelial cells is critical for mucosal immunity and tissue protection in response to lung infection. However, much of our current understanding of these pathways is derived from circumstantial evidence supporting functional roles for either factor or the signals required for their activation. There is presently limited detail about the precise signals which activate these transcription factors in epithelial cells during lung infection. More remarkably, epithelial-specific functions of either transcription factor during pneumonia are only beginning to be delineated, with knowledge at present based more on extrapolation than direct experimental interrogation. The processes downstream of these two signaling hubs and protecting the host constitute a major knowledge gap in mucosal immunity. The regulation and function of NF-κB and STAT3 in epithelial cells during acute bacterial pneumonia should prove to be a fruitful and exciting avenue for future investigation.

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Chapter 8
Innate Immune Responses in Ventilator-Associated Pneumonia

Megan N. Ballinger and Theodore J. Standiford

8.1 Introduction

Mechanical ventilation is a life-saving treatment of patients with acute and chronic respiratory failure. However, an adverse consequence of this intervention is the development of ventilator-associated pneumonia (VAP), which results in considerable morbidity and mortality in hospitalized patients (American Thoracic Society; Infectious Diseases Society of America 2005; Fujitani et al. 2011). VAP is defined as the development of pneumonia within 48–72 h after endotracheal intubation. Although the incidence of VAP is decreasing, still 9–27% of ventilated patients will develop this complication, with the highest incidence occurring in the first 10 days after intubation. Endotracheal intubation increases the risk of developing health care associated pneumonia by 6–20-fold. As compared to health care associated pneumonia (HAP) in non-intubated patients, both actual and attributable mortality is higher in VAP. Patients with certain underlying lung diseases, such as acute lung injury (ALI) and acute respiratory distress syndrome (Richardson et al. 1982), have a particularly high incidence of VAP (Richardson et al. 1982). Conversely, VAP represents a major risk factor for the development of ALI and ARDS.

8.2 Etiology of VAP

VAP can be caused by an array of Gram-negative and Gram-positive bacterial pathogens, and may be polymicrobial in up to a third of cases (American Thoracic Society; Infectious Diseases Society of America 2005; Fujitani et al. 2011).

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The most common cause of VAP is by the Gram-positive bacteria *Staphylococcus aureus*, with methicillin resistant *S. aureus* (MRSA) representing over 60% of the *S. aureus* isolates in VAP. Other VAP-causing pathogens include aerobic Gram-negative bacilli such as *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli*, *Enterobacter* species, *Acinetobacter* species, and *Stenotrophomonas maltiphila*. *Legionella pneumophila* is an obligate intracellular bacterial pathogen that is an etiologic agent in both community acquired pneumonia (CAP), HAP and VAP. Viruses and fungi are unusual causes of VAP, although these organisms can modulate innate mucosal responses predisposing to the development of VAP. While the bacterial pathogens that cause VAP are similar to those that cause HAP in non-intubated patients, VAP is more frequently caused by pathogens with intrinsic resistance to multiple antimicrobial agents, including *P. aeruginosa*, *Acinetobacter* species, *S. maltiphila*, and MRSA. Mortality is considerably higher in patients with VAP due to *P. aeruginosa* strains that express the type III secretion system required for the secretion of pseudomonal exotoxins S, T, U, and Y (Roy-Burman et al. 2001; Sadikot et al. 2005). A recent and disturbing trend is the increasing prevalence of community acquired stains of MRSA (CA-MRSA) as a cause of nosocomial infections, including VAP(Kashuk et al. 2010). CA-MRSA, which is typically the USA300 strain, produce an array of exotoxins that promote extensive tissue necrosis and cavity formation. The intrinsic antibiotic resistance of these Gram-positive and Gram-negative bacterial strains contributes to increased mortality in patients with VAP (Fujitani et al. 2011). However, these pathogens are generally less virulent and invasive than pathogens that cause pneumonia in otherwise healthy individuals in the community, and tend to be invasive in hosts with anatomic defects in the respiratory tract or substantial impairment in lung mucosal innate immunity. Therefore, the presence of these bacterial species as pathogens identifies patients with profound anatomic defects or defects in lung innate immunity.

8.3 Pathogenesis of VAP

The vast majority of VAP cases develop as a result of microaspiration of bacteria colonizing the oropharynx (American Thoracic Society; Infectious Diseases Society of America 2005). Oropharyngeal colonization occurs very rapidly in critically ill patients. For example, nearly 75% of patients with underlying lung disease and/or undergoing oropharyngeal intubation were found to be colonized by pathogenic bacteria within 24 h of admission to the intensive care unit (Garrouste-Orgeas et al. 1997). Reservoirs contributing to oropharyngeal colonization include the nasopharynx, sinuses, and stomach. Endotracheal tubes contribute to colonization by directly injuring mucosal surfaces of the upper respiratory tract, which facilitates bacterial adhesion. Organisms that cause VAP, including *P. aeruginosa* and *S. aureus*, promote biofilm formation with the endotracheal tube lumen, which can function as a nidus for direct inoculation of infected material into the distal airspaces. Less common sources of bacterial inoculation include colonization of the ventilator circuit or direct inoculation via infected aerosols or instrumentation, particularly suction catheters or

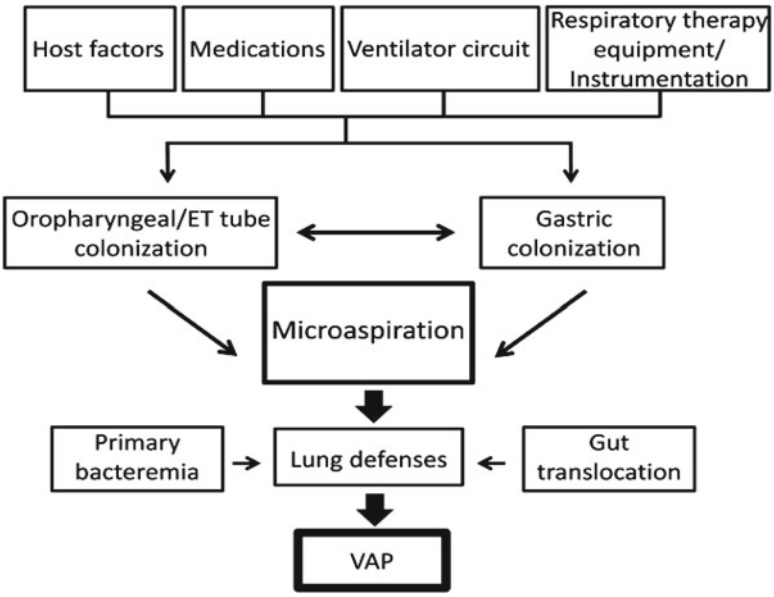

Fig. 8.1 Factors contributing to the pathogenesis of VAP. A variety of different contributing factors have been previously shown to contribute to the development of VAP. The end result from a combination of host factors, medication, and instrumentation is the introduction of infectious material into the sterile lung environment. These factors along with the immune state of host, contribute to the development of VAP

bronchoscopes. By comparison, hematogenous seeding of the lung as a cause of VAP is considerably less common, accounting for <15% of cases. Notable exceptions are hematogenous seeding from an intravascular *S. aureus* infection or gut bacterial translocation that can occur in immunocompromised patients with neutropenia.

Microaspiration is a common event in both healthy and critical ill patients. These events rarely result in infection in healthy subjects, primarily due to highly effective means to eradicate infectious or toxic insults of the respiratory tract, which include efficient mucocilliary clearance mechanisms and robust innate mucosal antimicrobial responses. In mechanically ventilated patients, impairments in both mucocilliary transport and innate cellular responses results in the establishment of pulmonary infection. A summary of factors contributing to the pathogenesis of VAP is shown in Fig. 8.1.

8.4 Structural Changes in the Respiratory Tract in Mechanically Ventilated Patients

Ciliated, pseudostratified columnar epithelial cells line the tracheobronchial tree. These ciliated cells are critical to effective mucocilliary transport and the cephalad movement of mucous, microbes, and acellular debris present within the conducting airways. Damage to ciliated cells can occur as a direct result of endotracheal intubation

or conditions that predispose the patient to respiratory failure (Nicholls et al. 2003; Piatti et al. 2005; Pittet et al. 2010). As discussed previously, denuding of columnar epithelial cells can result from the endotracheal tube or endotracheal tube cuff. Moreover, lung conditions that can result in mechanical ventilation, such as COPD, are associated with impaired mucocilliary transport (Piatti et al. 2005). Moreover, certain forms of infectious lung injury, including severe acute respiratory syndrome (SARS) is characterized by bronchial epithelial denudation and loss of cilia (Nicholls et al. 2003). Similarly, influenza infection predisposes to secondary bacterial infection, which is due not only to impairment in lung innate responses, but also disruption of mucocilliary transport mechanisms (Pittet et al. 2010).

8.5 Impairment in Innate Immunity

Many forms of critical illness result in a profound state of immune suppression affecting both the cellular and acquired arms of host immunity. This syndrome of immune suppression has been best characterized and is perhaps most severe in sepsis, but has also been described in trauma patients, burn injury patients, and patients during the peri-operative period. Sepsis is a complex clinical syndrome resulting from the interaction between microbe and host. Clinically, it is defined as the systemic inflammatory response syndrome (SIRS) with evidence of infection (Members of the American College of Chest Physicians/Society of Critical Care Medicine 2003). Changes in the population at risk for the development of sepsis, including an increase in the number of elderly and immunocompromised patients, has resulted in a steady rise in the incidence of severe sepsis (Martin et al. 2003). Despite improvements in supportive care and immunomodulatory therapies, the mortality rate from severe sepsis remains unacceptably high (Brun-Buisson 2000).

Host immune responses in critical illness, including sepsis can be conceptualized as occurring in distinct but overlapping phases. The initial response during critical illness, referred to as the systemic inflammatory response syndrome (SIRS), is characterized by the release of a number of pro-inflammatory mediators, including early responses cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin 12 (IL-12) leukocyte-active chemokines, adhesion molecules, and inflammatory leukotrienes (Dinarello 2000). SIRS is counter-regulated by the release of inhibitory molecules, including anti-inflammatory cytokines (e.g., interleukin 10 (IL-10), transforming growth factor-beta (TGF-β)), suppressors of pathogen recognition signaling cascades, immunomodulatory prostanoids and hormones. This counter-regulatory phase is referred to as the compensatory anti-inflammatory response syndrome (CARS) (Wesche et al. 1999; Bone 1996). Molecules released during CARS are believed to serve as a functional "brake" on systemic inflammation, and the expression of these mediators is induced by both microbial-derived and host-derived signals. SIRS and CARS overlap considerably, hence the overall immune status of the patient is dependent on which response predominates (Fig. 8.2) (van der Poll and van Deventer 1999). Recent evidence

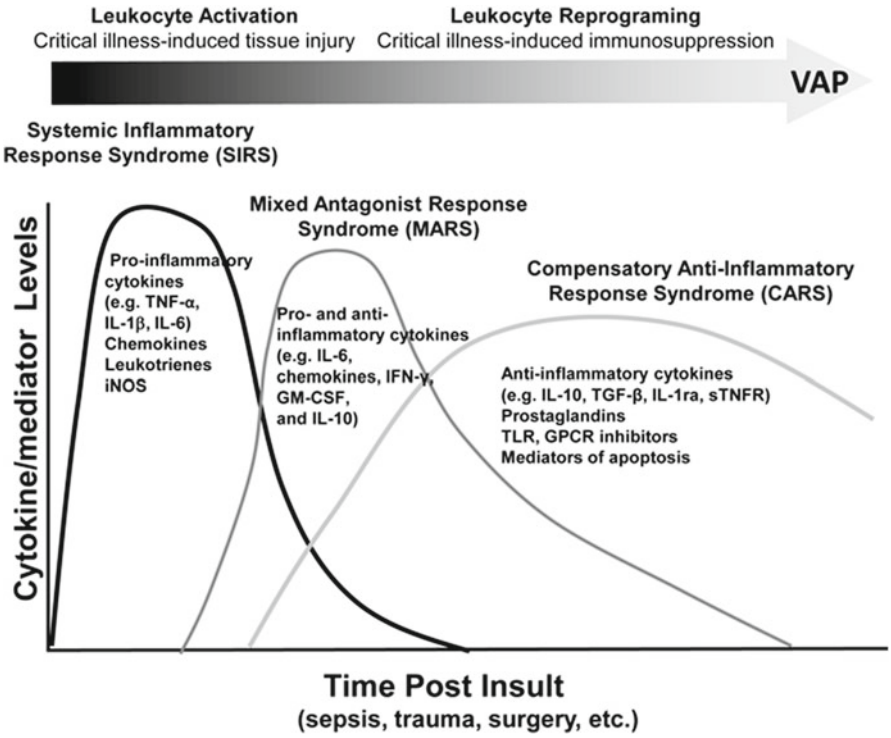

Fig. 8.2 Innate immune events in critical illness. The dysregulation of the innate immune system is a main factor in the development of VAP. The progression of leukocyte activation, along with SIRS, followed by leukocyte reprograming, including MARS and CARS, contributes to the overall dysfunctions leading to the development of VAP

suggests a third response to an inflammatory insult, referred to as the mixed antagonist response syndrome (MARS). This response is characterized by the secretion of both pro- and anti-inflammatory mediators (specifically IL-6, IL-8, monocyte chemotactic protein (MCP)-1, macrophage inflammatory protein (MIP)-1β, IFN-γ, granulocyte-macrophage colony stimulating factor (GM-CSF), and IL-10) (Tamayo et al. 2011). Consistent with this mixed systemic cytokine response, elevated levels of IL-6 in circulation has been shown to predict the development of VAP (Ramirez et al. 2009). Whether the initial SIRS response drives the expression of molecules that contribute to immune suppression or simply a marker of systemic inflammation remains to be determined. A summary of innate immune events in critical illness is shown in Fig. 8.2.

The compensatory release of anti-inflammatory molecules in sepsis is believed to mediate immunosuppression during the peri-septic or post-injury period, during which time immune cell function is substantially impaired (historically referred to as critical illness-induced leukocyte "deactivation" or "immunoparalysis"). Recently, since the altered leukocyte phenotype in critical illness involves selective regulation of some, but not all innate genes, this phenomenon is now more appropriated referred to as

reprogramming. Leukocyte reprogramming appears to be of considerable clinical significance, as higher rates of nosocomial infection and increased mortality are observed in postoperative, burn injury or septic patients who display evidence of monocyte deactivation, either in the form of decreased monocyte HLA-DR expression, ex vivo cytokine production or impaired delayed-type hypersensitivity responses (Appel et al. 1989; Munoz et al. 1991). Septic patients are especially susceptible to nosocomial infections of the lung, particularly pneumonia from multidrug-resistant Gram-positive and Gram-negative organisms, including *S. aureus* and *P. aeruginosa* (Richardson et al. 1982; Mustard et al. 1991). Sepsis-induced immunosuppression is particularly prominent in patients with preexisting deficiencies in innate and acquired immunity, including the elderly and patients with chronic medical conditions (Hotchkiss and Karl 2003).

8.6 Alterations of Leukocyte Function in Critical Illness and Mechanical Ventilation

Patients undergoing severe stress, including trauma, massive hemorrhage, burn injury, post-surgery, and sepsis exhibit significant defects in circulating and resident leukocyte populations. In addition, changes in the pulmonary microenvironment that occur as a result of mechanical ventilation substantially influence lung innate responses. Multiple leukocyte subtypes are affected and specific defects are shown in Fig. 8.3.

8.6.1 Monocytes/Macrophages

While sepsis and similar stress-associated events have been shown to influence the effector activity of a variety of immune cells, the majority of studies have focused on peripheral blood monocytes (PBM), and to a lesser extent tissue macrophages. Changes in monocyte/macrophage function in sepsis resemble but are not identical to those observed in endotoxin-tolerized macrophages. Endotoxin tolerance describes the phenomena whereby upon initial exposure to LPS, cells become refractory to a secondary stimulus with LPS. Pathogen-associated molecular patterns (PAMPs) other than LPS can also induce a tolerance phenotype, and PAMPs of one type can induce cross tolerance to a different PAMP. Induction of tolerance results in suppression of multiple inflammatory genes, including both NF-κB and mitogen-activated protein kinase (MAPK)-dependent genes (e.g., TNF-α, IL-6, iNOS). Tolerance does not cause global suppression of all genes, as genes encoding certain antimicrobial and phagocytic proteins, including cathelicidin antimicrobial peptide, lipocalin, the scavenger receptor MARCO and the fMLP receptor, are indeed super-induced in response to sequential exposure to LPS (Foster et al. 2007). It is also noteworthy that the induction of this phenotype is not restricted to myeloid

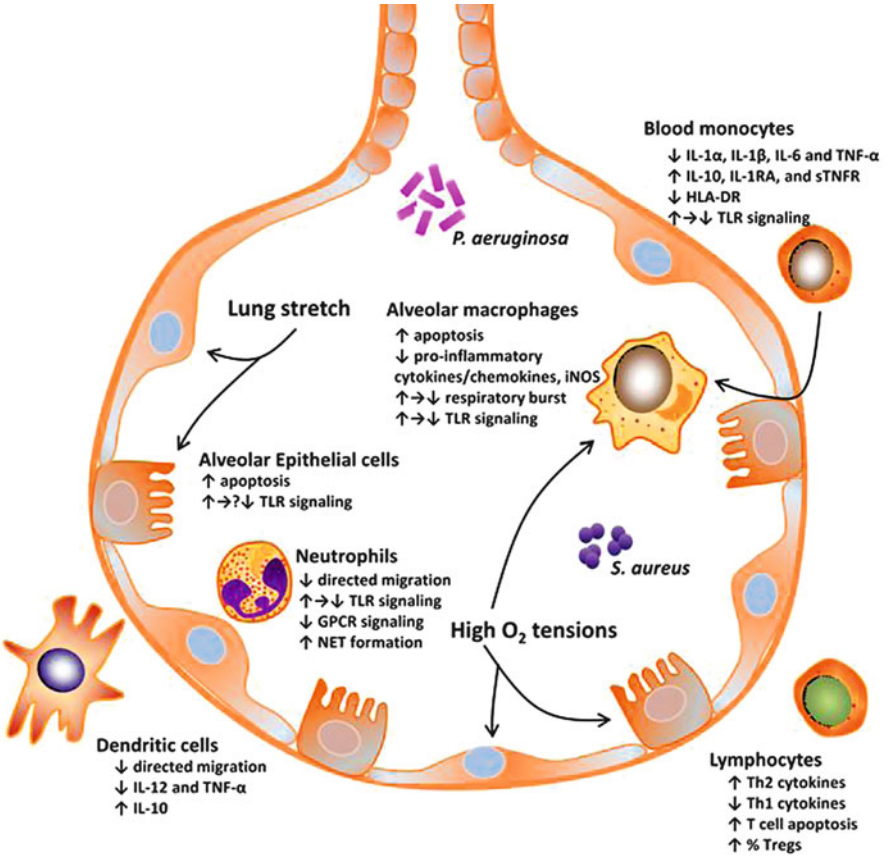

Fig. 8.3 Alterations and specific defects of leukocyte function in critical illness and mechanical ventilation. There are a variety of cellular, bacterial, and mechanical mediators which contribute to the impaired innate and acquired immune responses during critical illness. (*Upward arrow*) represent effects that enhance expression/function (*downward arrow*) represents effects that reduce expression/function

cells, as structural cells, including alveolar epithelial cells, have been shown to develop a tolerance response upon repeated exposure to PAMPs. The LPS or PAMP tolerized phenotype is transient in nature and entirely reversible, and has been associated with remodeling of chromatin in the promoter region of several tolerizable genes (Foster et al. 2007; Chan et al. 2005).

Critical illness, like endotoxin tolerance, leads to inhibition of a broad range of NF-κB-dependent inflammatory genes in monocytes. Most notably, a significant reduction in the ex vivo production of inflammatory cytokines, including IL-1α, IL-1β, IL-6, and TNF-α has been observed in monocytes isolated from patients with sepsis (Munoz et al. 1991). This change in cytokine production may be a predictor of outcome, as peripheral monocytes isolated from those who survived sepsis regained their ability to produce cytokines in response to LPS stimulation, and

monocytes isolated from the nonsurvivors did not (Munoz et al. 1991). Conversely, the production of certain anti-inflammatory proteins, including IL-10, IL-1 receptor antagonist, and the TNF soluble receptor I and II are enhanced in monocytes isolated from sepsis patients or patients with ventilator-induced lung injury (Frank et al. 2006). Patients with sepsis or early trauma have reduced monocyte HLA-DR expression (Appel et al. 1989; Adib-Conquy et al. 2006). This reduction in HLA-DR expression has been reported to directly correlate with the magnitude of sepsis (Volk et al. 2000) and may partially contribute to impaired cell-mediated immunity observed in patients with critical illness.

Similar critical illness-induced defects have been noted in macrophages residing in various tissues, which in some instances have been associated with evidence of enhanced macrophage apoptosis (Ayala et al. 1992; Gallinaro et al. 1994). In particular, alveolar macrophage function has been shown to be impaired in the setting of sepsis. For example, alveolar macrophages recovered from mice with abdominal sepsis (cecal ligation and puncture) display reduced production of inflammatory cytokines, chemokines, eicosanoids, nitric oxide, and respiratory burst (Reddy et al. 2001; Goya et al. 1992). Importantly, these phenotypic alterations in alveolar macrophage effector function are associated with a markedly enhanced susceptibility to intrapulmonary challenge with both Gram-positive and Gram-negative bacterial pathogens (Steinhauser et al. 1999; Deng et al. 2006). Little is known about alveolar macrophage phenotype in critically ill patients at risk for the development of VAP. However, we have performed Affymetrix microarray analysis on adherence purified alveolar macrophages recovered from patients with sepsis-induced ALI within 3 days of onset of sepsis. Relative to alveolar macrophages recovered from healthy subjects, lung macrophages from sepsis-induced ALI patients displayed a hybrid tolerized/alternatively activated phenotype, as characterized by minimal change or suppression of NF-κB-dependent genes (e.g., TNF-α, IL-1β, IL-6, iNOS), induction of antimicrobial genes (antimicrobial peptides, chemoattractant, and phagocytosis genes), and expression of makers of alternative (M2) rather than classical (M1) activation (high arginase, CCR2, IL-4Rα, MMP expression; low iNOS, interferon-γ, and IFN-inducible chemokine expression) (Gordon and Martinez 2010). Although this expression pattern may partially reflect the lung injury response, it is likely that the phenotype is shaped by systemic inflammation.

8.6.2 Neutrophils

Alterations in neutrophils (PMN), resembling those described in monocyte/macrophages, are present during the septic response and are predictive of adverse outcomes in these patients. Systemic inflammation promotes cytoskeletal changes in PMN cell membrane rigidity and reduced cellular deformability, resulting in impaired recruitment to sites of infection and deleterious accumulation and activation of PMN in vascular beds of distant organs. Directed migration is also impaired by nitric oxide-mediated inhibition of ICAM and VCAM-dependent adhesion and transmigration of PMN,

downregulation of the chemokine receptor CXCR2, and inhibition of G-protein coupled receptor signaling (Benjamim et al. 2000; Cummings et al. 1999; Czermak et al. 1999; Huber-Lang et al. 2001; Swartz et al. 2000). Microarray analysis of PMN isolated from septic patients within 24 h of onset reveals a global suppression of immune regulation and inflammatory response gene clusters, particularly genes regulated in an NF-κB-dependent fashion (Tang et al. 2007). Conversely, the expression of selected suppressive genes was enhanced, including the NF-κB inhibitor NFκBIA.

The discovery of neutrophil extracellular traps (NETs) has provided yet another role for neutrophils in the containment of infection. NETs are complex structures composed of nuclear chromatin, histones, a variety of granular antimicrobial proteins and some cytoplasmic proteins (Urban et al. 2009). Formation occurs in response to exposure of neutrophils to plasma from septic patients (Clark et al. 2007) as well as direct contact with microbial pathogens (Remijsen et al. 2011). Neutrophil elastase is released from azurophilic granules, assisting in the formation of NETs via decondensation of nuclear chromatin, which along with other serine proteases confer antimicrobial responses (Papayannopoulos et al. 2010). NET-associated myeloperoxidase directly contributes to bacterial killing of *Staphylococcus aureus* in the presence of H_2O_2 (Parker et al. 2012). NETs are capable of physically ensnaring bacteria and facilitating the interactions between bacteria and antimicrobial effectors, ultimately leading to enhanced bacterial killing (Mantovani et al. 2011). Despite their broad antimicrobial capacity, some bacteria express nucleases to degrade NETs, thus avoiding capture and bacterial cell death (Buchanan et al. 2006; Berends et al. 2010; Young et al. 2011). In some cases, NETs may exert detrimental effects to the host. Increasing evidence links NET formation to excessive inflammation and tissue damage in diseases such as sepsis (Clark et al. 2007). NET formation has recently been demonstrated in the alveoli of mice with influenza H1N1 pneumonia, and these structures contribute to acute lung injury responses in these animals (Narasaraju et al. 2011). While the presence of NETs has not been clearly established in experimental bacterial pneumonia or in patients with VAP, it is tempting to speculate that these structures may contribute to lung injury that can occur in this setting.

8.6.3 Dendritic Cells

Dendritic cells (DC) are the most efficient professional antigen-presenting cells (APC) in the lung and have the unique ability to induce primary immune responses in naïve T cells. DC are prevalent centrally within the spleen, lymphatics, and at mucosal surfaces, most notably in gut and respiratory tract. Systemic endotoxin administration in mice results in a brisk depletion in splenic DC by 24 h post-LPS. Similarly, there is a prolonged loss of DC out to 15 days post-induction of abdominal sepsis in both lung and spleen (Wen et al. 2008). In humans with lethal sepsis, follicular DC are substantially diminished early in the course of disease (Hotchkiss et al. 2002).

Similarly, reductions in blood myeloid DC and plasmacytoid DC (27 and 53% of controls, respectively) have been observed in patients admitted to the hospital with pneumonia, and numbers of DC inversely correlated with procalcitonin levels, a marker of systemic inflammation (Dreschler et al. 2012). Endotoxin-tolerized DC or DC isolated from animals or humans with sepsis produce low levels of IL-12 and TNF-α, but high levels of IL-10 (Wen et al. 2008; Wysocka et al. 2001). This shift in cytokine profiles can persist for up to 6 weeks post-abdominal sepsis (CLP), and has been associated with posttranslational epigenetic modifications of histones binding to the IL-12 p35 and p40 promoters and increased susceptibility to pulmonary fungal challenge (Wen et al. 2008). Regulatory DC, or "tolerogenic" DC, are a newly described DC population that can be induced by incubation of bone marrow-derived DC with IL-10, resulting in DC that preferentially secrete IL-10 rather than IL-12, and induce T cell tolerance. A naturally occurring DC_{reg} population has been identified in spleen ($CD11c^{low}$, $CD45RB^{high}$), and adoptive transfer of this cell population to septic mice diminished inflammatory cytokine production and sepsis-induced lethality (Fujita et al. 2006). Changes in the number, distribution, and function of these cells in lung, especially during critical illness, have not yet been explored.

8.6.4 Lymphocytes

Like other leukocyte populations, various lymphocyte populations are influenced by and likely contribute to the immunosuppressive effects of critical illness. This effect can be directly due to changes in lymphocytes numbers or effector functions, or indirectly due to changes in APC function, most notably DC. Studies consistently show that sepsis or other states of extreme stress (trauma, burn injury) generally result in anergy and a shift in T cell cytokine responses favoring a Th2-, rather than Th1-phenotype response.

Sepsis, trauma, and other critical states result in a substantial drop in the number of circulating lymphocytes. Lymphopenia develops early after the insult, and the persistence and magnitude of lymphopenia correlates with risk of nosocomial infection and death (Hotchkiss et al. 2001). Autopsy studies in septic patients revealed a profound loss of splenic B cells, CD4+ T cells, and follicular dendritic cells. No alterations in numbers of CD8+ T cells were observed. The loss of B and CD4+ T cells was mediated by caspase-9-dependent apoptosis. Similar changes, although not as uniform, could be observed in critically ill patients without sepsis (Hotchkiss et al. 2001).

In addition to changes in the absolute number of lymphocytes, the septic response can induce considerable alterations in lymphocyte effector function. For instance, the memory/effector CD8+/CD45RO+ T lymphocyte subset in nonsurviving septic patients demonstrate significantly decreased IFN-γ synthesis compared with survivors (Zedler et al. 1999). Similarly, T cell proliferative responses and cytokine production (IL-2, TNF-α) were significantly depressed in patients with abdominal sepsis, as compared to healthy controls, and the degree of IL-2 and TNF-α suppression directly correlated with patient survival (Heidecke et al. 1999). The proportion of Th2 T cells is increased in patients with sepsis, but not in non-septic critically ill

control patients and healthy subjects (Ferguson et al. 1999). Similar observations have been made in animal models of sepsis. Splenocytes isolated from mice undergoing CLP produced less IL-2, IL-12, and IFN-γ, and more IL-4 and IL-10 than splenocytes isolated from healthy animals (Ayala et al. 1994; O'Sullivan et al. 1995). Given the importance of Th1 phenotype responses in host defense against both intracellular and extracellular microbial pathogens, this shift away from an appropriate Th1- and towards a dysregulated Th2-phenotype response has obvious implications for antimicrobial host immunity.

Regulatory T cells (Treg), are a limited but important population of CD4+, CD25+ T cells that universally express the transcription factor Forkhead box p3 (Foxp3). Treg inhibit CD4+ and CD8+ T cell effector functions, resulting in negative regulation of both innate and acquired immune responses. Suppressive effects of Treg are mediated by both direct cell–cell contact and through the release of soluble mediators, including but not limited to TGF-β and IL-10. An increase in the percentage (but not absolute number) of Treg has been found in blood, lymphatics, or spleen in septic mice and humans with sepsis or trauma (Venet et al. 2008; Scumpia et al. 2006; Wisnoski et al. 2007). Moreover, there is evidence of enhanced Foxp3 expression and suppressive function of Treg in mice with abdominal sepsis, and adoptive transfer of Treg into septic mice reduced overzealous TNF-α production and improved mortality. However, the depletion of CD4+ CD25+ Treg in mice with polymicrobial sepsis had little impact on sepsis-induced mortality (Scumpia et al. 2006; Wisnoski et al. 2007). Thus, the role of Treg in controlling the systemic inflammatory response, or as a mediator of impaired innate and acquired immunity in critically ill patients at risk for VAP, is uncertain and requires further study.

A recently described B cell may play a critical role in innate responses during localized and systemic infection (Rauch et al. 2012). Innate response activator B (IRA-B) cells are a population of CD19+, B220+ cells that produce large quantities of GM-CSF during infection. This population expands in bone marrow and spleen in response to systemic LPS administration or abdominal sepsis, and the genetic deletion of these cells resulted in marked reduction of systemic cytokine responses, GM-CSF expression, and the ability to clear abdominal polymicrobial infection.

8.7 Alterations of Pathogen Recognition Receptors and/or Signaling Cascades in Critical Illness

Microbes and microbial components that initiate the septic response are recognized by both cell surface and intracellular pathogen recognition receptors (PRR), including Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLR). Toll-like receptors are a family of evolutionarily conserved type I transmembrane receptors that respond to PAMPs expressed by a diverse group of infectious microorganisms, resulting in activation of the host's immune system (Aderem and Ulevitch 2000; Akira and Hemmi 2003). There exist 13 distinct TLRs (10 in humans and 13 in mice) that have in common an extracellular domain with

leucine rich repeats and an intracytoplasmic domain shared with the IL-1 receptor (IL-1R). Binding of ligands to TLRs initiates a signaling cascade involving myeloid differentiation marker 88 (MyD88), IL-1R-associated kinases (IRAK 1 and 4), and TNFR-associated factor 6 (TRAF6), resulting in NF-κB translocation and MAPK activation, culminating in expression of genes involved in antimicrobial host defense (Aderem and Ulevitch 2000; Akira and Hemmi 2003). In addition, certain TLRs, such as TLR2, TLR3, and TLR4 can initiated a MyD88-independent signaling cascade that requires the adaptor proteins Toll-IL-1 receptor domain containing adaptor protein inducing interferon (TRIF) and TRIF-related adaptor molecule (TRAM), resulting in the expression of interferon responsive genes. The most relevant TLRs in lung antibacterial host defense include TLR2, which recognizes specific components of Gram-positive bacteria and fungi; TLR4, which is the major receptor for LPS; TLR5, which recognizes and is activated by bacterial flagellin; and TLR9, which is activated by unmethylated CpG motifs present in microbial but not mammalian DNA. In addition to PAMPs, TLRs can be activated by host-derived danger signals, referred to as damage-associated molecular patterns (DAMPs) or alarmins, and include heat shock proteins and matrix components (Ohashi et al. 2000). Also, high-mobility group box 1 protein (HMGB1) is a molecule released during the septic response that has recently been shown to activate TLR2 and TLR4 (Park et al. 2004). This is of particular relevance in the setting of sepsis and acute lung injury.

Multiple TLRs participate in lung host immunity against Gram-negative bacteria. For example, TLR4 recognizes the lipid A moiety of LPS, and is the major TLR mediating early innate responses and clearance of non-flagellated Gram-negative organisms that cause VAP, including *K. pneumoniae*, *H. influenza, and E. coli* (Schurr et al. 2005; Bhan et al. 2010; Wieland et al. 2005). In addition, mice deficient in TLR9 display impaired dendritic cell-mediated responses during experimental *Klebsiella* or *Legionella* pneumonia, culminating in reduced lung bacterial clearance and decreased survival (Bhan et al. 2007, 2008). Innate responses to the flagellated extracellular bacteria *P. aeruginosa* are mediated by several MyD88-dependent TLRs, predominantly TLR4 and TLR5 (Hajjar et al. 2005; Ramphal et al. 2008; Skerrett et al. 2004). Interestingly, both bone marrow-derived and stromal cells contribute to MyD88-dependent innate responses to *P. aeruginosa* in the lung (Hajjar et al. 2005).

Toll-like receptors appear to play a lesser role in host defense against *S. aureus*. For example, while TLR2 has been shown to mediate inflammatory responses to the staphylococcal toxin Panton-Valentine Leukocidin, neither TLR2, TLR4, nor MyD88 is required for effective anti-staphylococcal host immunity during respiratory infection (Skerrett et al. 2004; Zivkovic et al. 2011). The nucleotide-binding oligodimerization domain (NOD)-like receptors (NLR) NOD1 and NOD2, which recognize the peptidoglycan component muramyl dipeptide (MDP), have been shown to be important in inflammatory cytokine release and bacterial eradication in a murine *S. aureus* skin infection model(Hruz et al. 2009; Inohara et al. 2005). More recently, mice deficient in RIP2, the shared NOD1/2 adaptor molecule, are considerably more susceptible to intrapulmonary challenge with *S. aureus* than wild-type mice, an effect which is dependent on downstream activation of inflammasome-caspase-1-dependent IL-1β release (unpublished observations, J. Deng). These later

observations suggest that NLRs, rather than TLRs, may be the predominant contributors to anti-staphylococcal immunity in the lung

8.8 Suppression of PRR Expression, Binding or Downstream Signaling Cascades

8.8.1 Alterations of Cell Surface Expression of TLRs and LPS Binding Partners

Some, but not all studies have identified changes in the cell surface expression of various TLRs during the septic response. In particular, either enhanced or reduced cell surface expression of TLR2 and TLR4 have been described in monocytes from sepsis patients and in tissue macrophages during experimental sepsis (Deng et al. 2006; Brunialti et al. 2006). Moreover, changes in monocyte cell surface expression of LPS binding partners MD2, CD14, and CD71 have also been observed in sepsis (Brunialti et al. 2006; Wolfs et al. 2008; Williams et al. 1998). Disparate findings are likely attributable to temporal differences in assessment of TLR expression and the heterogeneity of patient populations studied and animal models employed. The extracellular domains of certain TLRs can be shed from activated macrophages, and serve as sinks to bind extracellular PAMPs, and as a consequence dampen TLR-mediated signal transduction. For instance, soluble TLR2 (sTLR2) is released by human peripheral blood monocytes (PBM) and diminishes the cellular response to the TLR2 agonist Pam3Cys without affecting cellular responses to LPS (LeBouder et al. 2003). Both naturally occurring and recombinant soluble TLR4 have been shown to diminish responses to LPS (Iwami et al. 2000; Hyakushima et al. 2004). The contribution of soluble TLR2 and TLR4 to impaired innate responses during critical illness remains to be determined.

Illuminating the importance of TLRs in lung innate immunity during critical illness, combined loss of function polymorphisms in both TLR4 and the TLR4 adaptor TIRAP/Mal, or a homozygous TIRAP/Mal polymorphism have been causally linked to reduced circulating inflammatory cytokine levels, reduced ex vivo monocyte cytokine expression, and increased risk for serious postoperative infections, including VAP (Ferwerda et al. 2009).

8.9 Inhibitors of TLR Signaling

8.9.1 Interleukin-1 Receptor-Associated Kinase-M

Molecules have been identified that inhibit TLR signaling at multiple sites downstream of the receptor. Interleukin-1 receptor-associated kinase (IRAK)-1 and -4 are key kinases necessary for both MyD88-dependent and IL-1 receptor-mediated

signal transduction. Consequently, interruption of IRAK-1 and -4 phosphorylation or trafficking can have profound effects on the downstream expression of both NF-κB and MAPK-dependent inflammatory or antimicrobial genes. Interleukin-1 receptor-associated kinase-M (IRAK-M), also named IRAK-3, is a member of the IRAK family. However, IRAK-M differs from IRAK-1 and IRAK-4 in that this protein lacks kinase activity and IRAK-M has been shown to be a negative regulator of TLR signaling by blocking the disassociation of IRAK-1 from the Toll-IL-1 signaling domain. Bone marrow-derived or lung macrophages lacking IRAK-M display enhanced MAPK kinase activation and inflammatory cytokine production in response to TLR agonists and live bacteria (Wesche et al. 1999; Kobayashi et al. 2002). Importantly, IRAK-M is induced by endotoxin, the NOD-2 ligand muramyl dipeptide (MDP), and other PAMPs and is required for the development of tolerance to endotoxin and peptidoglycan (Kobayashi et al. 2002; Hedl et al. 2007; Nakayama et al. 2004). We have found that IRAK-M is upregulated in alveolar macrophages during experimental sepsis in a MyD88-dependent fashion, and mediates both the suppression of macrophage cytokine responses and impaired lung clearance of *P. aeruginosa* in septic mice (Deng et al. 2006; Lyn-Kew et al. 2010). IRAK-M has also been shown to suppress TLR-mediated responses in murine primary alveolar epithelial cells (Seki et al. 2010). Emerging data suggests that IRAK-M may be a major mediator and perhaps a biomarker for severity of disease in sepsis. IRAK-M is substantially induced in monocytes from healthy subjects administered LPS intravenously (van't Veer et al. 2007). In patients with Gram-negative sepsis, blood monocytes demonstrate a more rapid and robust expression of IRAK-M when stimulated ex vivo with LPS (Escoll et al. 2003). Additionally, enhanced expression of IRAK-M mRNA has been noted in pediatric patients with sepsis, and high IRAK-M mRNA levels were associated with longer length of intensive care unit (ICU) stay, need for mechanical ventilation and death (Hall et al. 2007). We have also observed high constitutive expression of IRAK-M mRNA in alveolar macrophages and peripheral blood buffy coat cells isolated from patients with sepsis-induced ALI, as compared to similar cell populations from healthy subjects (T. Standiford, unpublished observations). In fact, IRAK-M was the only negative regulator of TLR signaling found to be significantly induced in this patient population.

8.9.2 Other Negative Regulators of TLR Signaling Cascades

Several other molecules have been causally linked with the development of endotoxin tolerance or hyperinflammatory responses to LPS in genetically deficient mice. Suppression of tumorigenicity 2 (ST2) is a transmembrane protein and soluble secreted protein that is expressed by a variety of cells, including T cells and macrophages. ST2 inhibits MyD88-dependent signaling by interfering with the ability of Mal/TIRAP and MyD88 to interact with downstream signaling molecules. This protein appears to contribute to sepsis-induced impairment in lung antibacterial

defense, at least in animal models (Holub et al. 2003). Specifically, CLP-induced impairment in anti-pseudomonal lung host defense is reversed in mice deficient in ST2. Interestingly, responsiveness of $ST2^{-/-}$ AM was not altered, whereas the expression of IFN-γ and TNF-α from CD4+ and CD8+ T cells was preserved in $ST2^{-/-}$ mice in the setting of abdominal sepsis, as compared to similarly treat wild-type animals.

Toll-like receptor signaling can also be modulated by both extracellular and intracellular decoys. Single immunoglobulin IL-1R-related protein (SIGIRR) is a member of the IL-1 receptor superfamily but is unable to signal. However, the extracellular domain of this molecule inhibits Toll-IL-1 signaling by interfering with binding of ligands to TLR4, TLR5, TLR9, and IL-1 receptor I, whereas the intracellular domain interferes with the complexing of IRAK-1 with TRAF-6 (Thomassen et al. 1999; Wald et al. 2003; Qin et al. 2005). SIGIRR is expressed predominantly by epithelial cells, including alveolar epithelial cells, but also to a lesser degree in monocytic populations. Mice deficient in SIGIRR have enhanced inflammatory responses to LPS challenge. Moreover, SIGIRR is upregulated in the PBM of septic patients, and is associated with the development of endotoxin tolerance in these cells (Adib-Conquy et al. 2006). MyD88 short (MyD88s) is an alternatively spliced variant of the parent molecule, MyD88. MyD88s functions as a dominant negative molecule by blocking recruitment of IRAK-4 to the toll-IL-1 signaling domain, resulting in reduced phosphorylation of IRAK-1 (Burns et al. 2003; Rao et al. 2005). The expression of MyD88s is induced in monocytes in response to LPS and is constitutively expressed in blood monocytes isolated from patients with sepsis (Adib-Conquy et al. 2006). Tollip disrupts IRAK-1 and IRAK-4 interactions, whereas microRNA 146 (miRNA 146) post-transcriptionally inhibits IRAK-1 and TRAF6 expression (Nahid et al. 2011). The suppressors of cytokine signaling (SOCS) are a family of molecules that predominately inhibit JAK-Stat signaling, but also disrupt TLR signaling cascades through a yet undefined mechanism. While these latter molecules could contribute to suppression of TLR-mediated responses during critical illness, there is no data to show enhanced expression and/or activity in blood monocytes or lung macrophages in patients at risk for the development of VAP.

8.10 Microenvironmental Factors that Regulate Innate Host Responses in VAP

8.10.1 Mechanical Ventilation

Initiation of mechanical ventilation (MV) is a vital therapeutic intervention in patients with respiratory failure. A consequence of mechanical ventilation is the inhomogeneous distribution of pressure and volumes to various regions of lung, resulting in excessive stretch in some alveolar units (referred to as volutrauma),

and repeated alveolar collapse in other regions (referred to as atelectotrauma) (Pugin et al. 1998). Excessive lung stretch results in activation of several transcriptional pathways, including the NF-kB and the MAPK kinase pathway (Fos, Jun), which contributes to the release of various inflammatory mediators such as TNF-α, IL-1β, IL-6, and IL-8 (Gharib et al. 2009; Halbertsma et al. 2005; Jaecklin et al. 2011). These cellular mediators not only trigger deleterious lung injury responses and possibly multiple organ dysfunction (An et al. 2011), but may also promote the reprogramming of leukocytes and structural cells that occurs in critical illness. Importantly, MV at moderate to high lung volumes can also prime the lung for enhanced lung injury or systemic organ failure in response to an infectious challenge (e.g., second hit). For instance, as compared to spontaneously breathing animals, the intrapulmonary administration of *S. aureus* or *E. coli* to mechanically ventilated mice results in enhanced lung inflammation and lung injury, without changes in lung bacterial clearance (Dhanireddy et al. 2006). Likewise, the i.p. administration of LPS to mice undergoing high tidal volume MV substantially increased lung and systemic cytokine expression and extrapulmonary organ injury, as compared to non-mechanically ventilated controls (O'Mahony et al. 2006). Mechanisms accounting for synergistic interactions between lung stretch and infectious challenge have not been clearly defined. However, previous work has shown that stretch of human alveolar epithelial cells increases the expression of TLR2 by sixfold (Charles et al. 2011). Moreover, mechanical ventilation increased the relative expression of TLR2 and TLR4 in lung tissue and increased the generation of endogenous ligands for TLR4 in bronchoalveolar lavage fluid (Vaneker et al. 2008). Recent work has shown that mechanical ventilation also generates other TLR4-independent and MyD88-dependant endogenous TLR ligands (Chun et al. 2010). Hyperinflation of the lung with high tidal volume not only promotes a significant increase in the expression of TLR4, but also paradoxically induces the expression of IRAK-M, an important negative regulator of TLR signaling (Villar et al. 2010).

A frequent consequence of mechanical ventilation and diseases that cause acute respiratory failure is alveolar collapse and atelectasis. Alveolar collapse is due, in part, to reductions in surfactant that occur in patients receiving mechanical ventilation and in patients with VAP (Nakos et al. 2003). Atelectasis has been shown to promote bacterial overgrowth, and use of open ventilation strategies and administration of exogenous surfactant can reduce bacterial numbers in an animal model of VAP (van Kaam et al. 2004). Moreover, administration of positive end-expiratory pressure (PEEP) at 5–8 cmH_2O to non-hypoxemic mechanically ventilated patients can reduce the incidence of VAP (Manzano et al. 2008). Surfactant proteins A and D can agglutinate *P. aeruginosa*, and SP-D can serve as an opsonin to enhance phagocytosis of *P. aeruginosa* (McCormack 2006). Pseudomonal elastase has been shown to degrade SP-A and SP-D, and these proteins are decreased in the lungs of patients with cystic fibrosis (Mariencheck et al. 2003). However, changes in SP-A and SP-D levels during mechanical ventilation and/or VAP have not been well characterized.

8.10.2 High Ambient Oxygen Concentrations

Administration of high concentrations of oxygen (FIO_2 >50%) used during transient or prolonged mechanical ventilation is a common treatment for patients with respiratory failure (Gore et al. 2010). Although therapeutically necessary, hyperoxia results in the generation of reactive oxygen species (ROS), which promote the breakdown of critical barriers leading to systemic cellular and organ injury (Lee and Choi 2003). In the lung, ROS cause severe cellular damage and death, exposure of the basement membrane and disruption of the alveolar capillary membrane leading to increased pulmonary permeability, influx of inflammatory cells, and impaired gas exchange (Bhandari and Elias 2006). Hyperoxic exposure can also exacerbate alveolar epithelial injury and apoptosis in response to infectious challenge with *P. aeruginosa* or *L. pneumophila*, resulting in increased bacterial dissemination (Kikuchi et al. 2009). Moreover, high oxygen tensions inhibit the function of innate immune cells. For instance, macrophages exposed to elevated concentration of oxygen both in vitro and in vivo display reduced phagocytosis and killing of Gram-negative bacteria which correlated with changes in cell morphology and actin polymerization (O'Reilly et al. 2003). In addition, in vivo hyperoxia exposure increased the susceptibility to *K. pneumoniae* lung infections, an effect that was partially attributed to reduced BAL GM-CSF levels and cell surface expression of TLR4 by AM (Baleeiro et al. 2003). Importantly, systemic treatment of these mice with GM-CSF during hyperoxia preserved macrophage functionality and decreased the severity of lung infection (Baleeiro et al. 2006). Taken together, hyperoxia is detrimental to the host by promoting greater alveolarcapillary injury, impairing local antibacterial responses, and increasing the risk of bacterial dissemination.

8.10.3 Microbial Flora Within the Lung Microenvironment

Emerging clinical and epidemiological data suggests a possible link between colonization with *Candida* species and susceptibility to *P. aeruginosa* pulmonary infection. *Candida* species is among the most common organisms recovered from endotracheal tube biofilm and tracheal secretions in patients with VAP (Adair et al. 1999). Historically, *Candida* has been considered a commensal organisms rather than a true pathogen, and therefore believed to play no role in VAP disease pathogenesis. However, an observational study found a statistical association between airway colonization with Candida species and the development of *P. aeruginosa* VAP (Azoulay et al. 2006). In a rat model of *P. aeruginosa* pneumonia, prior bronchial instillation of live but not heat-killed *C. albicans* resulted in increased susceptibility to subsequent bacterial challenge (Roux et al. 2009). Mechanisms accounting for impaired in vivo clearance responses were not identified, but *C. albicans* was found to inhibit AM respiratory burst ex vivo. While these intriguing findings require confirmation in other experimental model systems, they do raise the

possibility that Candida and perhaps other commensal organisms may contribute meaningful to VAP pathogenesis.

8.11 Novel Therapeutic Approaches to Reverse Critical Illness-Induced Immunosuppression

Antibiotics, prophylactic measures to reduce oropharyngeal colonization and microaspiration, and approaches to stimulate mucocilliary transport are the mainstay of therapy to prevent and treat VAP. While these treatments are effective in some patients, adjuvant therapies are needed in others to bolster innate host responses, especially in the elderly and in patients with chronic immunosuppressive therapy. The recognition that critical illness can induce a profound state of immune dysregulation has prompted a reevaluation of potential immunologic approaches being used in the treatment of sepsis and other forms of critical illness (Pockros et al. 2007a). Effective immunoadjuvant therapy must necessarily promote antimicrobial effects without exacerbating deleterious lung inflammatory responses.

8.11.1 Immunostimulatory Therapy (Interferon-γ and GM-CSF)

Common features of both endotoxin tolerance and immune dysregulation of critical illness is impaired TLR signaling, NF-κB-dependent responses, reduced APC function, and a shift toward type 2 rather than type 1 immune responses. Two cytokines that have been shown to partially reverse these changes in vitro and in vivo are IFN-γ and GM-CSF. In endotoxin-tolerized monocytes, treatment with IFN-γ or GM-CSF can reverse the tolerance phenotype, in part by facilitating interactions between IRAK and MyD88, resulting in enhanced downstream activation of NF-κB (Adib-Conquy and Cavaillon 2002). Similarly, ex vivo treatment of blood monocytes from trauma patients with IFN-γ or GM-CSF, but not G-CSF, enhanced LPS-induced cytokine production, and HLA-DR expression (Lendemans et al. 2007).

These preclinical studies served as the foundation for several small clinical trials in patients with sepsis. Docke and colleagues administered IFN-γ to patients with sepsis in an attempt to reverse the cytokine imbalance and restore monocyte function (Docke et al. 1997). In this uncontrolled study, nine patients with evidence of sepsis-induced immunosuppression (decreased blood monocyte HLA-DR expression) were administered IFN-γ at a dose of 100 μg subcutaneously daily. Treatment with IFN-γ resulted in increased monocyte HLA-DR expression in all patients, along with a restoration of monocyte TNF-α production to levels observed in monocytes isolated from healthy subjects. Resolution of sepsis occurred in eight of the nine treated patients (Docke et al. 1997). In two small single center clinical trials, the i.v. administration of hrGM-CSF to patients with sepsis resulted in improvements

in ex vivo effector responses in PBMs or neutrophils (Nierhaus et al. 2003; Presneill et al. 2002). Moreover, one of the studies revealed improvements in PaO_2/FIO_2 ratios, as a measure of pulmonary gas exchange, suggesting reduced lung injury in the GM-CSF treated group (Presneill et al. 2002). Prevention of lung injury may be due, in part, to the fact that GM-CSF is an alveolar epithelial cell mitogen and can protect the alveolar epithelium against hyperoxic and bleomycin-induced injury (Baleeiro et al. 2006; Moore et al. 2000) and in a murine model of influenza pneumonia (Sever-Chroneos et al. 2011). These preclinical and clinical findings served as the basis for a multicenter randomized placebo controlled trial of subcutaneous GM-CSF administration in 38 patients with severe sepsis and evidence of monocyte deactivation (reduced HLA-DR expression). As compared to the placebo group, GM-CSF administration resulted in improved monocyte function (restored cell surface TLR2/4 expression, TNF production, and HLA-DR expression) and improved clinical outcomes, including reduced APACHE II scores, shorter time of mechanical ventilation, and a trend toward decreased length of ICU and hospital stay. These studies and others suggest that immunostimulatory therapy for treatment of critical illness-induced immune dysregulation or even end-organ injury appears to be a potentially viable therapeutic option that warrants larger controlled trials (Luedke and Cerami 1990). An obvious concern of immunostimulatory therapy in patients with severe sepsis and/or pneumonia is the potential of exacerbating the "cytokine storm" of SIRS. Fortunately, neither IFN-γ nor GM-CSF has precipitated worsening of hemodynamic compromise or multiorgan failure, even in patient with severe sepsis or septic shock (Docke et al. 1997; Nierhaus et al. 2003; Meisel et al. 2009). Additional consideration could be given to compartmentalized immunostimulatory therapy (e.g., aerosolized delivery) to prevent or treat VAP. However, this approach may be limited substantially by ventilation-perfusion mismatching that occurs in patients with lung disease, and the concern that the leukocyte reprogramming that occurs during critical illness is not limited to the lung microenvironment but almost certainly occurs more broadly in leukocyte populations systemically.

8.11.2 Inhibitors of Apoptosis

Activation of the PI3K/Akt pathway in certain leukocyte populations can lessen NF-κB-mediated pro-inflammatory responses while stimulating pro-survival and antimicrobial responses (Williams et al. 2006; Wrann et al. 2007; Zhang et al. 2007). For example, the administration of selective activators of the PI3K/Akt signaling pathway (e.g., glucan, α-lipoic acid) to LPS-challenged mice or mice undergoing CLP reduced apoptosis, inflammatory cytokine release, and improved mortality (Wrann et al. 2007; Zhang et al. 2007).

Interleukin 15 is a pleurapotential cytokine that regulates DC, T, and NK cell activation, proliferation, and survival. The administration of IL-15 to mice with abdominal sepsis (CLP) has been shown to block sepsis-induced apoptosis of NK cells, DC, and CD8 T cells, and to restore NK cell production of IFN-γ (Inoue et al.

2010). Treatment with IL-15 also mitigated sepsis-induced apoptosis of gut epithelium. Importantly, IL-15 not only reduced mortality in CLP, but also in mice administered *P. aeruginosa* i.t.

Finally, caspase inhibitors have been shown to reduce lymphocyte apoptosis and increase survival in murine models of sepsis (Hotchkiss et al. 2000). A pan-caspase inhibitor (IDN-6556) have been employed in the treatment of liver disease in patients with Hepatitis C (Pockros et al. 2007b). However, trials targeting caspases or other pro-apoptotic molecules or administration of pro-survival factors (e.g., AKT activators, IL-15) in patients with sepsis or nosocomial pneumonia have not yet been reported.

8.12 Summary

In this review, we have defined the clinical features of VAP, and described the impact of critical illness and microenvironment factors introduced during mechanical ventilation on susceptibility to VAP, with special attention to specific molecules as potential mediators of immunosuppression and tissue injury. Increases in microbial resistance, combined with a burgeoning population of patients at risk, are trends that clearly make VAP a major clinical problem now and in the future. Preventative strategies and optimal ventilator management have been paramount in reducing the incidence of VAP. However, critical illness-induced reprogramming of leukocyte innate immune responses clearly contributes to susceptibility to VAP and VAP-induced tissue injury. Given our past failures, a paradigm shift in how we approach patients with evidence of immune dysregulation is required. In order for novel therapies to proceed, better clinical markers are needed to distinguish a deleterious innate response (e.g. SIRS) from a state of immunoparalysis (CARS) or mixed antagonist response syndrome (MARS) as the inflammatory response evolves (Wesche et al. 1999). Differentiating these quite disparate but overlapping responses in a patient-specific fashion will allow for better selection of patients in which immunoadjuvant therapy is more likely to be beneficial.

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Chapter 9
Streptococcus pneumoniae: The Prototype of Lung Responses in Pneumonia

Jessica Humann, Kim LeMessurier, and Elaine Tuomanen

9.1 Introduction

Streptococcus pneumoniae causes infections as diverse as meningitis, sepsis, and otitis media, but its species name "*pneumoniae*" refers to its dominant role in pneumonia. Pneumonia is broadly divided into three classes: community-acquired, hospital-acquired, and pneumonia in an immunocompromised host (Reynolds et al. 2010; Watson et al. 1993). Since its initial identification by Louis Pasteur and George Sternberg over 130 years ago, *S. pneumoniae* remains the leading causative agent of community-acquired pneumonia worldwide (Watson et al. 1993). Host immune responses must contend with over 90 pneumococcal capsular serotypes. Children <2 years old who lack the ability to mount effective immunoglobulin responses to capsular antigens are the most susceptible to invasive pneumococcal disease (Reynolds et al. 2010). Lobar pneumonia, a classic description in clinical medicine, presents with five cardinal signs: sudden onset chest pain, a shaking chill, cough, fever, and production of blood tinged sputum (Heffron 1939). In pediatric patients, presenting symptoms include fever, cough, labored breathing with grunting, and cyanosis (Tan et al. 1998). In adults, community-acquired pneumococcal pneumonia often presents with opacity in a lobar pattern on chest radiographs (Reynolds et al. 2010). In both children (Don et al. 2010) and adults (Cilloniz et al. 2011), most cases of pneumococcal pneumonia resolve after treatment with a 5 % mortality rate. However, complications such as pleural effusion, empyema, and multi-lobar consolidation can arise (Cilloniz et al. 2011).

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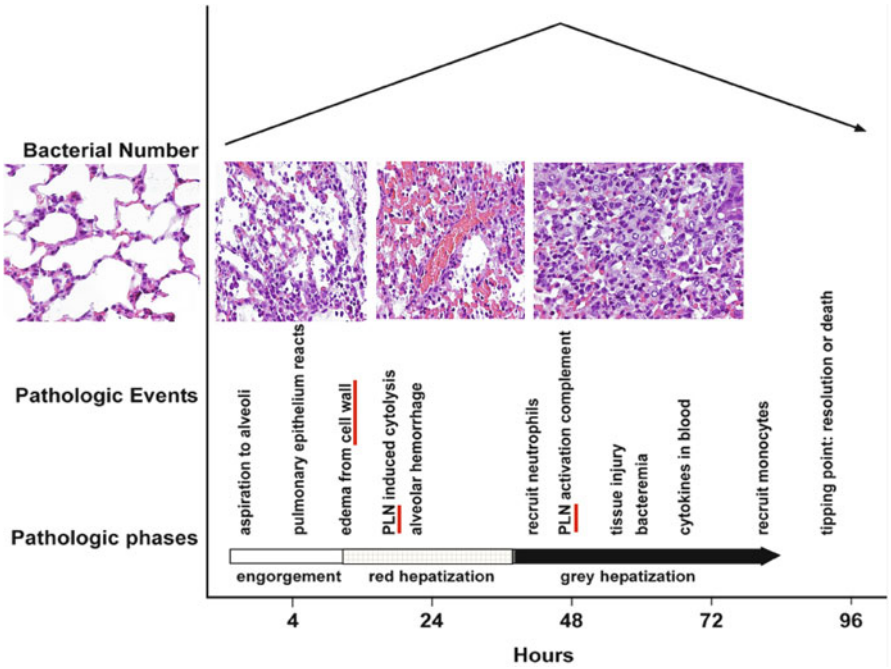

Fig. 9.1 Kinetics of events during progression of pneumococcal pneumonia. Hematoxylin and eosin stained sections of lung depict the indicated three stages of consolidation in pneumonia progressing from *engorgement* to *red* to *grey hepatization*. Bacterial multiplication proceeds unimpeded during the stages of *engorgement* and *red hepatization*, peaking at 36 h in the stage of *grey hepatization.* Bacteremia is a result of pneumococcal adherence to and invasion of alveolar cells. The edema characteristic of *engorgement* arises from cell wall induced signaling in epithelial cells and activation of the alternative pathway of the complement cascade by cell wall. Cytokines begin to appear in BAL fluid in the first few hours of engorgement but do not reach a maximum until the phase of *red hepatization* (18–24 h). At this stage, the cytolytic activity of pneumolysin is prominent. During the stage of *grey hepatization*, polymorphonuclear leukocytes are recruited and begin to control pneumococcal multiplication. Complement activation by pneumolysin (PLN) aids in this clearance. The outcome of the infection depends, at least in part, on the ability of the host to withstand the inflammation associated with bacterial death (i.e. tipping point). Adapted from (Tuomanen et al. 2000) with permission the American Society of Microbiology

S. pneumoniae is the prototypical extracellular pulmonary pathogen that multiplies in the alveolar space and then crosses the epithelial barrier to disseminate via the bloodstream (Fig. 9.1). The molecular details of the progression of bacterial pneumonia to bacteremia, first described for pneumococci, are known collectively as “innate invasion” since they apply to virtually every bacterial respiratory pathogen (Thornton et al. 2010). A key common element is decoration of the bacterial surface by phosphorylcholine (PCho). By molecular mimicry, PCho not only thwarts local mucosal defenses that initiate the recognition of bacteria in the airways, such as surfactant and C-reactive protein (CRP), but also

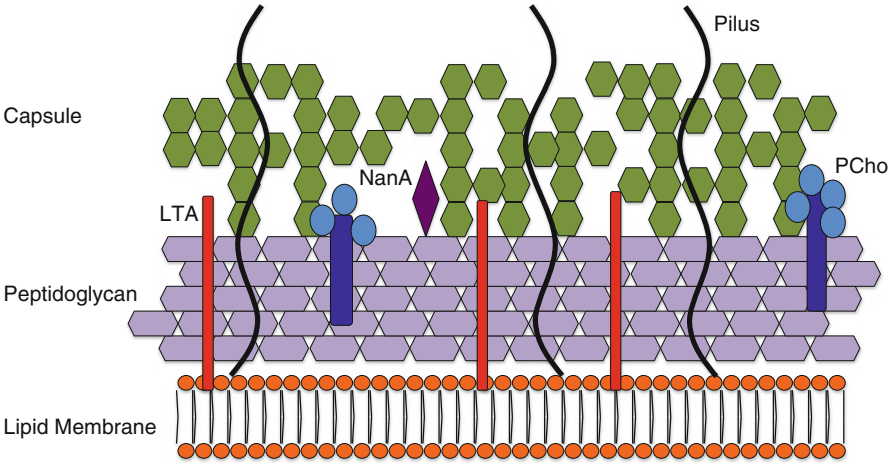

Fig. 9.2 Select pneumococcal cell wall PAMPs and proteins. *S. pneumoniae* is a Gram-positive pathogen with a cell wall composed of a lipid membrane, a thick layer of peptidoglycan, and an outer polysaccharide capsule layer. Within the cell wall there are many teichoic acid moieties, including lipoteichoic acid (LTA) and teichoic acids with phoshporylcholine (PCho). There are also multiple proteins linked to the cell wall, such as the exoglycosidase NanA. Many of these cell wall components contribute to the pathogenesis of pneumococcal infections in the lung

serves as an adherence and invasion ligand (Cundell et al. 1995). Inflammation accelerates as host epithelial cells and resident alveolar macrophages respond to pneumococcal pathogen-associated molecular patterns (PAMPs) such as PCho, peptidoglycan (PGN), lipoteichoic acid (LTA), bacterial DNA, and toxins (pneumolysin) (Fig. 9.2). Pattern recognition receptors (PRRs) that are activated by PAMPs include platelet activating factor receptor (PAFr), Toll-like receptors (TLRs) 2, 4, and 9 and Nod-like receptors (NLRs) (Cundell et al. 1995; Kawai and Akira 2006) (Fig. 9.3). The endpoint of PRR binding to pneumococcal PAMPs is the activation of transcription factors such as NFκB, which regulate the induction of inflammatory cytokines including IL-1 and IL-6 (Kawai and Akira 2006) (Fig. 9.4). Cytokine production heralds the influx of neutrophils, an essential step in controlling bacterial multiplication. Cellular influx and fluid accumulation in alveoli are common results of an active inflammatory immune response. Clearance of pneumococci is substantially attenuated in the absence of neutrophils, but exuberant neutrophil accumulation can also contribute to tissue damage in the lung (Balamayooran et al. 2010a). Reduction in the intensity of neutrophil recruitment to the lungs, but not total neutropenia, is associated with improved outcome of pneumonia (Karlstrom et al. 2011; Marks et al. 2007). A comprehensive understanding of PAMP recognition and signaling underlies ongoing studies directed at mitigating the symptoms of acute pneumonia and developing vaccines to prevent pneumococcal disease.

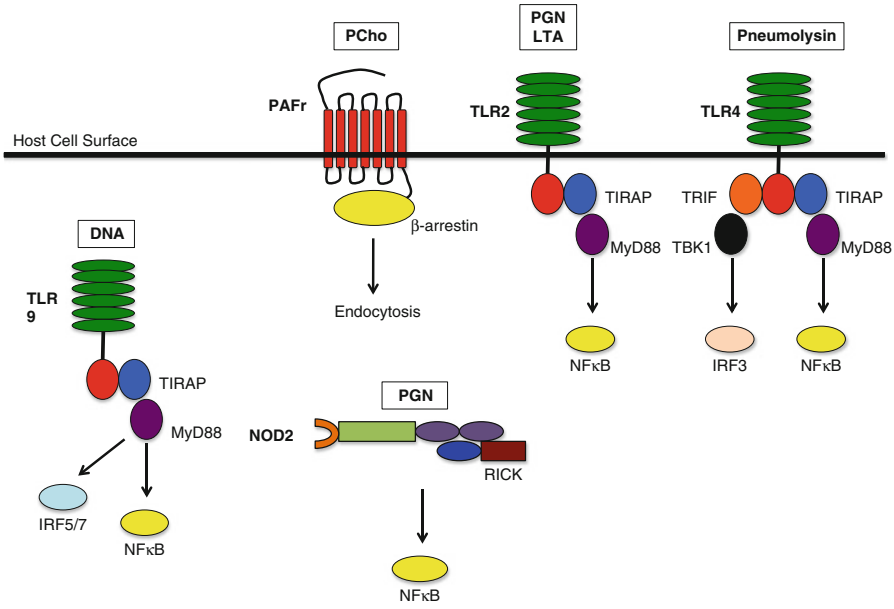

Fig. 9.3 Pattern recognition receptors for pneumococcal PAMPs. At the cell surface of epithelial cells and innate immune cells such as neutrophils and macrophages are multiple pattern recognition receptors. PAFr, TLR2, and TLR4 are three such receptors commonly associated with pneumococcal infection of the lung. PAFr binds to PCho and facilitates endocytosis and transmigration of the pneumococcus through the epithelium. Cell wall peptidoglycan (PGN) and LTA likely signal via TLR2, and the virulence factor pneumolysin has been associated with TLR4. In addition to surface receptors, there are cytosolic host receptors that bind to cell wall or other bacterial components. Pneumococcal PGN can bind to the NLR family member NOD2. DNA released from the bacteria can bind to TLR9

9.2 Earliest Interactions of Pneumococci with the Airway Epithelium

9.2.1 Nasopharynx

The pneumococcus is commonly carried asymptomatically in the nasopharynx of up to 60 % of young children and it spreads from person to person via droplets (Hedlund et al. 2003). Several bacterial surface elements contribute to nasopharyngeal colonization. These elements are coordinately regulated in a process of phase variation, switching between a transparent colony morphology adapted to colonization and an opaque morphology adapted to the bloodstream (King et al. 2004; Weiser et al. 1994). The transparent phenotype expresses more surface adhesins and less capsular polysaccharide while the reverse is true for the opaque phenotype. Colonization is associated with the formation of a biofilm propagated by PsrP, a very large serine-rich protein (Sanchez et al. 2010). Prior influenza infection may

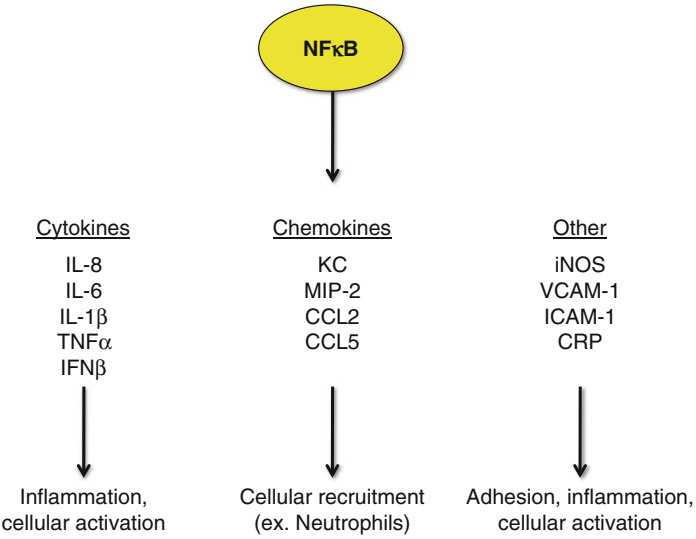

Fig. 9.4 Signaling downstream of NFκB**.** TLRs and other pattern recognition receptors utilize the NFκB signaling cascade. Targets of the cascade include multiple cytokines involved in inflammation (such as IL-6 and IL1β); chemokines such as CCL2 that attract monocytes and neutrophils; and adhesion molecules and other inflammatory mediators (CRP, iNOS) that contribute to cellular activation and response during infection

promote the formation of pneumococcal biofilms secondarily leading to enhanced colonization and a greater incidence of pneumonia (Trappetti et al. 2009). This coinfection stimulates a synergistic Type 1 interferon response that reduces bacterial clearance and increases morbidity (Nakamura et al. 2011).

A variety of surface elements contribute to nasopharyngeal colonization. A major protein adhesin is choline-binding protein A (CbpA). CbpA is a member of the choline-binding protein family, a set of a dozen secreted proteins that harbor a domain that binds non-covalently to PCho on the pneumococcal cell wall teichoic acid. CbpA binds to the polymeric immunoglobulin receptor (pIgR) (Hammerschmidt et al. 1997; Zhang et al. 2000), an interaction that triggers uptake of bacteria into vesicles that transit from apex to base of human nasopharyngeal cells (Zhang et al. 2000; Radin et al. 2005). CbpA is upregulated in the transparent colony phenotype and is an important antigen recognized during the immune response to colonization (McCool et al. 2002). Two serine proteases, HtrA and an IgA protease, are covalently anchored in the cell wall and strongly promote colonization (Sebert et al. 2002; Weiser et al. 2003). In addition to surface proteins, ~20 % of pneumococcal strains display pilus surface structures that extend beyond the capsule layer and have been associated with pneumococcal adherence to airway epithelial cells (Barocchi et al. 2006). The *rlrA* pathogenicity islet encodes the multiple covalently linked pilus subunits (Telford et al. 2006) with the tip bearing the adhesin RgrA (Nelson et al. 2007a).

Clearance and prevention of colonization involve IL-17 and Th-17 cell adaptive responses (Lu et al. 2008). Recruitment of CCR2-bearing monocytes and macrophages to the upper airways mediated by the interactions of TLR2, $CD4^+$ T-cells, and IL-17A is important for pneumococcal clearance (Zhang et al. 2009). In a model of immunization using a whole cell pneumococcal vaccine with cholera toxin as an adjuvant, IL-17A and $CD4^+$ T-cells were found to be necessary for pneumococcal clearance following subsequent nasopharyngeal challenge (Lu et al. 2008). Similarly, IL-17A receptor-deficient mice are not protected from pneumococcal challenge after immunization compared to control mice (Lu et al. 2009).

9.2.2 Airways

As transparent pneumococci interact with the nose and upper airway epithelium, capsular polysaccharide production is downregulated exposing surface adhesins. However, bacterial entrance into the alveolus and bloodstream induces the opaque colony morphotype (Weiser et al. 1994). At this stage capsule polysaccharide production is induced, making capsule the major element counteracting host responses in these niches. Each of the >90 serotypes is defined by a biochemically unique capsular composition and antibody to capsule is critical to defense against invasive disease. Capsule shields the pneumococcal cell wall from the host, partially inhibiting recognition of bacterial PAMPs and limiting the ability of host to eliminate bacteria via phagocytosis, deposition of complement, or mucous clearance (Heffron 1939; Nelson et al. 2007b).

As bacteria transit the large airways to the alveoli, early innate defense involves antimicrobial peptides (Lehrer and Ganz 2002). These cationic peptides include those secreted by epithelial cells, such as LL-37 and β-defensins, or α-defensins expressed primarily by neutrophils and stored in large amounts in the azurophilic granules (Lehrer and Ganz 2002; Selsted et al. 1985; Goldman et al. 1997; Harder et al. 1997; Stolzenberg et al. 1997). Defensins kill Gram-negative as well as Gram-positive bacteria, fungi, and some enveloped viruses by insertion into cell membranes forming voltage-regulated channels (Lehrer et al. 1989). α-defensins are more active against encapsulated bacteria (Beiter et al. 2008) and block hemolysis by cholesterol-dependent cytotoxins like pneumolysin (Lehrer et al. 2009).

9.3 Defense of the Alveolus

9.3.1 Surfactant

In the early pneumonic lesion, bacteria are freely floating in the serous fluid of the alveolar space where they are bathed in surfactant (Rake 1936; Wood et al. 1946).

Surfactant, composed of PCho, apoplipoproteins, and surfactant proteins A and D, not only regulates surface tension and alveolar compliance but also competitively inhibits bacterial attachment to the alveolar epithelium. PCho in surfactant blocks interactions of PCho on the pathogen surface with host receptors. PCho is a ligand on most respiratory pathogens that is critical to the progression of pneumonia to bacteremia (Cundell et al. 1995). Phagocytosis of pneumococci by lung cells, such as alveolar macrophages (AMs), can be augmented by surfactant protein A (SP-A), a 28- to 36-kDa collectin (Haagsman et al. 1987) produced primarily by alveolar type II cells, tracheobronchial gland cells, and non-ciliated bronchiolar cells. SP-A has been ascribed broad roles in innate lung defense, such as stimulating the chemotaxis of AMs to the site of infection (Wright and Youmans 1993), and eliciting the production of oxygen radicals (van Iwaarden et al. 1990) and pro-inflammatory cytokines (Kremlev and Phelps 1994) by AMs. Although radioactively labeled SP-A has been shown to bind pneumococci directly, it may indirectly affect the phagocytosis of pneumococci by causing the localization of the phagocytic receptor SR-A (scavenger receptor A) to the cell surface of AMs (Kuronuma et al. 2004). Like SP-A, the 43kDa surfactant protein SP-D stimulates AM oxidative activity (Van Iwaarden et al. 1992) and is capable of binding pneumococci in the presence of calcium (Jounblat et al. 2004). Binding of SP-D to pneumococci results in bacterial agglutination, the extent of which is partially influenced by the capsule (Jounblat et al. 2004). SP-D-deficient mice exhibit greater bacterial burdens in the lung following intranasal challenge, compared to control animals (Jounblat et al. 2005). Thus, the activities of surfactant proteins within the host lung serve to enhance bacterial phagocytosis by host cells by both direct and indirect interaction with the pneumococci.

9.3.2 C-Reactive Protein

A key mucosal innate immune molecule in to lungs is C-reactive protein (CRP). It was the observation that the protein bound to cell wall C-polysaccharide of *S. pneumoniae* that earned it the name "C-reactive protein." CRP is produced locally in the human upper respiratory tract (Gould and Weiser 2001) and binds PCho moieties on the bacterial cell wall (Volanakis and Kaplan 1971) as well as FcγRI (CD64) and FcγRII (CD32) (Marnell et al. 1995; Bharadwaj et al. 1999; Stein et al. 2000; Devaraj et al. 2005; Tron et al. 2008). As PCho is present on most respiratory pathogens, this interaction is an important element of the innate defense against invasion. CRP is able to act as an antagonist for PCho-mediated bacterial adherence to epithelial PAFr (Gould and Weiser 2002), thereby reducing bacterial invasion of the lung epithelium. Ligation of CRP elicits TNFα production by peripheral blood mononuclear cells (Mold and Du Clos 2006), macrophages, and neutrophils (Stein et al. 2000). Pneumococcal-bound human CRP also interacts with C1q and activates the classical complement pathway (Kaplan and Volanakis 1974; Siegel et al. 1974; Claus et al. 1977).

Another surface-exposed choline-binding protein, PspA (pneumococcal surface protein A), interferes with opsonization via the alternative complement pathway by limiting C3b deposition on the bacterial surface (Ren et al. 2004, 2003; Tu et al. 1999). PspA has also been shown to bind lactoferrin (Hammerschmidt et al. 1999), and PspA significantly inhibits the killing of pneumococci by apolactoferrin (Shaper et al. 2004). Strains secreting truncated PspA lacking the choline-binding domain are as attenuated as strains expressing no PspA (Ren et al. 2003; Yother et al. 1992), indicating PspA must be attached to the pneumococcal surface to contribute to pneumococcal virulence.

9.3.3 Alveolar Epithelial Surface Carbohydrates

Alveolar type 2 epithelial cells display surface carbohydrates suitable for loose bacterial attachment (Tuomanen et al. 1995), particularly N-acetylglucosamine β1-3 galactose that is targeted by many respiratory pathogens (Krivan et al. 1988). Pneumococcal modification of host carbohydrates further promotes adherence, invasion, and manipulation of the immune response. At least ten extracellular glycosidases are produced by *S. pneumoniae*, many of which are surface-exposed (King 2010). These glycosidases cleave both terminal and internal carbohydrate linkages on *N*-linked or *O*-linked glycans and glucosaminoglycans (King 2010). Neuraminidase A (NanA) is a surface-associated pneumococcal exoglycosidase (Camara et al. 1994) which cleaves terminal sialic acid moieties (King et al. 2004) such as those found on IgA. Pneumococcal strains lacking NanA are severely attenuated in their ability to colonize the host (Manco et al. 2006; Orihuela et al. 2004). Pneumococcal strains unable to transport sialic acid also exhibit attenuated colonization (Marion et al. 2011).

9.4 Bacterial PAMPs: The Progression to Pneumonia

The three stages of evolution of pulmonary consolidation during pneumococcal pneumonia is a classic description in clinical medicine (Laennec 1932; Loosli 1940) (Fig. 9.1). Initially, as bacteria fill the alveolus, the host responds to bacterial PGN PAMPs (Rich and McKee 1939; Sutliff and Friedemann 1938; Tuomanen et al. 1987a), capillaries, and epithelial cells become leaky, and edema fluid spreads through the pores of Kohn (Hamburger and Robertson 1940). This stage of *engorgement* is largely asymptomatic but characterizes the leading edge of the pneumonic lesion as it progresses through lung parenchyma in a pattern demarcated by lobar architecture. The appearance of erythrocytes in the alveolus around day 2 heralds the stage of *red hepatization* characterized by a clotted exudate throughout the lobe and a dense lung with little internal air. Mice become bacteremic at this stage, i.e. ~36 h post-challenge coincident with peak bacterial multiplication and prior to peak host response at ~72 h (Dallaire et al. 2001). In the final stage of *grey hepatization*,

leukocytes migrate into the lesion. The patient appears toxic with poor pulmonary perfusion (Osler 1897). This represents the tipping point at which the host response either overcomes bacterial multiplication or the patient succumbs.

9.4.1 TLR2: Recognition of Peptidoglycan and LTA

Bacterial PGN fragments and lipoproteins are ligands for TLR2 (Kawai and Akira 2006; Yoshimura et al. 1999) (Fig. 9.3). It is thought that TLR2 distinguishes between diacyl and triacyl lipopeptides by its associations with TLR1 and TLR6, respectively (Mitchell et al. 2007; Takeda and Akira 2005). In addition to TLR2, the acute-phase protein LPS-binding protein can also bind to PGN (Weber et al. 2003). Within the lung, alveolar epithelial cells express TLR2 (Basu and Fenton 2004) and engagement of TLR2 by pneumococcal LTA can result in p38 MAPK activation and TGFβ signaling, which is sufficient to disrupt polarized lung epithelial cells in vitro (Beisswenger et al. 2007). Intra-tracheal instillation of purified LTA induces an increase in the total number of neutrophils in bronchoalveolar lavage within hours; $TLR2^{-/-}$ mice do not exhibit this neutrophil recruitment and also show a decreased production of inflammatory cytokines, including IL-6, IL-1β, MIP2, and TNFα (Dessing et al. 2008).

9.4.2 TLR9 and DNA

TLR9, a cytosolic TLR that recognizes un-methylated CpG DNA, is also important in the response to pneumococcal infections in the lung (Fig. 9.3) (Kawai and Akira 2006; Hemmi et al. 2000). Pneumococcal DNA is released during natural autolysis of the bacteria or after contact with bacteriolytic antibiotics during treatment of infection. In cell culture, live *S. pneumoniae* induce TLR9-dependent NFκB activation and IL-8 production (Mogensen et al. 2006). In an animal model of infection, mice lacking TLR9 demonstrate impaired clearance of pneumococcal infection from the lower respiratory tract (Albiger et al. 2007).

Pneumococcal DNA fragments also signal via intracellular receptors other than TLR9. Pores formed as a result of pneumolysin permit entry of DNA into the cytoplasm of host cells. Once inside, pneumococcal DNA is thought to trigger IFNβ production through DAI, a double-stranded DNA-sensing molecule (Parker et al. 2011).

9.4.3 Non-TLR Pattern Recognition Receptors

TLR-independent signaling pathways are linked to inflammatory cytokine production during pneumococcal infection of the lungs. In addition to NFκB, downstream

signaling of TLRs includes the p38 MAPK pathway (O'Neill 2000; Xu et al. 2008). Inhibition of p38 MAPK correlates with a decrease in inflammatory cytokine production that is more significant than blocking either TLR2 or TLR4.

Pneumococcal PGN fragments are also recognized by intracellular NOD2 (Fig. 9.3). NOD2 binds the muramyldipeptide (MDP) fragment of PGN (Girardin et al. 2003) and initiates the NFκB signaling cascade (Fig. 9.4) (Mook-Kanamori et al. 2011). Both TLR and NOD-mediated NFκB signaling have been implicated to play positive roles for host defense against *S. pneumoniae,* with NFκB being described as "essential" for host defense against pneumococcus (Balamayooran et al. 2010b). NOD1 and NOD2 are upregulated in pulmonary epithelial cells in vitro after pneumococcal infection, as well as in vivo after lung infection (Hippenstiel et al. 2006). NOD2 has also been reported as necessary for NFκB activation in response to internalized pneumococcus (Opitz et al. 2004).

9.5 Damage to the Alveolar Epithelium

9.5.1 Pneumolysin

Pneumolysin is a cholesterol-dependent pore-forming toxin that is produced by virtually all pneumococcal clinical isolates (Paton et al. 1993). Its pore-forming activity causes significant damage to lung cells, and bacterial mutants lacking pneumolysin are severely attenuated in mice. Pneumolysin induces vascular permeability independent of recruited neutrophils, alveolar macrophages, or leukocytes (Maus et al. 2004). The role of pneumolysin in creating "leakiness" in the vasculature of the lung promotes both bacterial invasiveness and influx of recruited neutrophils and macrophages (Maus et al. 2004). Other hypotheses for pneumolysin-induced neutrophil influx include cytokine production as a response to tissue damage and activation of complement (Hirst et al. 2004).

In vitro, pneumolysin has been shown to elicit TNFα and MIP-2 by a mouse alveolar macrophage cell line (Dessing et al. 2009). This translates to the in vivo setting, where intranasal administration of pneumolysin elicits production of TNFα, MIP-2, KC and IL-1β in mice (Dessing et al. 2009). These cytokines augment local cytokine production by activating NFκB (Fig. 9.4) leading to the recruitment of neutrophils and macrophages to the bronchoalveolar space. Pneumolysin-dependent inflammatory cytokine induction (TNFα) in macrophages is synergistically enhanced by active TLR2 signaling (Malley et al. 2003). Pneumolysin signaling has also been linked to the NLRP3 inflammasome, resulting in induction of IL-1β (McNeela et al. 2010; Witzenrath et al. 2011). In the absence of NLRP3, the lungs of mice infected with *S. pneumoniae* exhibited enhanced permeability compared to wild-type mice (Witzenrath et al. 2011). These data suggest that NLRP3 may play a role in modulating lung repair in response to pneumolysin-induced damage, but this function of NLRP3 has yet to be fully characterized.

Independent of its pore-forming activity, pneumolysin has been associated with TLR4 signaling (Malley et al. 2003). Thus, pneumolysin is both a bacterial virulence factor and an agonist for TLR4-mediated host defense (Kadioglu and Andrew 2004). It is suggested that TLR4 expression is only protective during low dose *S. pneumoniae* challenge (Branger et al. 2004).

9.5.2 Hydrogen Peroxide

In addition to direct toxicity from pneumolysin, pneumococci produce H_2O_2 in amounts equivalent to leukocytes. Since pneumococci do not produce catalase, this reactive oxygen species remains highly toxic (Pericone et al. 2000). Mutants lacking pyruvate oxidase produce greatly reduced amounts of H_2O_2 and are highly attenuated in virulence in animal models of pneumonia (Spellerberg et al. 1996). The effects of pneumolysin and H_2O_2 are additive and lead to epithelial cell death and arrest of ciliary beating (Feldman et al. 2002).

9.6 Cellular Host Responses

Bacterial PAMP signaling via TLRs and other host receptors and toxic damage to alveolar cells serves to recruit and activate innate immune cells to the site of infection. Alveolar epithelial cells express functional TLRs and produce inflammatory cytokines in response to TLR stimulation (Gribar et al. 2008; Schmeck et al. 2006; Armstrong et al. 2004). In addition to resident airway epithelial cells, macrophages, neutrophils, and specific T-cell subsets are recruited to the lung and airways during pneumococcal pneumonia (Wissinger et al. 2008; Kirby et al. 2007a; Nakasone et al. 2007). These cellular subsets promote inflammation and bacterial uptake as a result of direct and indirect PAMP signaling (Fig. 9.5). The host response is greatly amplified if antibiotic therapy kills bacteria by autolysis, which releases PAMPs on cell wall fragments and intracellular DNA and toxins (Cabellos et al. 1992).

Using a mouse model of pneumococcal pneumonia, Bergeron et al. described stages of the pulmonary cellular immune response over the initial 96 h of infection (Bergeron et al. 1998). In the first few hours, there is partial phagocytosis of bacteria by AMs with concurrent cytokine release. TNF, IL-6, and nitric oxide predominate in bronchoalveolar lavage fluid while TNF, IL-6, and IL-1 appear in lung tissue and only IL-6 appears in serum. The second step (4–24 h) is marked by the multiplication of pneumococci in the alveoli and neutrophil chemotaxis into lung tissue associated with high release of TNF, IL-1, IL-6, and leukotriene B_4 in BAL fluid and tissue, as well as transient spillover of IL-1 into serum. This recruitment involves complement activation by pneumolysin and cell wall teichoic acid. Step 3 (24–48 h) is marked by lung damage with injury to alveolar ultrastructure and interstitial edema allowing bacteria to move from alveoli to lung tissue and into the bloodstream. In step 4 (48–72 h) a

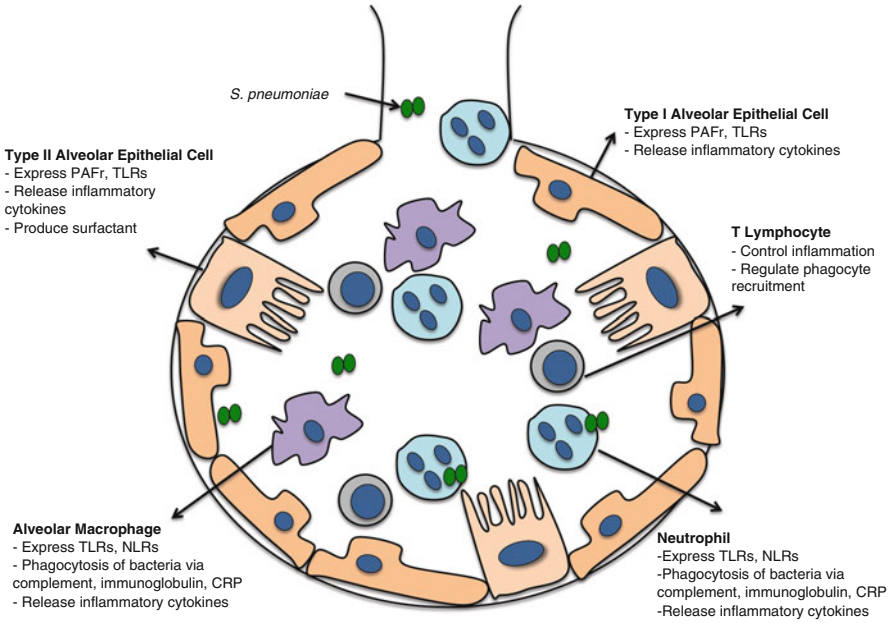

Fig. 9.5 Roles of lung cell populations in pneumococcal recognition and response. Multiple cell populations within the lung contribute to the progression and resolution of pneumococcal pneumonia. Alveolar epithelial cells express TLRs and PAFr. Epithelial cells can release inflammatory cytokines, as well as serve as points of entry for the pneumococcus to cross the cellular barrier of the lung by PAFr–Pcho interactions. Type II alveolar epithelial cells also produce surfactant. Alveolar macrophages and neutrophils take up bacteria that have been opsonized by immunoglobulin, CRP, and complement. These cells also express TLRs and other pattern recognition receptors that can promote an inflammatory response to PAMP stimulation

pronounced leukopenia and thrombocytopenia arises by apoptosis of circulating cells (Kemp et al. 2002). Pneumolysin induces apoptosis of dendritic cells (DC) by a rapid caspase-independent mechanism and TLR2 signaling induces DC apoptosis by a caspase-dependent mechanism (Colino and Snapper 2003). The end result (72–96 h) is a loss of alveolar architecture and diffuse tissue damage with high nitric oxide concentrations. Apoptotic damage is accompanied by strong recruitment of monocytes and lymphocytes from blood to alveoli promoting phagocytosis and bacterial killing (Dockrell et al. 2001). Survival depends on efficient bacterial killing by the $CD49d^{+}$ subset of neutrophils followed by clearance of the inflammatory debris by macrophages (Tsuda et al. 2004; Garrison et al. 2010).

9.6.1 Alveolar Macrophages

Upon entering the lungs, pneumococci encounter AMs, the resident phagocytes in this niche. The detection of pneumococci by AMs has three broad ramifications: phagocytosis of pneumococci, apoptosis of AMs, and activation of AMs, which

leads to the release of pro-inflammatory cytokines and reactive oxygen species and cellular recruitment (Fig. 9.5). Alveolar macrophages take up pneumococci after the bacteria are opsonized by complement and IgG. Once ingested, the membrane-associated TLRs (TLR2, TLR4) come into contact with bacterial cell wall components, initiating a signaling cascade and contributing to the release of inflammatory mediators such as IL-8 and CXC chemokines (Martin and Frevert 2005). This initial recognition is a significant step in effecting early bacterial clearance from the lung. Mice lacking AMs have greater bacterial numbers in the lung after intra-tracheal challenge with a low dose of pneumococci (Dockrell et al. 2003) and significantly less TNFα in bronchoalveolar lavages.

Befitting the importance of early bacterial recognition in preventing the development of pneumonia and invasive pneumococcal disease, recognition of pneumococci by AMs occurs in several ways: direct binding of pneumococci to AM surface receptors and indirectly, with the help of secondary factors such as antibodies, complement components, and surfactants. AMs possess two defined PRRs that can bind pneumococci: MARCO (macrophage receptor with collagenous structure) and SR-AI/II, both of which belong to the class A scavenger receptor family (Arredouani et al. 2004, 2006). AMs that lack MARCO bind approximately half the pneumococci of MARCO-sufficient AMs. MARCO-deficient AMs are also attenuated in phagocytosis (Arredouani et al. 2004). A similar deficit has been shown in vivo for SR-AI/II, with SR-AI/II-deficient AMs taking up fewer FITC-labeled pneumococci following intranasal challenge (Arredouani et al. 2006). Mice that lack either MARCO or SR-AI/II are unable to efficiently clear pneumococci from their lungs, and experience enhanced pulmonary inflammation and decreased survival (Arredouani et al. 2004, 2006). MARCO expression by AMs is downregulated in response to IFNγ (Sun and Metzger 2008). IFNγ is produced during the recovery stage of lung infection of mice, and pneumococci administered at this time are poorly cleared by AMs (Sun and Metzger 2008), a contributing mechanism to the greatly enhanced susceptibility of individuals with influenza to a subsequent pneumococcal infection.

9.6.2 Neutrophils

Neutrophils are key elements of the host response to pneumococcal infection, as they take up and kill invading bacteria. The inflammatory milieu of the lung in response to pneumococcal invasion serves to recruit neutrophils to the site of infection. At the site of infection, neutrophils take up bacteria by phagocytosis, often mediated by Fc receptors. FcγRIIa has two alleles, both of which can bind IgG2. However, R131 binds IgG2 far more weakly than the H131 allele (Warmerdam et al. 1991). The receptor binding avidity correlates to phagocytosis of *S. pneumoniae* in vitro, with neutrophils from R131 homozygous donors exhibiting poorer uptake of pneumococci than neutrophils from H131 donors (Rodriguez et al. 1999). In the clinical setting, patients diagnosed with bacteremic pneumococcal pneumonia are more likely to possess the R131 allele that those with nonbacteremic pneumonia (Yee et al. 2000).

While neutrophils typically use CD18/CD11a (LFA-1), CD18/CD11b (Mac-1), or CD18/CD11c (p150, 95) to adhere to and migrate through an activated vascular endothelium (Tonnesen et al. 1989), CD18-deficient mice retain neutrophil emigration to the lung during pneumococcal infection (Mizgerd et al. 1997). Equal percentages of $CD18^+$ and $CD18^-$ neutrophils are found in the lungs of pneumococcal-infected mice possessing mixed bone marrow from wild-type and CD18-deficient donors (Mizgerd et al. 1999). These observations indicate that a CD18-independent neutrophil emigration pathway exists and is active during pneumococcal lung infection. The mechanism of this pathway likely involves PAFr, since it is inhibited by PAFr antagonists (Cabellos et al. 1992). Interestingly, the strong phenotype observed in animal models is weaker in vitro, with a blocking antibody against CD18 reducing 79 % neutrophil transmigration to pneumococci over polarized human lung endothelial cells (Moreland et al. 2004). Although neutrophil VLA4 (very late antigen 4) contributes to CD18-independent neutrophil influx into the airways, its role is minor during pneumococcal pneumonia (Tasaka et al. 2002). Additionally, mice deficient in both E- and P-selectin, which promote recruitment and rolling by binding neutrophil ESL-1 and PSGL-1, are not deficient in neutrophil emigration to the lung following pneumococcal infection (Mizgerd et al. 1996). Thus, there exists a CD-18 independent mechanism of recruitment of neutrophils that is unique to both the lung as a site of infection and the pneumococcus as the causative agent.

Despite being a hallmark of early cellular influx to the lung during pneumococcal disease, the mechanism by which neutrophil emigration occurs remains poorly characterized. CXC chemokine production is one mechanism of neutrophil recruitment that has been explored. Recruitment of both neutrophils and exudate macrophages (precursors to AMs) to the lung occurs via CXCR2, which binds CXCL1 and CXCL2/3 (Herbold et al. 2010). Purified pneumolysin has been shown to elicit IL-8 expression and release by human neutrophils in vitro (Cockeran et al. 2002), thus the initial recruitment of neutrophils to the site of pneumococcal infection is likely to promote further migration of neutrophils.

In some cases, activated neutrophils can undergo a unique, caspase-independent form of cell death that involves the loss of the nuclear membrane and granules (Fuchs et al. 2007) and the eventual expulsion of neutrophil extracellular traps (NETs) comprised of DNA, histones, and granule proteins (Brinkmann et al. 2004). These NETs are then able to trap bacteria. NETs are not bactericidal for either encapsulated or unencapsulated pneumococci (Beiter et al. 2006; Wartha et al. 2007). However, capsule can impair the initial capture and immobilization of bacteria by NETs (Wartha et al. 2007). Additionally, *S. pneumoniae* also possesses a surface endonuclease EndA, that has been shown to degrade NETs in vitro (Beiter et al. 2006), thus liberating already-bound bacteria and allowing for further infection to occur.

9.6.3 Beyond the Phagocyte: T-Cells and Acute Pneumonia

$CD4^+$ T-cells are recruited to the lungs early after intranasal challenge with pneumococci (Kadioglu et al. 2004). Pneumolysin is important in promoting the

migration of $CD4^+$ T-cells to the site of infection. In vitro, pneumolysin-positive pneumococci and sub-lytic concentrations of purified pneumolysin are able to elicit the chemotaxis of human $CD4^+$ T-cells, whereas pneumolysin-deficient mutants are far less capable (Kadioglu et al. 2004). Once recruited, the contribution of $CD4^+$ T-cells to innate defense in the lungs is unclear, with reports demonstrating $MHCII^{-/-}$ mice (which are inherently deficient in $CD4^+$ T-cells) possess greater bacterial loads in the lungs than wild-type mice following intranasal infection (Kadioglu et al. 2004), versus the absence of $CD4^+$ T-cells not affecting pulmonary bacterial numbers (Weber et al. 2011; LeMessurier et al. 2010). The role of $CD4^+$ T-cells in the resolution of lung infection may be influenced by factors such as mouse model, challenge dose, capsular serotype, or bacterial strain. Indeed, serotype- and strain-specific T-cell responses have been reported for $CD8^+$ T-cells during pneumococcal disease. Mice lacking $CD8^+$ T-cells exhibit more severe lung pathology and inflammation, and are more prone to developing bacteremia than wild-type mice following intranasal infection with serotype 3 strains WU2 and A66, but not with serotype 2 or serotype 8 pneumococci (Weber et al. 2011).

During the resolution phase of pneumococcal lung infection, there is a dramatic increase in the γδ T-cell population, predominantly the pulmonary-associated Vγ1, Vγ4, and Vγ6 subsets (Sim et al. 1994; Wands et al. 2005; Kirby et al. 2007b). Infected mice that lack γδ T-cells ($TCR\delta^{-/-}$) accumulate AMs and DCs in the lungs, even after pneumococci have been cleared. As γδ T-cells are able to kill AMs and pulmonary DCs, their elevated numbers in the lung most likely serve to curtail the influx of professional antigen-presenting cells during the latter stages of infection (Kirby et al. 2007b).

9.7 Advanced Pneumonia and Invasion to Bacteremia

In most cases, the innate immune response is able to recognize and clear pneumococcal infection in the lungs without significant long-term tissue damage or progression to sepsis. However, in patients with disregulated immune responses, including the immunosuppressed or those with coinfections, the delicate balance of the inflammatory response can become disrupted and lead to excessive immune signaling and contribute to lung pathology and the spread of infection. Inflammatory insult may be further exacerbated by release of PAMPs during antibiotic therapy (Karlstrom et al. 2011). Apoptosis of innate effector cells and cellular and fluid influx into the lung during pneumococcal infection are contributors to overall lung pathology and disease outcome.

While apoptosis of specific cell populations, such as macrophages, augments bacterial killing, apoptosis of "bystander" epithelial cells is less desirable for the host (Dockrell et al. 2003). Significant apoptosis of epithelial cells is characteristic of pneumococcal pneumonia (Marriott and Dockrell 2006). However, the mechanism by which this occurs is only partially elucidated. Cellular apoptosis can be mediated by Fas/FasL interactions, and indeed ligation of Fas (using recombinant sFasL or anti-Fas antibody) has been shown to increase both alveolar permeability

and epithelial cell death in mouse models (Hagimoto et al. 1997; Matute-Bello et al. 2001a, 2001b). During pneumococcal pneumonia, however, preventing the Fas/FasL interaction by prophylactic administration of DcR3-a (a decoy receptor for soluble Fas) or by using mice deficient in Fas (*lpr*- mice), does not reduce the alveolar permeability and subsequent development of systemic disease, although pneumococci are more efficiently cleared from the lungs compared to control animals (Matute-Bello et al. 2005).

High doses of purified pneumolysin cause massive disruption to the integrity of the lung (Dessing et al. 2009). Alveolar epithelial cell damage as a result of pneumolysin pore-forming activity contributes to tissue damage during pneumococcal pneumonia, and likely promotes dissemination of *S. pneumoniae* into the bloodstream in severe cases of infection (Rubins et al. 1993). Direct binding of pneumolysin to TLR4 has been associated with increased apoptosis in both epithelial cells and macrophages (Srivastava et al. 2005). Compared to TLR4 deficient mice, pneumolysin-producing pneumococci induced increased apoptosis in cells of the upper respiratory tract of wild-type mice in an in vivo infection model (Srivastava et al. 2005), although this interaction is not solely responsible for epithelial cell death as apoptosis still occurs in the absence of pneumolysin (Schmeck et al. 2004).

As excessive inflammation produces tissue damage and pathology during pneumococcal pneumonia, regulating inflammation during treatment of *S. pneumoniae* infection is key in preventing serious complications. It has been shown that inflammatory damage as a result of ampicillin-induced lysis of bacteria and innate immune recognition of PAMPs in the released debris is dampened by the administration of cyclooxegenase inhibitors (Tuomanen et al. 1987b). In regards to pneumonia-induced lung damage, late stage administration of corticosteroids improves lung function and reduces CRP levels in patients with acute lung injury (Varpula et al. 2000). Targeted control of inflammatory responses is also being studied as a method to reduce the use of broad spectrum inhibitors such as steroids (Wissinger et al. 2008). Studies of *S. pneumoniae*-infected mice suggest that IL-10 inhibition in concert with ceftriaxone antibiotic therapy increases survival from severe pneumonia (Wang et al. 2005). Understanding the PAMP-mediated inflammatory pathways involved in *S. pneumoniae* infection of the lungs would aid in the development of treatments that both target the bacteria and prevent and repair inflammatory-mediated lung damage.

9.7.1 Innate Invasion: PAFr, PCho, and Bacteremia

Several points in the progression of pneumococcal pneumonia highlight the critical interaction of bacterial surface PCho and its target receptor PAFr (Fig. 9.3). PAFr, a G-protein-coupled receptor, is widely expressed on host cells including the pulmonary epithelium and vascular endothelium (Cundell et al. 1995). PAFr is known to be rapidly internalized following binding of its natural ligand, the phospholipid chemokine PAF, which contains PCho as its active determinant. Thus, by mimicry,

pneumococcal PCho subverts this pathway that triggers transmigration through the lung mucosa into the bloodstream (Cundell et al. 1995; Radin et al. 2005; Le Gouill et al. 1997).

The major early innate pulmonary defenses target PCho in order to protect against invasive disease. PCho in surfactant and CRP produced in the acute phase response competitively inhibit pneumococcal binding and uptake via PAFr (Gould and Weiser 2002). Thus innate immunity targeting PCho directly counteracts PAFr-mediated innate invasion, a fundamental defense process that applies to the many pathogens that use this pathway to cause pneumonia. Mycoplasma (Deutsch et al. 1995), lipopolysaccharide of *Haemophilus influenzae*, *Pseudomonas* surface protein and pili of *Neisseria* (Weiser et al. 1998a) demonstrate modification by PCho in a phase variable manner (Weiser et al. 1994, 1998b, 1997). If host innate defenses fail, then PCho on the pathogen supports spread from lung into the bloodstream by PAFr ligation and transmigration. In the bloodstream, PCho is detrimental as it attracts CRP and complement. Thus, the opaque colony morphotype found in the blood is characterized by removal of surface PCho by a phosphorylcholine esterase (Vollmer and Tomasz 2001).

The PAFr is encoded by an NFκB target gene (Chaqour et al. 1999; Ishizuka et al. 2001), suggesting the initial production of TNFα, IL-1β, and other NFκB targets during pneumococcal infection up-regulates PAFr on the lung epithelium and endothelial cells. Loss of PAFr severely inhibits neutrophil recruitment and inflammatory cytokine production in response to intra-tracheal administration of purified LTA (Knapp et al. 2008). Mice lacking PAFr or mice treated with PAFr antagonists fail to progress from pneumonia to sepsis implicating the PCho-PAFr interaction as a major determinant of progression of infection from lung to other organs (Radin et al. 2005).

9.8 Concluding Remarks

The pneumococcus is a prototype pulmonary pathogen of major medical importance. It is a commensal of the nasopharynx that progresses to invasive disease via the lung. The alveolus reacts to PGN and LTA, classic Gram-positive PAMPs, with edema, cytokine production and recruitment of leukocytes. The toxins pneumolysin and H_2O_2 cause direct toxicity to the alveolar epithelium while the inflammatory response can indirectly amplify damage, particularly as antibiotic therapy releases more PAMPs from lysing bacteria.

The molecular mechanism of progression to bacteremic pneumococcal pneumonia illustrates an innate invasion strategy shared by pathogens entering the host bloodstream through the lungs. These pathogens decorate their surfaces with PCho, a moiety that mimics surfactant and the chemokine PAF. Bacteria use PCho to bind to host epithelial PAFr, which is then internalized in a vacuole and bacteria traverse the alveolar epithelium into the bloodstream. In defense, airway CRP binds PCho and opsonizes the bacteria for phagocytosis by resident AMs and neutrophils.

To ensure resolution of pneumococcal pneumonia, the innate immune response needs to attain balance. A poor response may result in sub-optimal cellular recruitment and inefficient clearance of bacteria from the lung, whereas a high response risks damaging the lung mucosa and promoting the development of invasive disease. Indeed, much of the tissue injury and vascular leakage that are characteristic of acute pneumococcal pneumonia can be attributed to an over-exacerbated innate response to bacterial components, rather than direct damage by the pneumococcus.

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Chapter 10
Staphylococcus aureus Infection in the Respiratory Tract

Alice Prince

S. aureus is a component of the upper respiratory flora of up to 30% of normal individuals, although such innocuous colonization is widely considered to predispose to invasive infection (Shukla et al. 2010). While skin and soft tissue infection is exceedingly common, lower respiratory infection due to *S. aureus* is much less frequent but associated with high mortality (Klevens et al. 2007a). *S. aureus* pulmonary infection is associated with different types of pathology including pleural effusions, empyema, abscesses, and pneumatoceles, in addition to the necrotizing pneumonia characteristic of USA300 infection (Quadri and Hock 2007; Francis et al. 2005). Historically staphylococcal pneumonia was observed in young infants and has long been recognized as a severe and often fatal complication of influenza, dating back to the 1918 pandemic (Morens et al. 2008). *S. aureus,* especially the epidemic USA300 strains of MRSA, is now the major cause of hospital-associated infection in the United States, often causing ventilator-associated pneumonia as a complication of intensive care (Kollef et al. 2005) (Sandiumenge 2012, p. 802). Staphylococcal pneumonia has been associated with significantly higher morbidity and mortality, up to 37% in a recent study in Detroit, much more than other hospital-associated pneumonias, despite "appropriate" antimicrobial therapy, indicating that host clearance mechanism are often inadequate in clearing the infection (Haque et al. 2012). There are a relatively limited number of strains of *S. aureus* circulating worldwide although there is a constant evolution and adaptation of these clones in response to environmental pressures and local antimicrobial usage. In the United States most of the current invasive infections are due to the USA300 lineage of MRSA and much of the following discussion will be related to that strain, with its expression of specific virulence factors (Klevens et al. 2007b).

Given the ubiquity of nasal colonization with *S. aureus*, relatively few individuals develop lower respiratory tract infection (Gorwitz et al. 2008). The pathogenesis of *S. aureus* respiratory infection seems highly dependent upon the nature of the host

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response to expression of specific staphylococcal gene products. While skin and soft tissue infection affect entirely well adults and children, respiratory infection are especially common in hosts with specific alterations in immune function as well as immunodeficiency syndromes (von Bernuth et al. 2008; Casanova and Abel 2007; Rosenzweig and Holland 2011). The USA300 MRSA often associated with nasal colonization; in the setting of influenza or severe trauma, can be aspirated and cause a severe necrotizing, frequently fatal pneumonia. The propensity of the USA300 strains as well as other widespread epidemic clones of *S. aureus* to cause such severe infections may be due to its evolution in response to immune pressure and the selection of a clone optimized for persistence and avoidance of innate immune clearance. In these strains of *S. aureus* circulating worldwide, there are multiple gene products with redundant functions, that when expressed in a conducive setting, facilitate respiratory tract infection.

10.1 Nasal Colonization

There are substantial epidemiological data indicating an association between nasal (or other mucosal site) colonization and subsequent *S. aureus* infection (Lederer et al. 2007). While persistent nasal colonization may be associated with the development of antibody, this is not protective (Verkaik et al. 2009). The association between nasal colonization and infection is sufficient to drive decolonization procedures prior to major orthopedic and cardiac surgery in many large medical centers. The specific gene products that are essential for human colonization are not well defined. The ability to withstand the effects of host antimicrobial peptides and mucociliary clearance as well as bacteriocins expressed by the resident microbial flora are important characteristics. Bacterial interference, the inoculation of susceptible hosts with an "avirulent" strain of *S. aureus* was used successfully in the 1960s to prevent colonization and subsequent infection with the highly virulent phage type 80/81 *S. aureus* associated with newborn nursery outbreaks of infection (Light et al. 1967). The large genome of *S. aureus* includes a number of variably expressed surface proteins the MSCRMMs (microbial surface components recognizing adhesive matrix molecules) that recognize specific eukaryotic receptors including the ubiquitously expressed matrix proteins fibrinogen and fibronectin (Patti et al. 1994). Direct transcript analysis from human nasal isolates suggests a distinctive pattern of gene expression in vivo, regulated by the *wkl* and *agr* loci (Burian et al. 2010). The distribution and accessibility of host receptors is a key factor in mucosal colonization (Foster and Hook 1998). Bacterial factors that promote attachment to abiotic surfaces, such as catheters are also likely to be important in the pathogenesis of specific respiratory infections, especially those in a critical care setting.

The expression of many *S. aureus* virulence determinants is regulated by the *agr* locus (Montgomery et al. 2010; Cheung et al. 1992, 2011, 1992). The *agr* system controls the divergent expression of surface proteins and secreted toxins through the production of an RNAIII (Xiong et al. 2002). Whilst initial colonizing strains are

likely to have increased protein A and FnBP (fibronectin binding protein) production, as stationary phage of growth is achieved, there is decreased expression of *spa* and increased production of Hla and similar secreted toxins (Cheung et al. 1997), all of which are involved in different stages of pathogenesis of pulmonary infection. Host factors that influence *agr* behavior may also influence susceptibility to nasal colonization and predisposition to invasive infection. In the initial stages of colonization, staphylococci adhere to mucins, either tethered to the mucosal epithelium or as secreted products. If not cleared by local antimicrobial peptides they can persist presumably through binding interactions with mucosal surfaces, but without eliciting inflammation.

10.2 Adhesins and Colonization

Among the many *S. aureus* surface proteins that specifically recognize host matrix components, a few have been directly shown to promote colonization. The expression of ClfB (clumping factor B) (Wertheim et al. 2008) is perhaps the best characterized MSCRMM associated with nasal colonization, binding to cytokeratin 10 and fibrinogen (O'Brien et al. 2002) (Ganesh et al. 2011). Additional staphylococcal proteins involved include teichoic acid, capsule formation, iron binding proteins, and sortase A, which is involved in their surface expression. Proteomic analyses of carrier versus noncarrier *S. aureus* strains reveals a number of differences in surface proteins involved both in attachment and immune function. These include the epidemiologically important surface protein A (Muthukrishnan et al. 2011). Host factors are clearly important in *S. aureus* colonization. The availability of human hemoglobin may be involved, acting through its affect on *agr* expression which regulates both protein A and ClfB expression (Torres et al. 2007). The ability of certain staphylococcal strains to colonize mucosal surfaces requires resistance to antimicrobial peptides produced by both competing commensal flora and the host epithelium. This may entail alteration of surface structures effecting charge, as the many of the antimicrobial peptides are highly cationic and their effects salt-sensitive. The production of antimicrobial peptides appears to be a major host factor in determining susceptibility to staphylococcal colonization. Diminished induction of hBD-3, but not RNAse-7 has been associated with nasal carriage (Zanger et al. 2011). The composition of the host microbiota also has a major impact upon the ability of *S. aureus* to colonize (Frank et al. 2010).

10.3 Genes Involved in Biofilm Formation

Once attached to a biological surface, *S. aureus* grow in a biofilm with growth and bacterial community structure regulated through *agr*-dependent quorum sensing (Periasamy et al. 2012). This mode of growth is typically associated with staphylococcal infection of catheters but is likely relevant to the respiratory tract as

well and is associated with local induction of mucosal immune responses (Prabhakara et al. 2011). Within the biofilm staphylococci increase the production of extracellular carbohydrates and especially the PSMs, a group of surface proteins that are important in both the structural properties of the biofilm and detachment of organisms from the biomass (Gonzalez et al. 2012). The extracellular polysaccharides prevent macrophage phagocytosis and clearance (Thurlow et al. 2011). There is some debate as to whether staphylococcal colonization of the upper airway represents a true biofilm, as this is likely to depend upon multiple factors including the density of bacterial growth and the production of quorum sensing systems.

10.4 Phenol Soluble Modulins

Phenol soluble modulins (PSMs) are a surface component of *S. aureus* that contribute to many aspects of pathogenesis. Several PSMs have been identified in the USA300 strains, 4 α- or short- 20 amino acids in length, and 2 - β- 40 amino acids peptides. Although their role in the pathogenesis of pulmonary infection has not been formally assessed to date, their contribution to biofilm formation, neutrophil chemotaxis, and antimicrobial activities are likely to be relevant (Joo et al. 2011). PSMs are ribosomally encoded surface peptides that have antimicrobial activity towards other species. They also possess surfactant—like properties that contribute to biofilm formation and degradation. These *psm* genes, expressed by strains of *S. epidermidis* strains as well as the USA300 MRSA clones (Gonzalez et al. 2012) are a major factor in staphylococcal growth in biofilms. The antimicrobial activity of specific PSMs has been documented and is likely to contribute to the ability of *S. aureus* strains to colonize the upper respiratory tract by decreasing the competing local flora. There are local environmental signals that initiate biofilm formation that are likely initiated at mucosal surfaces (Sadykov and Bayles 2012). In vitro studies using *psm* deletion mutants have established the contribution of PSMs to both biofilm formation and the ability of *S. aureus* to disseminate from biofilms and cause disseminated infection (Periasamy et al. 2012).

10.5 Internalization Versus Superficial Adhesion

S. aureus strains are targeted for ingestion and killing by phagocytes, primarily neutrophils, and macrophages. Staphylococci can evade phagocytic clearance through internalization by phagocytes as well as nonprofessional phagocytes including the mucosal epithelial cells that line the upper airway, endothelial cells, and keratinocytes. Agr-dependent patterns of staphylococcal gene expression are involved in internalization (Wesson et al. 1998). Surface adhesion to airway cells has been shown in some settings to involve the fibronectin binding protein FnBPB (Mongodin et al. 2002). This may be followed by internalization mediated by fibronectin which is recognized by the α5β1 integrin on the cells lining the mucosal

surface. Once internalized, biopsies from patients with chronic mucopurulent rhino-sinusitis demonstrate that staphylococci can persist intracellularly for extended periods of time. Alternatively *S. aureus* can stimulate apoptosis or pyroptosis of the host cell (Soong et al. 2012).

Often the staphylococci that are able to persist intracellularly are small colony variants (SCVs) (Atalla et al. 2011); mutants that have slow replication rates and limited expression of virulence factors (Kriegeskorte et al. 2011; Mitchell et al. 2011). These SCVs also fail to activate proinflammatory signaling (Tuchscherr et al. 2011). A very different pattern of staphylococcal gene expression is activated when the organisms persist within eukaryotic cells (Garzoni et al. 2007). Thus both phenotypic changes in staphylococci that are able to persist intracellularly, as well as their protected niche, enable them to evade phagocytic clearance (Sadykov and Bayles 2012).

10.6 Pathogenesis of *S. aureus* Pulmonary Infection

A small fraction of patients with nasal colonization by *S. aureus* will aspirate these organisms and develop pneumonia. Lower respiratory tract infection, caused by the epidemic *S. aureus* USA300 strains typically consists of a severe hemorrhagic alveolitis with extensive fluid accumulation in the alveoli as well as tracheobronchitis (David and Daum 2010). Exactly how a sufficient inoculum of colonizing organisms are aspirated into the lower airways remains unclear, but is a common complication of mechanical ventilation, loss of the cough reflex and impaired mucociliary clearance. MRSA pneumonia is also a major complication and cause of death in patients following influenza infection, both sporadic cases and in epidemics (Louie et al. 2009; Louria et al. 1959). In murine models a significantly lower staphylococcal inoculum is required to cause severe pneumonia in mice with antecedent influenza (Lee et al. 2010), which may be associated with changes in innate immunity, especially type I interferon induction associated with the viral infection. Innate immune defenses are especially important in the clearance of *S. aureus* from the respiratory tract. Nasal colonization with these organisms does not induce protective adaptive immunity and even patients with invasive infection are not necessarily protected from subsequent staphylococcal infection. Despite the intense interest in the pathogenesis of these infections, there has been difficulty correlating the epidemiological data obtained from large studies of human infection to more tractable animal models. This discrepancy has also impaired progress in vaccine development (Spellberg and Daum 2012).

10.7 Models of *S. aureus* Pneumonia

A great deal of information regarding host–pathogen interactions has been generated using mouse models of pneumonia. These are valuable in comparing the importance of both bacterial strains and specific virulence factors in pathogenesis and in defining

host immune components that are involved in host defense, by using the many available transgenic mouse models. As staphylococcal infection is also a major veterinary issue, a major cause of bovine mastitis, there is also a wealth of literature examining factors relating to bovine susceptibility to infection. Common to both murine models and severe pneumonias in humans, is the observation that most severe pathology occurs in the early stages of infection; if the mouse (or patient) survives the initial proinflammatory response, they generally will clear the infection eventually. Thus, the mouse in some ways does approximate important aspects of human disease.

It is important to recognize that mouse models are limited by the relative resistance of mice to *S. aureus* infection. Staphylococci are unable to obtain iron from murine hemoglobin, in contrast to human Hgb (Pishchany et al. 2010) which significantly limits their ability to cause infection. In addition, murine leukocytes are less susceptible to *S. aureus* toxins, such as PVL, which has resulted in the use of rabbit models of infection (Diep et al. 2010). However, in even the "susceptible" rabbit the intratracheal instillation of 10^8 cfus of staphylococci, which is not representative of the mode of acquisition in humans, is required to cause severe pneumonia. It has been possible to examine nasal colonization in murine models, although this data must of course be corroborated in human experimental systems. Similarly, the evaluation of the effects of specific toxins in mouse and rabbit models is not straightforward, as these also have involved the instillation of large amounts of toxins directly into the lung, which is unlikely to mimic the situation in an in vivo infection. Nonetheless, particularly when the observations from animal models does correlate with human pathology or even if human cell lines or cells in primary culture can be used to verify observations stemming from murine models, the ability to identify specific components of host immune signaling that contribute to disease has provided major new insights into the importance of host mucosal immune signaling in the pathogenesis of staphylococcal infection.

10.8 Neutrophils and *S. aureus* Clearance from the Lung

It is well recognized that neutrophils are critical for the clearance of *S. aureus* from the respiratory tract; however their influx also causes host damage (Rigby and DeLeo 2012). Although staphylococci have multiple mechanisms to thwart opsonization by host proteins such as IgG and complement, they are nonetheless, readily phagocytosed by human macrophages and neutrophils. In most of the model systems as well as in humans, the inflammation triggered by *S. aureus* contributes to pathology. Once *S. aureus* are aspirated into a normally sterile site in the respiratory tract, they activate a brisk proinflammatory response mediated by the airway epithelium as well as the resident and recruited immune cells (Gomez and Prince 2008). The intensity of this inflammation has often been cited as the mechanism for *S. aureus* induced pathology; the accumulation of neutrophils and their products in the airways impedes respiration and results in local damage due to reactive oxygen

intermediates, neutrophil elastase, and their toxicities. The biology of neutrophils in the pathogenesis is detailed in Chapter 4 by C. Doerschuk. The importance of neutrophil cidal activity against *S. aureus* is well illustrated by the immunodeficiency syndromes that become clinically manifest through recurrent or persistent staphylococcal infection. While many of these infections involve skin and soft tissue, severe staphylococcal pneumonias are often described in patient with mutations in the genes required for superoxide production (chronic granulomatous disease), the Stat-3 mutations associated with the hyper IgE syndromes and other immunodeficiency syndromes involving neutrophil function.

Despite the importance of inflammation both in the pathology associated with *S. aureus* and in its clearance, several murine models targeting specific components of immune signaling demonstrate that pathology and even mortality do not directly correlate with the numbers of PMNs recruited into the airway in response to *S. aureus* inoculation, suggesting that additional factors are involved. Despite abundant human data establishing that neutrophils are critical in staphylococcal clearance, it is less clear how "excessive" PMN recruitment necessarily causes respiratory failure. For example, mice lacking the ability to respond to the type I IFNs are protected from a lethal dose of USA300 MRSA (Martin et al. 2009), yet this protection is independent of the numbers of PMNs recovered from BAL fluid. Thus, while functional neutrophils are critical to handle *S. aureus* infection, beyond a threshold number, more PMNs do not improve outcome and in fact, may interfere with efficient bacterial clearance by contributing to lung damage.

Mice lacking alveolar macrophages have significantly increased mortality from *S. aureus* pneumonia (Martin et al. 2011), but this does not correlate with the numbers of PMNs mobilized to the site of infection. Instead, it may reflect the activation state of recruited macrophages and dendritic cells. Macrophage-mediated phagocytosis is enhanced by in presence of surfactant protein A-which binds to staphylococcal surface protein Eap and functions as an opsonin (Sever-Chroneos et al. 2011).

The ability of these recruited neutrophils to actually kill *S. aureus* is a major factor in the host response to pneumonia. As discussed below, *S. aureus* have several surface proteins that interfere with efficient opsonization. However, even once the organisms are phagocytosed, they have a remarkable ability to resist phagocytic killing through the production of proteases such as staphylokinase and other mechanisms (Jin et al. 2004; Rigby and DeLeo 2012). Several studies have demonstrated systemic dissemination of *S. aureus* by virtue of its ability to persist within phagocytes, both neutrophils and macrophages (Kapral and Shayegani 1959; Kubica et al. 2008; Watanabe et al. 2007). It should be noted that nonprofessional phagocytes, such as endothelial cells and keratinocytes, also can ingest and kill staphylococci via classical endocytic routes. (Garzoni and Kelley 2009) This is thought to entail the interactions of the MSCRMMs FnBPA/B (fibronectin binding proteins), Fn and the $\alpha 5\beta 1$ integrin receptor which delivers the attached bacteria into the endosome. It remains unclear exactly how *S. aureus* then resist killing within the phagolysosome or their efficiency gaining access to the cytoplasm, although toxins such as Hla and PVL could contribute.

10.9 Interactions with Complement

The complement system provides opsonization for many bacterial pathogens in the absence of preformed IgGs. *S. aureus* express a number of proteins that function to interfere with complement activation (Jongerius et al. 2007; Lambris et al. 2008; Foster 2005). These include Ecb which binds to the C3d domain of C3 (Rooijakkers et al. 2005) and Efb (extracellular fibrinogen binding protein) which blocks complement activation and neutrophil binding. SCIN (staphylococcal complement inhibitor) and CHIPS (chemotaxis inhibitory proteins of staphylococci) are phage encoded proteins that interfere with phagocytic function blocking C3 convertases and C5a respectively (Haas et al. 2005; Postma et al. 2004). The details of these complement evasion proteins have been well described in previous reviews. *S. aureus* also produce the Sbi proteins (Haupt et al. 2008; Smith et al. 2011a). Sbi blocks IgG-mediated opsonization, in a manner similar to that described for protein A as well as interacting with the C3 component of complement (Smith et al. 2012).

Once ingested by professional phagocytes, *S. aureus,* itself, is relatively resistant to killing, although this varies widely among different strains. Despite expression of leukocidins it appears that many strains of staphylococci induce PMN cell death that is not directly mediated by pore forming (Rigby and DeLeo 2012). *S. aureus* activate the expression of *agr*-regulated genes, include Hla once within phagocytes, which is important in staphylococcal escape from phagocytic killing (Pang et al. 2010).

10.10 Activation of Host Signaling Cascades Associated with Lung Damage

From the analyses of murine models of *S. aureus* pneumonia, the contribution of the host response to staphylococcal infection, in causing pathology has become apparent. Despite the production of numerous toxins, there is little indication that *S. aureus* in vivo, directly destroys tissues. Instead, several of these toxins target host receptors to activate what are often pathological immune responses. Among the many staphylococcal gene products that have major effects on host signaling are those that are highly conserved among virtually all clinical isolates of *S. aureus*, such as the α hemolysin (Hla) and protein A, as well as the PAMPs conserved among many bacterial species, such as peptidoglycan, cell wall lipoproteins and DNA.

The ability of *S. aureus* to activate the many classes of receptors that function in innate immune defenses have been well described and the participation of TLRs in innate immune defenses in the lung are reviewed by S. Skerrett (Chapter 5). These include TLRs 2 and 9—which recognize cell wall lipoproteins and DNA as well as Nod2 that is activated by staphylococcal peptidoglycan (Girardin et al. 2003). These signal transduction cascades generally result in the activation of NF-κB and proinflammatory cytokine production. In addition, staphylococci are also capable of initiating more complex modes of eukaryotic signaling. Many signaling cascades, such as the induction of TNF or IFN-β, for example, are not only dispensable for host clearance in murine models, they appear to increase mortality (Fig. 10.1).

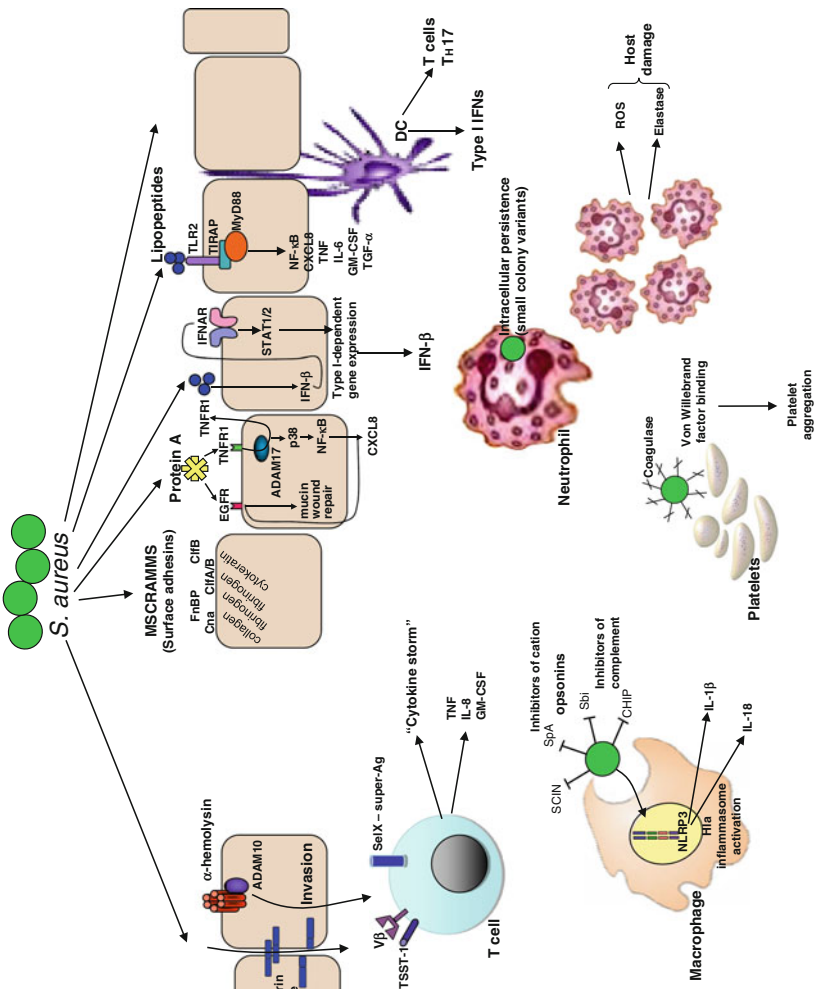

Fig. 10.1 *S. aureus* has multiple gene products that directly interact with various components of the human innate immune system. Depicted in this cartoon are the epithelial targets of these virulence factors as well as interactions with macrophages, platelets, neutrophils, T cells, and DCs

As illustrated by these studies, there is tremendous redundancy in the signaling pathways that function to recruit and activate neutrophils and other phagocytes in response to *S. aureus* airway infection. Although each of the experiments depicted have somewhat differing methodologies, each demonstrates that the deletion of a seemingly critical component of innate immune signaling, does not significantly impair the initial immune clearance of these virulent organisms. Note that even heat killed *S. aureus* elicit inflammation in the lung, indicating that staphylococcal PAMPs, cell wall components, DNA as well as specific toxins all contribute to inflammatory signaling. These studies illustrate that *S. aureus* have multiple gene products that serve to activate host clearance mechanisms and the potentially pathological consequences of this immune activation.

10.11 Hla

The α-hemolysin is a heptameric pore forming toxin that penetrates eukaryotic lipid bilayers. Expressed by most clinical isolates of *S. aureus*, it can contribute to pathology through interactions with epithelial and endothelial cells as well as with macrophages and keratinocytes. Production of the α-hemolysin (Hla) has been demonstrated to contribute significantly to lung pathology in numerous models of pulmonary infection. Hla recognizes ADAM-10, a matrix metalloprotease in the lung (Wilke and Bubeck Wardenburg 2010), as a receptor and ADAM-10$^{-/-}$mice were found to be significantly less susceptible to fatal pneumonia than wild type mice. As ADAM10 participates in the cleavage of the junctional protein E-cadherin this interaction may facilitate staphylococcal dissemination throughout the lung (Bubeck Wardenburg et al. 2007) or at least contribute to alveolar hemorrhage and fluid accumulation. In addition, expression of ADAM 10 in endothelial cells may contribute to the invasion of the nonmotile staphylococci through small blood vessels and into tissues during dissemination of pulmonary infection (Powers et al. 2012).

An additional mechanism of Hla participation in pathology is through the activation of the NLRP3 inflammasome (Munoz-Planillo et al. 2009). It has been well documented in several experimental systems that *S. aureus* expressing Hla escape from the phagolysosome and activate inflammasome signaling. This results in the expression of IL-18 and IL-1β which is associated with pyroptosis, a highly proinflammatory mechanism of cell death. Of note, some studies suggest that the contribution of NLRP3 inflammasome activation to lung pathology is largely independent of IL-1 (Craven et al. 2009). However, in other model systems the recruitment of neutrophils has been closely linked to the activation of IL-1β by *S. aureus* (Miller et al. 2007). These studies do not discriminate between the relative amounts of inflammasome activity in immune cells as opposed to stromal cells. There appears to be much less inflammasome activity in the airway epithelium, as opposed to the CD11b cells recruited to the site of infection. The multiple targets and consequences of Hla in the lung illustrate the multiple mechanisms of pathogenesis initiated by both the organism and its eukaryotic targets.

The *S. aureus* β-toxin, which has sphingomyelinase activity, is another factor associated with lung damage and inflammation (Huseby et al. 2007). β-toxin induces injury through a vascular leak syndrome associated with the shedding of syndecan-1 (Hayashida et al. 2009). In concert with the β-toxin, a staphylococcal δ-toxin (Janzon et al. 1989) facilitates bacterial escape from the endosome (Giese et al. 2011).

10.12 Panton Valentine Leukocidin

In contrast to Hla, exactly how the Panton Valentine Leukocidin participates in the pathogenesis of human pneumonias is much less well understood (Labandeira-Rey et al. 2007; Olsen et al. 2010; Otto 2011). Panton Valentine Leukocidin(PVL) is the product of two genes, *lukS-PV* and *lukF-PV* that together form an octamer pore forming structure in host membranes (Finck-Barbancon et al. 1993). The toxin has long been known to target leukocytes (Loffler et al. 2010) and it appears to cause neutrophil death by targeting mitochondrial homeostasis, inducing apoptotic caspases (Genestier et al. 2005). Now widespread due to its expression by the common USA300 MRSA strains, PVL had been found sporadically in clinical isolates of *S. aureus* over the past decades. While epidemiologically linked to strains of *S. aureus* causing severe pneumonia (Gillet et al. 2002), PVL null strains are also capable of causing a similar necrotizing pneumonia in specific animal models (Montgomery and Daum 2009). Introduction of purified PVL into the susceptible rabbit model, recapitulated the pathology generated by the intact bacteria, but involved amounts of toxin unlikely to be generated in vivo (Diep et al. 2010). Additional mechanistic studies indicate that PVL can stimulate TLR2/CD14 signaling also contributing to its proinflammatory capabilities (Zivkovic et al. 2011). It is also possible that PVL expressed intracellularly may facilitate *S. aureus* escape from the endosome and contribute to pyroptosis and host cell death (Soong et al. 2012).

10.13 Superantigens

S. aureus express a number of superantigens, gene products that activate T cells through direct binding to specific Vβ chains stimulating a significant percentage of T cells and causing widespread tissue damage as a result (Marrack and Kappler 1990; Strandberg et al. 2010). This phenomenon was first associated with staphylococcal production of TSST-1 and the toxic shock syndrome, characterized by multisystem involvement associated with widespread T cell activation and shock. Several additional putative superantigens have been identified by analysis of the USA300 genome (Diep et al. 2006). Selx is a superantigen expressed by USA300 (which does not usually express TSST-1). SelX expression is associated with the expected Vβ T cell activation and contributes to necrotizing pneumonia in a rabbit

model of infection (Wilson et al. 2011). The superantigens SEA and SEB have also been linked to pulmonary inflammation, primarily in models of asthma (Bachert et al. 2002). These are structurally related to TSST-1 and also enhance proinflammatory signaling via effects on T cells.

10.14 Immunological Properties of Protein A

Protein A (SpA) is a well characterized and abundant surface protein of *S. aureus* that has many interactions with innate immune signaling, especially in the lung (Foster 2005). Perhaps best studied and exploited, is the affinity of the 5 IgG binding domains of SpA for the Fc component of IgG. This interaction is widely exploited in immunoprecipitation reactions in the laboratory and long thought to provide protection from phagocytosis. The SpA–IgG interaction coats the organism in antibody that cannot interact with the appropriate receptors on the neutrophil. However, several recent in vivo and in vitro studies suggest that SpA does not have a major antiphagocytic function in vivo, which is not surprising, given the similar activity of Sbi, which could function in its place (Smith et al. 2011b).

Multiple properties of SpA contribute to virulence. The IgG binding domains of SpA recognize TNFR1 (Gomez et al. 2006) and activate the expected TNF signaling cascade (Gomez et al. 2004). This results in a brisk proinflammatory response that is important in the pathogenesis of pneumonia in murine models. Excessive TNF signaling is clearly detrimental in the clearance of staphylococci as TNFR1 mice clear staphylococci from the lungs significantly more than do wild type controls. In additional models of murine pneumonia, levels of TNF inversely correlate with outcome (Martin et al. 2011). The IgG binding domains of SpA also activate EGFR signaling and ADAM 17, a matrix metalloproteinase that targets many immune components including TNF and TNFR1 (Gomez et al. 2007). ADAM17 activation is also associated with gp130 trans-signaling that entails the ADAM17-mediated release of soluble gp130 that neutralizes IL-6 in the airway, acting as an anti-inflammatory (Gomez et al. 2005). ADAM 17-mediated release of TNFR1 similarly functions to neutralize TNF in the airway. Thus, despite the initial activation of neutrophil chemokines and activators, *S. aureus* also stimulate cascades to diminish airway inflammation as well.

Protein A interactions with TNFR1 and EGFR are also associated with invasion across the airway epithelium (Soong et al. 2011). Both TNF and EGFR signaling cascades are linked to the RhoGTPases that are essential for cytoskeletal responses to environmental signals. SpA was found to activate RhoGTPases causing acto-myosin contraction via myosin light chain kinase activity that provides a mechanism for *S. aureus* intercalation between adjacent airway epithelial cells and access to the matrix components below. This, as well as the effect of ADAM-10 on E-cadherin and potentially other junctional proteins, likely provides a mechanism for pulmonary invasion, by these otherwise, nonmotile organisms.

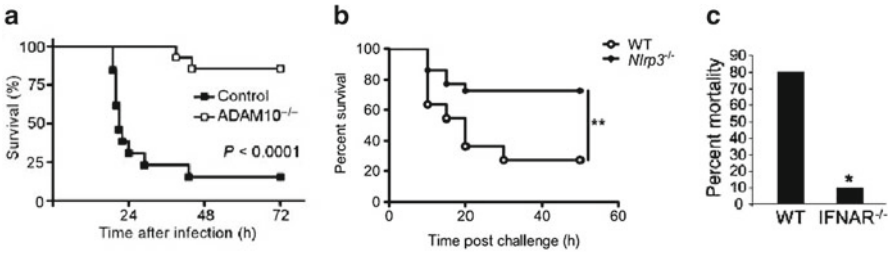

Fig. 10.2 Proinflammatory signaling activated by *S. aureus* often contributes to pathology. *S. aureus* infection in mice lacking: (**a**) ADAM 10, (Pishchany et al. 2010); (**b**) NALP3 (Kebaier et al. 2012) and IFNAR (Rooijakkers et al. 2005) all demonstrate *decreased* mortality in response to *S. aureus* infection

Adjacent to the multifunctional IgG binding region of SpA, the Xr domain of *spa* is a locus of considerable diversity at the DNA sequence level that has been useful for epidemiological studies serving as the basis for spa-typing (Koreen et al. 2004) (Ridom spaserver). The Xr domain may participate in immune function by activating type I IFN signaling in airway epithelial cells. Organisms expressing SpA are able to stimulate IFN-β expression via IFNAR and Stat-3 phosphorylation in airway epithelial cells, in a manner similar to what has been shown for viral infection (Martin et al. 2009). However, as the *Ifnar*$^{-/-}$ mouse is resistant to an otherwise fatal pneumonia caused by USA 300, type I IFN signaling actually potentiates staphylococcal virulence in the setting of pneumonia. This may contribute to the dramatically increased morbidity and mortality associated with *S. aureus* pneumonia as a complication of influenza, an infection that results in a tremendous type I IFN response.

In addition to producing T cell superantigens, *S. aureus* protein A functions as a B cell superantigen (Sasso et al. 1989). It has the ability to indiscriminately activate B cells, interacting with IgM B cell receptors causing clonal expansion and cell death. This B cell superantigen activity is thought to block the development of appropriate B cell responses to staphylococcal antigens, and can be overcome by using strains of *S. aureus* with modified SpA as immunogens (Kim et al. 2010).

10.15 Coagulopathy

Although the activation of the clotting cascade is not usually included as a component of innate immune defense in the lung, the numerous interactions of staphylococci and platelets contribute to virulence and the failure of local innate immunity. The ability of staphylococci to interact with components of the clotting cascade are likely important in the pathogenesis of pneumonia. Production of coagulase can help to provide a barrier from neutrophil-mediated clearance (Guggenberger et al. 2012).

Activation of clotting factors in the lung not only results in local hemorrhage, but also prevents the recruited neutrophils from reaching the site of staphylococcal replication in the airway. The IgG binding domains of SpA activate von Willebrands factor—causing platelet agglutination (Hartleib et al. 2000). A recently described virulence factor, von Willebrands binding protein, in combination with the expression of coagulase and ClfA cause Staphylococcal agglutination in a murine model of sepsis (McAdow et al. 2011) which is also characterized by thromboembolic phenomena. The numerous staphylococcal surface components that can interact with either platelets, fibrinogen, or von Willebrands factor all are likely to contribute to the coagulopathy that often accompanies serious systemic *S. aureus* infection.

The success of *S. aureus* as a human pathogen is likely due to its multiplicity of interactions with host immune signaling, especially in the respiratory tract. In addition to the many well characterized genes that contribute to pathogenesis, there is an increasing appreciation for the ability of this pathogen to use RNAs to modify gene expression and rapidly adapt to the host (Felden et al. 2011). While we have focused this review mainly upon the features of the currently epidemic USA300 strains of *S. aureus*, undoubtedly these organisms will continue to evolve in response to host mucosal immune pressures. Host responses to these gene products, selectively expressed in response to the environmental signals recognized in the lung, can result in either efficient clearance of pathogens from the airway or a potentially fatal inflammatory response with subsequent loss of pulmonary function. Exploring the complexities of the host response to these bacteria and how epithelial cell immune function is co-regulated along with that of the recruited phagocytes and lymphocytes may provide useful targets for prevention and treatment of pneumonia.

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Chapter 11
Pseudomonas aeruginosa and Mucosal Defenses in the Lung

Taylor S. Cohen

Commensal and opportunistic pathogens that populate the respiratory tract evolve within the host in response to the innate and adaptive immune clearance mechanisms. *Pseudomonas aeruginosa*, an opportunist, is not normally a component of the airway flora but is ubiquitous in the environment and especially common in health care-associated facilities (Richards et al. 1999; Lynch 2001). Although the focus of this review is not upon the pathogenesis of *P. aeruginosa* infection in cystic fibrosis (CF), there are substantial data examining host innate immune signaling in response to this organism in CF as compared to normal cells. These data have been important in the understanding of host–pathogen interactions in the airway.

Aspiration or contamination of the airways with *P. aeruginosa* is an infrequent cause of pneumonia in a normal host, but a common pathogen in immunocompromised and mechanically ventilated patients (Craven and Hjalmarson 2010). Due to its large genome and genetic flexibility, *P. aeruginosa* rapidly adapts to the milieu of the airway and adopts a biofilm mode of growth that favors persistence and evasion of phagocytic clearance (Smith et al. 2006; Hoboth et al. 2009; Huse et al. 2010). Environmental organisms responsible for initial colonization elicit a highly proinflammatory host response, and express gene products that facilitate iron scavenging, carbohydrate and amino acid utilization and motility. Once within the airways, many of these gene products activate host immune clearance pathways, providing selective pressure for the organisms that lack expression of these genes.

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11.1 *P. aeruginosa* Communities: Biofilms

Once a critical mass of bacteria is present in the airways, in vivo data indicate that they form biofilms which aid in evasion of the host's innate immune clearance mechanisms (Davies et al. 1997; Singh et al. 2000). This is relevant to infections in mechanically ventilated patients as well as the chronic infections in CF. The switch to the biofilm phenotype, however, does not necessarily indicate a lack of proinflammatory signaling. When serially isolated CF strains of *P. aeruginosa* were instilled into mice, isolates from late stage infection did not kill the animal as did the early isolates, but were equally capable of inducing excessive lung inflammation and establishing a chronic infection (Bragonzi et al. 2009). Biofilms are associated with an enrichment of extracellular DNA. Host pattern recognition receptors respond to the increase in DNA by upregulating inflammatory signaling, which does not necessarily correlate with increased bacterial clearance (Fuxman Bass et al. 2010). This is just one example of a bacterial adaptation to the host promoting colonization while maintaining the bacteria's ability to activate local mucosal signaling.

Biofilm formation also facilitates the coordinate expression of numerous genes throughout the microbial population through secretion of highly soluble quorum sensors, such as the *Pseudomonas* homoserine lactones and the quinolones which act in concert with specific transcriptional activators (Wade et al. 2005). The Las system produces the homoserine lactone *N*-3-oxo-dodecanoyl that activates the transcriptional regulator LasR (Passador et al. 1993; Pearson et al. 1994). This pathway controls expression of elastases and proteases involved in host damage. A second lactone, *N*-butanoyl, activates an alternate transcriptional regulator RhlR, which represses expression of the proteins that compose a major virulence factor, the type III secretion system (TTSS). Furthermore, these signaling pathways regulate each other as LasR upregulates expression of RhlR.

P. aeruginosa produced quinolones are utilized by the bacteria to communicate within a biofilm, and production is upregulated in chronic infection (Guina et al. 2003). The Pseudomonas quinolone signal (PQS) activates its receptor PqsR. In turn, downstream signaling increases production of PQS and other proteins including the phenazine and pyocyanin (Fito-Boncompte et al. 2011). PQS signaling is regulated by homoserine lactones, though through different mechanisms as LasR positively regulates and RhlR negatively regulates PQS production. Importantly, PQS correlates with bacterial virulence in animal models, potentially due to a relationship between PQS production and expression of inflammatory lipopolysaccharide (Guina et al. 2003).

Pyocyanin, regulated by transcription regulators LasR and OxyR, is produced by all *P. aeruginosa* strains, but at increased levels in biofilms (Schaber et al. 2004; Vinckx et al. 2010). It can directly interact with host cells inducing inflammatory signaling and reducing the function of antioxidants GSH and *N*-acetylcysteine by blocking the dual oxidase-based antimicrobial system (Look et al. 2005; Rada et al. 2008). Pyocyanin may also negatively affect the function of the chloride channel cystic fibrosis transmembrane conductance regulator (CFTR), perhaps enhancing

disease progression in cystic fibrosis (CF) (Schwarzer et al. 2008). Mutations in CFTR underlie the failure of innate immune to clear pathogens from the lung in CF (Cohen and Prince 2012). It is unclear if the immune deficiency is directly or indirectly linked to CFTR channel function; however, this is yet another potential mechanism through which *P. aeruginosa* attempts to evade eradication by the host immune system.

Biofilm formation also supports the spontaneous selection of MucA mutants that over express the exopolysaccharide alginate (Martin et al. 1993; Silo-Suh et al. 2002). These mucoid organisms elude phagocytosis and have been virtually pathognomic for CF (Bjarnsholt et al. 2009). Although alginate is immunogenic and elicits antibody production, this amplification of the inflammatory immune response further contributes to oxidative stress without resulting in clearance of the organisms (Schiøtz et al. 1978; Pedersen 1992). An indication of well-established *P. aeruginosa* infection, these alginate producing organisms represent the end result of failed innate immunity and effective bacterial adaptation to the host.

11.2 *P. aeruginosa* Adaptation to the Host

An intrinsic property of *P. aeruginosa* is its high mutation rate during infection (Hoboth et al. 2009). The bacterium downregulates expression of genes involved in the mismatch repair system (mutS, mutL, and uvrD) promoting increases in mutation rate and genetic diversification, and modifications of global regulators such as Vfr and OprF can reduce expression of numerous virulence factors (Oliver et al. 2000, 2002; Fuchs et al. 2010; Fito-Boncompte et al. 2011). The diversification of the bacterial population's collective gene pool potentiates survival against selective pressure applied by the host (Rodríguez-Rojas et al. 2012). The large range of gene expression within the community allows for the bacteria to utilize difference sources of nutrition, survive in aerobic and anerobic conditions, and withstand antimicrobial peptides produced by the host (Williamson et al. 2012; Zhao et al. 2012). Furthermore, selective pressure also favors mutations that suppress virulence factor expression, potentially inhibiting the ability of the host's immune system to recognize and eradicate the bacteria.

Inhaled bacteria encounter an array of host receptors that activate innate immune signaling pathways. Epithelial and immune cells express toll-like receptors (TLRs) on the cell surface that recognize bacterial lipoproteins (TLR2), LPS (TLR4), and flagellin (TLR5), as well as intracellular receptors for double-stranded RNA (TLR3), single-stranded RNA (TLR7), and CpG DNA (TLR9). These cells also express nod-like receptors (NLRs) that recognize bacterial peptidoglycans, flagellin, genomic material (DNA and RNA), and components of the TTSS, resulting in caspase activation, cytokine release, and a form of inflammatory cell death called pyroptosis (Akira et al. 2006). Pathogens must either successfully evade activation of these pathways or harness innate immune signaling to facilitate colonization. *P. aeruginosa* is a successful opportunist not only due to its many virulence factors but also due to its ability to adapt to the host environment (Bianconi et al. 2011) (Fig. 11.1).

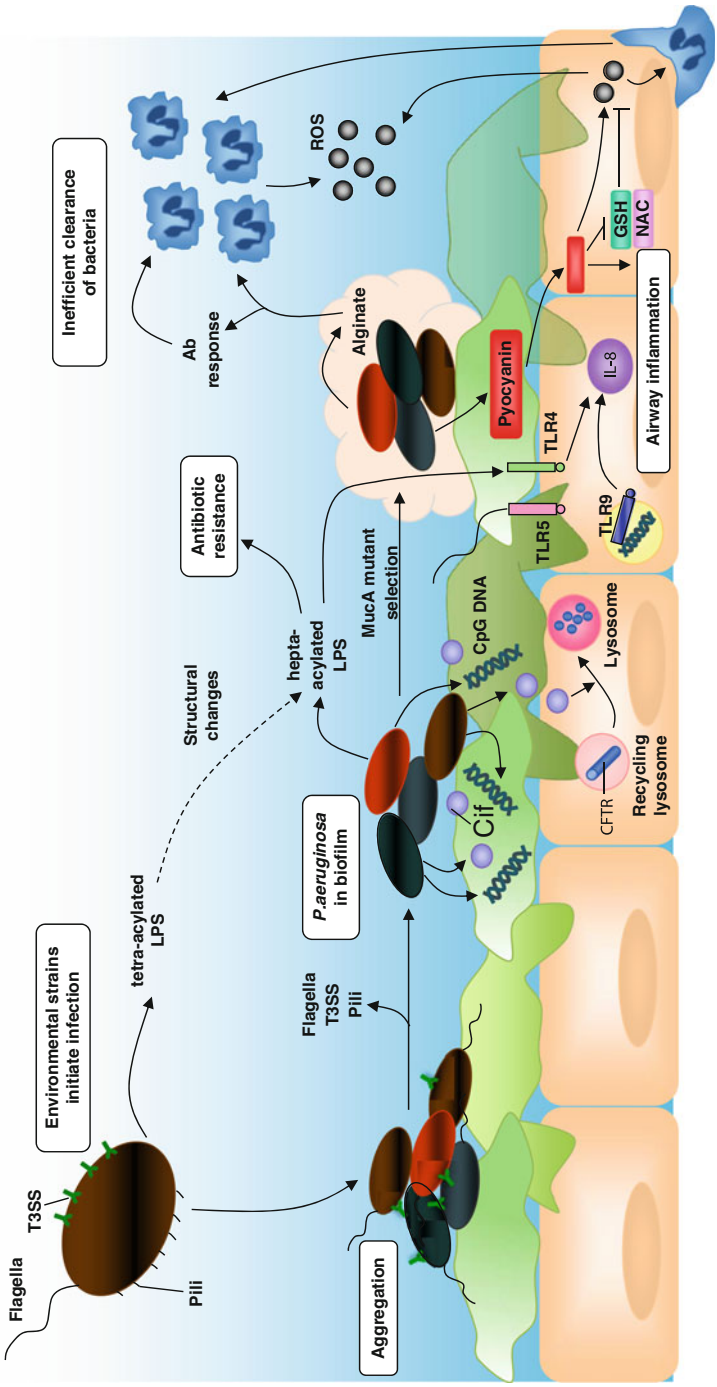

Fig. 11.1 Adaptation of *P. aeruginosa* during colonization of the airway

LPS, a major cell wall component all Gram-negative organisms, is shed from the surface of bacteria into the airway and is recognized by TLR4 on host cells (Poltorak et al. 1998). The LPS–TLR4 interaction, mediated by adaptor proteins MD2 and CD14, results in activation of NF-κB and Trif-dependent signaling pathways (Visintin et al. 2001; Rowe et al. 2006; Kagan et al. 2008). The core signaling component of LPS, lipid A, is structured in a manner such that the number and chemical composition of its acylated side chains can be modified, altering the ability of LPS to stimulate host signaling. These differences allow for variation between the immunostimulatory capacity of LPS from various Gram-negative bacteria (Gangloff et al. 1999; Zughaier et al. 1999). Bacteria mediate lipid A alterations via transcriptional regulation (PhoP and PhoQ) of PagP, PagL, and LpxO genes that are responsible for acylation, deacylation, and hydroxylation of lipid A, respectively (Kawasaki et al. 2004; Geurtsen et al. 2006). The affect of LPS structure on both acute and chronic infection has been clarified by analysis of isolates from newly infected infants with cystic fibrosis and sequential isolates from colonized patients. Comparison of the LPS produced by *P. aeruginosa* isolates from infants with cystic fibrosis (CF) or non-CF patients with sepsis or bronchiectasis demonstrated that bacteria initially infecting the lungs of CF patients predominantly had a penta-acylated LPS lipid A with additional palmitate and aminoarabinose incorporated into the side chains (Ernst et al. 1999). These additions confer protection against cationic antimicrobial peptides (CAMPs) giving these organisms a significant survival advantage (Guo et al. 1998).

Interestingly, the lipid A from CF isolates was more immunostimulatory when applied to human endothelial cells, resulting in more of an interleukin-8 response than that of non-CF isolates. The differences in signaling were also attributed to palmitate additions to the side chains. Growth of laboratory strain PAK in low Mg^{2+} media resulted in similar modifications to the side chain, indicating that these modifications could be in response to specific environmental stimuli within the CF lung.

P. aeruginosa lipid A structure not only has an affect on the acute infection, but once in the CF lung further adaptations in side chain composition promote colonization. Analysis of sequential isolates from CF patients (isolation at first positive culture, 1–5 years, and 7–16 years post colonization), confirmed to be clonal variants by pulsed-field gel electrophoresis and single nucleotide polymorphism, demonstrated an enhanced ability of the later isolates to persist within the mouse lung (Hajjar et al. 2002). While the in vivo data associated with these studies is controversial considering reports that murine TLR4 is unable to recognize alterations in lipid A, activation of NF-κB signaling in culture human epithelial cells was reduced in groups stimulated with later isolates. Analysis of the lipid A structure showed a blend of tetra- and penta-acylated lipid A in the early and mid isolates. Some samples from the mid isolate also contained hexa-acylated lipid A, while the late isolate contained primarily hepta-acylated lipid A. These modifications persisted through serial passaging. Altered lipid A in the late isolate correlated with a mutation in the *pagL* gene that is responsible for deacylation, which was also observed in an independently published study of early and chronic infection in cystic fibrosis (Ernst et al. 2007).

In addition to changes in LPS structure, *P. aeruginosa* modifies expression of other virulence factors over the course of infection. The *mucA* mutant is associated with biofim formation and increased alginate production. Alginate and specifically the gene *algT* negatively regulate flagella production through inhibition of *fleQ* expression (Garrett et al. 1999; Cobb et al. 2004; Tart et al. 2005). The result is nonmotile *P. aeruginosa* that lack flagella, but are also better able to evade immune clearance. Strains of *P. aeruginosa* lacking flagella have reduced virulence as they cannot activate TLR5 or NLRC4 signaling pathways, have an impaired ability to bind and colonize epithelial cell surfaces, and are resistant to macrophage engulfment (Saiman et al. 1990; Hazlett et al. 1991; Saiman and Prince 1993; Miao et al. 2008; Morris et al. 2009). Alternate regulation of flagella is also downregulated in colonizing infection (Mahenthiralingam et al. 1994). Flagellin and pilin synthesis are regulated by RpoN and the majority of clinical isolates from long colonized patients are RpoN negative. Complementation of RpoN on a plasmid partially restores swimming ability. Therefore it seems that the presence of flagella, a highly inflammatory virulence factor, is not required for long-term survival within the host, and a selective advantage exists for bacteria that lose flagellin expression.

Another group of virulence factors associated with strains of *P. aeruginosa* isolated from acute infections is those delivered by the TTSS, which directly injects effector proteins into host cells and interrupts host signaling processes. The TTSS needle is composed of numerous proteins including Psc, capable of activating the NLRC4 inflammasome independent of effector protein secretion. Once the pore has formed, the bacteria inject a cocktail of proteins made up of a combination of ExoU, ExoY, ExoS, and ExoT. Individual bacterial isolates do not necessarily express each effector but they have major effects on the host.

While important for bacterial dissemination during acute infections, expression of TTSS components are reduced in colonizing strains of *P. aeruginosa* (Jain et al. 2004). The bacterium is able to sense the host and regulate expression and activation of the TTSS through the RsmAYZ regulatory cascade (O'Callaghan et al. 2012). Furthermore, *mucA* is part of the regulatory pathway upstream of TTSS expression, and selection of *mucA* mutants also selects for bacteria lacking TTSS expression (Wu et al. 2004). Therefore, as with other virulence factors such as flagella, expression of TTSS needle and effector proteins is repressed facilitating the selection of mutants that evade detection by the innate immune system and eradication by the host.

11.3 *P. aeruginosa* Alters Host Protein Expression and Signaling

P. aeruginosa, in addition to changing the expression of virulence factors, can directly interact with host structural proteins and signaling pathways to promote colonization and dissemination from the airways. There are multiple consequences of the toxins delivered through the TTSS proteins directly into the cytoplasm of host

cells. More commonly expressed effectors such as ExoS or ExoT interfere with activation of the Rho GTPases preventing host cells from internalizing bacteria, promoting reorganization of key tight junction proteins, and allowing invasion of the bacteria across epithelial barriers (Geiser et al. 2001). The GTPase-activating protein domain of ExoS in addition to targeting the Rho pathway also prevents ERM protein phosphorylation preventing their interaction with the actin cytoskeleton, and disrupting epithelial barrier function (Soong et al. 2008). ExoU, a phospholipase, is only found in a small fraction of isolated bacteria, but is the most virulent of the effector proteins and is commonly associated with strains causing ventilator-associated pneumonia (Schulert et al. 2003). Following injection into host cells, it activates signaling through MAPK signaling cascades and activates transcription factor AP-1 (McMorran et al. 2003; Cuzick et al. 2006; Diaz and Hauser 2010). Furthermore, ExoU and ExoS can inhibit activation of caspase-1, limiting production of inflammasome dependent cytokines and cell death pathways (Sutterwala et al. 2007; Galle et al. 2008).

Infection of polarized epithelial monolayers is thought to require bacterial access to components of the epithelial basal membrane. During infections in which epithelial integrity is not compromised, *P. aeruginosa* is able to recruit components of the basal membrane to the apical surface of the cell (Kierbel et al. 2007). Rapidly following bacterial interaction with confluent epithelial monolayers, PI3K is recruited to the apical surface, resulting in local PIP3 production and localized increases in actin. Through a proposed vesicle transport mechanism components of the basal membrane are then transported to the apical surface without disrupting tight junction integrity. *P. aeruginosa* are then able to bind and invade epithelial monolayers at these sites. It is currently unclear how the bacterium is able to induce this localized depolarization.

Outer membrane vesicles (OMVs) of *P. aeruginosa* containing immunostimulatory bacterial components are released during infection and can directly interact with the host (Bomberger et al. 2009). OMVs are endocytosed in a lipid raft dependent manner where they localize with markers of the early endosome. One protein found in OMVs, Cif, was shown to redirect CFTR from recycling endosomes to the lysosome where it is degraded (Bomberger et al. 2011). Reduced availability of CFTR due to Cif mediated degradation can potentially result in defects in innate immunity such as those attributed to genetic mutations in this same channel although the in vivo significance of Cif activity has yet to be determined.

The accumulating literature indicates that opportunists such as *P. aeruginosa* are tremendously successful in adapting to the multiple innate immune effectors encountered in the airway. While resistance to antimicrobial agents is oft cited as the major barrier to effective eradication of these organisms the lung, it is clear that their tremendous genetic and metabolic flexibility facilitate their ability to colonize, persist, and occasionally cause invasive infection and sepsis. Preventative strategies must take into account these intricate interactions with the host and the involvement of epithelial and endothelial cells as well as the phagocytes and immune cells that target these organisms.

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