Chapter 1
Alveolar Macrophages

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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).

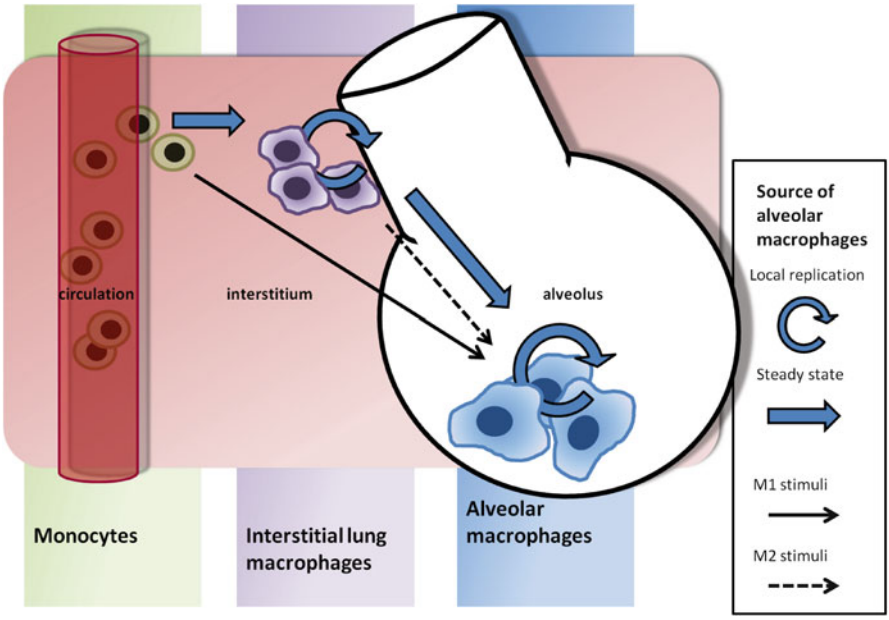

Fig. 1.1 Development and differentiation of the alveolar macrophage. Monocytes from the peripheral blood differentiate first into interstitial (parenchymal) lung macrophages and then further differentiate into alveolar macrophages. Each of these macrophage populations is capable of local replication 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

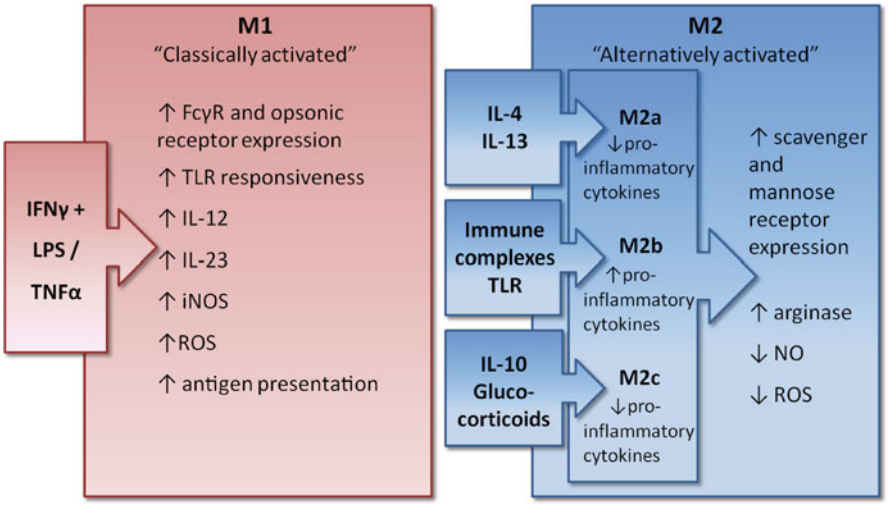

Fig. 1.2 Macrophage polarization. M1 or classical activation of macrophages is engendered by interferon γ (IFN-γ) plus either lipopolysaccharide (LPS) or tumor necrosis factor-α (TNF-α). M2 or alternatively activated macrophages can be subdivided according to the activating stimuli into M2a, M2b, and 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

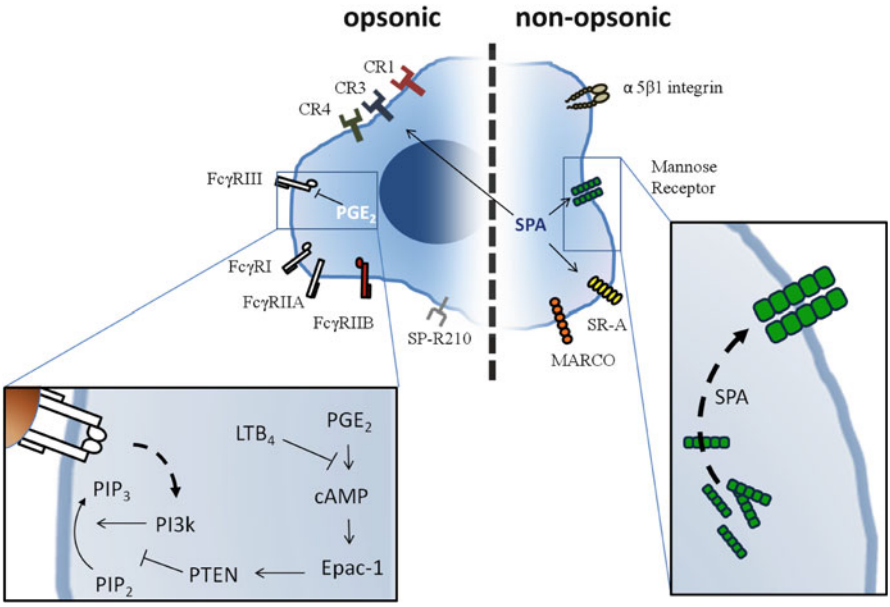

Fig. 1.3 Major phagocytic receptors of the AM. Major phagocytic receptors expressed by alveolar macrophages that are involved in recognition of extracellular bacteria (*Streptococcus pneumoniae, Staphylococcus aureus, Pseudomonas aeruginosa, Haemophilus influenzae and Klebsiella pneumoniae*) discussed 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).

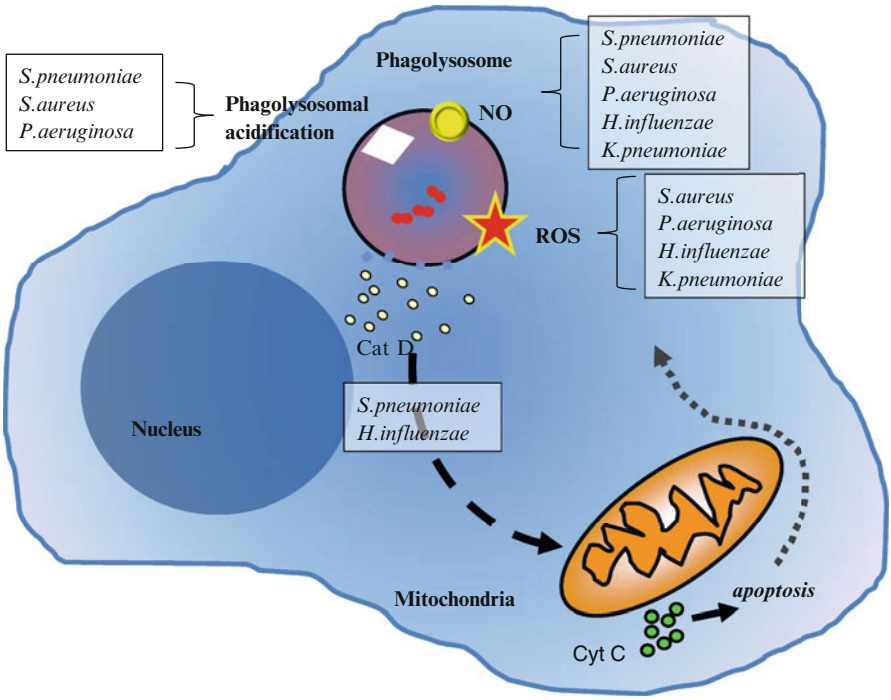

Fig. 1.4 Alveolar macrophage killing mechanisms for extracellular bacteria. Nitric oxide (NO), reactive oxygen species (ROS) and reactive nitrogen species (RNS) are generated in the AM in response to different pathogens as indicated. Phagolysosomal acidification provides an additional level of degradation 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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