Pulmonary immune response during (myco)bacterial infection
Leemans, J.C.

Citation for published version (APA):
Leemans, J. C. (2002). Pulmonary immune response during (myco)bacterial infection

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Alveolar Macrophages have a Protective Anti-Inflammatory Role during Murine Pneumococcal Pneumonia by Phagocytosing Apoptotic Neutrophils

Sylvia Knapp, Jaklien C. Leemans, Sandrine Florquin, Judith Branger, Nico A. Maris, Jennie Pater, Nico van Rooijen and Tom van der Poll
ABSTRACT

Alveolar macrophages (AM) are considered major effector cells in host defense against respiratory tract infections by virtue of their potent phagocytic properties. In addition, AM may regulate the host inflammatory response to infection by production of cytokines and by their capacity to phagocytose apoptotic polymorphonuclear cells (PMN). To elucidate the \textit{in vivo} contribution of AM to host defense against pneumococcal pneumonia we depleted mice of AM via intrapulmonal application of liposomal dichloromethylene-bisphosphonate (AM\textsuperscript{mice}) before inoculation with \textit{Streptococcus pneumoniae}; controls received saline (AM\textsuperscript{+sal}) or liposomal PBS (AM\textsuperscript{+lip}) before bacterial inoculation. AM\textsuperscript{mice} displayed a significantly higher mortality compared to AM\textsuperscript{+controls} whereas bacterial clearance did not differ. Detrimental outcome of AM\textsuperscript{mice} was accompanied by a pronounced increase of local pro-inflammatory cytokine production as well as strongly elevated and prolonged pulmonary PMN accumulation. Closer examination of infiltrating PMN in AM\textsuperscript{mice} disclosed high proportions of apoptotic and secondary necrotic cells reflecting the lack of efficient clearance mechanisms in the absence of AM. Furthermore, caspase-3 staining showed only slightly higher activity in AM\textsuperscript{mice}, arguing against accelerated apoptosis per se. These data suggest that AM are indispensable in the host response to pneumococcal pneumonia by means of their capacity to terminate inflammation via elimination of apoptotic PMN.
**INTRODUCTION**

*Streptococcus pneumoniae* is a leading causative pathogen in community acquired pneumonia (1-3). Despite adequate antimicrobial therapy, pneumococcal pneumonia remains a major cause of morbidity and mortality. Further fuelled by increasing antibiotic resistance in this pathogen, there is urgent need to expand our knowledge on pathogenetic and host defense mechanisms in *S. pneumoniae* pneumonia (2, 4).

Alveolar macrophages (AM), located at the interphase between air and lung tissue, provide the first line of cellular defense against air-borne microbials upon initial encounter (5, 6). Most *in vivo* data about the role of AM in pulmonary host defense come from studies in which AM were depleted by administration of liposome-encapsulated dichloromethylene bisphosphonate (Cl2MBP) to the pulmonary tract. Thereby, AMs traditional role as phagocyte has been demonstrated in mice challenged with *Pseudomonas aeruginosa* or *Klebsiella pneumoniae*. Mice lacking AM showed a delayed and impaired bacterial clearance as compared to control mice (7, 8), although conflicting data exist about the phagocytic properties of AM in a mouse model with unopsonized *P. aeruginosa* (9). In sharp contrast to unencapsulated bacteria, *S. pneumoniae* are known to bind poorly to macrophages without prior opsonization (10-12). Moreover, mouse AM show minimal expression of receptors for C3b/iC3b (CR1/CR3/CR4) (13), indicating that AM use other, nonopsonic phagocytosis mechanisms. Alternatively, the contribution of AM to pulmonary host defense in *S. pneumoniae* pneumonia may rely on different – so far not examined – mechanisms.

Until recently, most investigations dealing with lung inflammation concentrated on initiation events. Within the past few years more effort has been made to study mechanisms responsible for resolution of inflammation and restitution of tissue homeostasis. By this, it has become evident that persistent inflammation, leading to tissue injury and organ malfunction, may not solely rely on prolonged proinflammatory events but may equally likely arise from inefficient resolution processes (14). AM have been implicated as major effector cells in this resolution process, mainly by phagocytosing apoptotic PMN (15). The rapid elimination of extravasated, apoptotic PMN by AM may provide an injury-limiting mechanism since the membrane of PMN remains intact, preventing potential injurious granule contents to become released. As such AM can be regarded as major modulators of pulmonary host defense: upon encounter they readily
anti-inflammatory role of AM

phagocytose and eliminate certain inhaled pathogens, whereas later AM represent important effector cells in the equally important resolution process (14, 15).

To our knowledge, the putative dual function of AM, facilitating both the clearance of bacteria from and the resolution of the ensuing inflammatory response in the pulmonary compartment, has never been investigated directly in a model of bacterial pneumonia. We therefore sought to obtain insight into the in vivo contribution of AM to the host response to pneumococcal pneumonia in a well established animal model. For this purpose, mice were depleted of AM by administration of liposomal Cl2MBP prior to intranasal (i.n.) infection with S. pneumoniae. Intranasal Cl2MBP administration selectively depletes AM without damaging other cells (16). We thereby investigated AM's traditional role as phagocytes as well as their contribution to resolution processes. In this study we show for the first time, that the major role of AM in host defense in pneumococcal pneumonia relies on their ability to eliminate apoptotic PMN and to restore tissue homeostasis.

MATERIAL & METHODS

Mice
Pathogen-free 6-8 wk-old female BALB/c mice were obtained from Harlan Sprague-Dawley (Horst, The Netherlands). The Animal Care and Use Committee of the University of Amsterdam (Amsterdam, The Netherlands) approved all experiments.

In vivo AM depletion
Cl2MBP was a gift from Roche Diagnostics (Mannheim, Germany). Preparation of liposomes containing Cl2MBP was performed as described previously (16). 100μl Cl2MBP was administered intranasally (i.n.) (AM mice) 48 hrs prior to bacterial inoculation. Our laboratory previously showed that this dose results in >70% AM depletion within 2 days (17). Control-mice received 100μl liposomal PBS (AM+ lip) or saline i.n. (AM+ sal mice), respectively.

Induction of pneumonia
Pneumonia was induced as described previously (18, 19). Briefly, S. pneumoniae serotype 3 was obtained from American Type Culture Collection (ATCC 6303; Rockville, MD). Pneumococci were grown for 6 hours to midlogarithmic phase at 37°C in 5% CO2 using

72
Todd-Hewitt broth (Difco, Detroit, MI) was harvested by centrifugation at 1500 x g for 15 min, and washed twice in sterile isotonic saline. Bacteria were then resuspended in sterile isotonic saline at a concentration of approximately 5x10^4 CFUs/50 μl, as determined by plating serial 10-fold dilutions on sheep-blood agar plates. Mice were lightly anesthetized by inhalation of isoflurane (Upjohn, Ede, The Netherlands) and 50 μl (approximately 5x10^4 CFU) were inoculated intranasally.

**Determination of bacterial outgrowth**

At 20 h and 44 h after infection, mice were anesthetized with Hypnorm® (Janssen Pharmaceutica, Beerse, Belgium) and midazolam (Roche, Meidrecht, the Netherlands) and sacrificed by bleeding out the vena cava inferior. Blood was collected in EDTA containing tubes. Whole lungs were harvested and homogenized at 4°C in 4 volumes of sterile saline using a tissue homogenizer (Biospec Products, Bartlesville, OK). CFUs were determined from serial dilutions of lung homogenates and blood, plated on blood agar plates and incubated at 37°C at 5% CO₂ for 16 h before colonies were counted.

**Preparation of lung tissue for cytokine measurements or FACS**

For cytokine measurements, lung homogenates were diluted 1:2 in lysis buffer containing 300 mM NaCl, 30 mM Tris, 2 mM MgCl₂, 2 mM CaCl₂, 1% Triton X-100, and Pepstatin A, Leupeptin and Aprotinin (all 20 ng/ml; pH 7.4) and incubated at 4°C for 30 min. Homogenates were centrifuged at 1500 x g at 4°C for 15 minutes, and supernatants were stored at -20°C until assays were performed. For FACS analysis, pulmonary cell suspensions were obtained using an automated disaggregation device (Medimachine System; Dako, Glostrup, Denmark) and resuspended in FACS buffer (PBS supplemented with 0.5% BSA, 0.01% NaN₃, and 100 mM EDTA) exactly as described previously (17).

**Bronchoalveolar lavage**

The trachea was exposed through a midline incision and canulated with a sterile 22-gauge Abbocath-T catheter (Abbott, Sligo, Ireland). Bronchoalveolar lavage (BAL) was performed by instilling two 0.5 ml aliquots of sterile saline. Approximately 1 ml of lavage fluid (BALF) was retrieved per mouse. Total cell numbers were counted from each sample using a hemocytometer (Türck chamber), BALF differential cell counts were done on cytofuge preparations stained with Giemsa.
**FACS analysis**

For analysis of apoptotic PMN, BALF and lung cells were gated for PMN by forward and side scatter and stained with Annexin-V PE and 7-Amino-Actinomycin D (7-AAD). Binding to Annexin-V in Hepes buffer containing calcium was carried out to assess the exposure of phosphatidylserine. To assess plasma membrane permeability cells were exposed to 7-AAD. Annexin V positive and 7-AAD negative cells were considered apoptotic; Annexin V positive and 7-AAD positive PMN were considered necrotic (20). To correct for specific staining, an appropriate isotype control Ab was used. Staining was performed in the presence of 20% normal mouse serum to block nonspecific binding to FcγR. All reagents were purchased from Pharmingen (San Diego, CA) and used in concentrations recommended by the manufacturer. Samples were analyzed by flow cytometry using a FACScan (Becton Dickinson, San Jose, CA).

**Histologic examination**

Lungs for histologic examination were harvested at 20 h and 44 h after infection, fixed in 4% formalin and embedded in paraffin. 4 μm sections were stained with hematoxylin and eosin (H&E), and analyzed by a pathologist who was blinded for groups. Granulocyte staining was done as described previously (21). Briefly, slides were deparaffinized and endogenous peroxidase activity was quenched by a solution of methanol/0.03% H₂O₂ (Merck, Darmstadt, Germany). After digestion with a solution of pepsine 0.25% (Sigma, St Louis, MO) in 0.01M HCl, the sections were incubated in 10% normal goat serum (Dako, Glostrup, Denmark) and then exposed to FITC-labelled anti-mouse Ly-6G mAb (Pharmingen, San Diego, CA). Slides were incubated with a rabbit anti-FITC antibody (Dako) followed by a further incubation with a biotinylated swine anti-rabbit antibody (Dako), rinsed again, incubated in a streptavidin-ABC solution (Dako) and developed using 1% H₂O₂ and 3,3'-diaminobenzidin-tetra-hydrochloride (Sigma) in Tris-HCl. The sections were mounted in glycerin gelatin without counter staining and analyzed. To detect apoptotic bodies, deparaffinized slides were boiled 2 x 5 min. in citrate buffer (pH 6.0). Non-specific binding and endogenous peroxidase activity were blocked as described, followed by an incubation with rabbit anti-human active caspase 3 polyclonal antibody (Cell Signaling, Beverly, MA) followed by a further incubation with a biotinylated swine anti-rabbit antibody (Dako). The slides were further revealed as described above. All antibodies were used in concentrations recommended by the manufacturer's.
Cytokines

Cytokines and chemokines (TNF-α, KC, IL-10, IL-1β) were measured using specific ELISAs (R&D Systems, Minneapolis, MN) according to the manufacturers' instructions. The detection limits were 31 pg/ml for TNF-α and IL-10, 12 pg/ml for KC and 8 pg/ml for IL-1β.

Myeloperoxidase (MPO) assay

MPO activity was measured as described previously (22). Lung tissue was homogenized in potassium phosphate buffer, pelleted at 4,500 x g for 20 min. Pelleted cells were lysed in potassium phosphate buffer pH 6.0 supplemented with hexadecyltrimethyl ammoniumbromide (HETAB) and 10mM EDTA. MPO activity was determined by measuring the H₂O₂ dependent oxidation of 3,3',5,5'tetramethylbensidine (TMB). Briefly, serial dilutions of samples in potassium phosphate buffer were mixed with tetramethylbensidine substrate N,N’dimethylformamide. The reaction was stopped with Glacial Acetic Acid followed by OD reading at 655nm. MPO-activity is expressed as activity per gram lung tissue per reaction time. All reagents were purchased from Sigma (St.Louis, MO).

Statistical analysis

Differences between groups were calculated by Mann-Whitney U test. For survival analyses, Kaplan-Meier analysis followed by log rank test was performed. Values are expressed as mean ± SEM. A p-value ≤ 0.05 was considered statistically significant.

RESULTS

**AM** mice are more susceptible to *S. pneumoniae* pneumonia

To determine the role of AM in host defense against pneumonia we first assessed survival of mice depleted of AM (AM) and mice pre-treated with saline (AM’sal) or liposomal-PBS (AM’lip) after i.n. inoculation with 5x10⁴ *S. pneumoniae* CFU. All AM mice died within 5 days after the bacterial challenge, whereas 33% (4/12) of AM’lip (p=0.04 versus AM) and 50% (6/12) of AM’sal mice (p=0.001 versus AM) survived (Fig.1). All mice surviving 5 days appeared permanent survivors.
The increased mortality of AM mice is not related to an impaired bacterial clearance

Since AM are considered an important first line phagocytic defense against inhaled pathogens, we next sought to investigate whether the increased lethality of AM mice was related to an impaired clearance of *S. pneumoniae*. Both at 20 and 44 hours post inoculation with *S. pneumoniae* the number of CFUs in lungs was similar in all three groups (Fig. 2). Of interest, the lowest bacterial outgrowth at 44h was found in AM^- mice, although the difference with the two control groups did not reach statistical significance. In addition, blood from all 3 groups contained the same amount of bacteria at 20 and 44 hours postinoculation (data not shown). Hence, bacterial clearance was not impaired in the absence of AM and therefore can not explain differences in survival.

*AM* mice display an increased inflammatory response within the pulmonary compartment

Neutrophilic influx to the lungs as well as the production of proinflammatory cytokines and CXC-chemokines are regarded as major host defense mechanisms in bacterial pneumonia (23, 24). Via their secretory capacity, AM are thought to play a key role in the initial recruitment of PMN. We therefore investigated these early host responses to obtain insight in the mechanism by which AM^- mice are more susceptible to
pneumococcal pneumonia. The highest level of pulmonary cell recruitment was found in AM+ mice (Table 1).

Table 1: Cellular Composition of Lungs

<table>
<thead>
<tr>
<th>Lung</th>
<th>Cells/ml x10^4</th>
<th>PMN</th>
<th>Macrophage</th>
<th>Lymphocyte</th>
</tr>
</thead>
<tbody>
<tr>
<td>AM+ sal</td>
<td>150 ± 29</td>
<td>39 ± 5</td>
<td>35 ± 2</td>
<td>27 ± 5</td>
</tr>
<tr>
<td>AM+ lip</td>
<td>378 ± 108 A</td>
<td>59 ± 6 A</td>
<td>31 ± 3</td>
<td>10 ± 4 A</td>
</tr>
<tr>
<td>AM-</td>
<td>454 ± 95 A</td>
<td>86 ± 2 A,B</td>
<td>9 ± 1A,B</td>
<td>5 ± 1 A</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>44h post-infection</th>
<th>Cells/ml x10^4</th>
<th>PMN</th>
<th>Macrophage</th>
<th>Lymphocyte</th>
</tr>
</thead>
<tbody>
<tr>
<td>AM+ sal</td>
<td>633 ± 151</td>
<td>27 ± 6</td>
<td>44 ± 3</td>
<td>29 ± 4</td>
</tr>
<tr>
<td>AM+ lip</td>
<td>1285 ± 187 A</td>
<td>39 ± 4</td>
<td>42 ± 3</td>
<td>19 ± 2</td>
</tr>
<tr>
<td>AM-</td>
<td>2985 ± 422 A,B</td>
<td>65 ± 4 A,B</td>
<td>26 ± 3 A,B</td>
<td>10 ± 2 A,B</td>
</tr>
</tbody>
</table>

Data are mean ± SEM of 8 mice per group for each timepoint. Mice received 5x10^4 CFU S. pneumoniae i.n. at t=0, preceded (-48h) by i.n. saline (AM+sal), liposomes (AM+lip) or Cl2MBP (AM). Cell counts and differentials were done on whole lung suspensions as described in the Methods. A p ≤ 0.05 AM+ vs AM+sal, B p ≤ 0.05 AM+ vs AM+lip.

Differential counts of whole lung cell suspensions displayed an impressive predominance of PMN. This observation was confirmed by elevated MPO-activity in lungs of AM+ mice (p=0.0002 versus AM+sal and p=0.04 versus AM+lip) and a profoundly elevated PMN number in BALF of AM mice (p=0.002 versus AM+sal and p=0.002 versus AM+lip) (Fig. 3).

Figure 3 Increased pulmonary PMN influx in AM+ mice. A: Increased MPO activity in lungs of AM+ mice 44h after bacterial challenge. B: PMN counts obtained in BALF 44h post inoculation with S. pneumoniae. Data are from eight mice per group (mean ± SEM); * p ≤ 0.05 AM+ mice vs AM+lip, * p ≤ 0.05 AM+ vs AM+sal (A and B).

Fourtyfour hours after S. pneumoniae inoculation both groups of AM+ control mice presented a predominantly interstitial inflammatory infiltrate (Fig. 4A and C), composed of monocytes, lymphocytes and only few granulocytes (Fig 4B and D) - compatible with the clearance phase of the infection. In sharp contrast, lungs of AM mice displayed
dense and diffuse inflammatory infiltrates with focal destruction of the lung parenchyma (Fig 4E). These inflammatory infiltrates mostly composed of PMN as demonstrated by immunostaining with the mouse granulocyte marker Ly-6G (Fig 4F).

This increased PMN influx in AM+ mice was accompanied by an early and strong increase in pulmonary TNFα, IL-1β and KC levels, whereas in both AM− control groups a less impressive and less sustained elevation in these cytokines was found (Fig. 5). Of interest, lung concentrations of the anti-inflammatory cytokine IL-10 were significantly
lower in AM mice than in AM⁺ mice. Hence, together these data suggest an exaggerated inflammatory response to be responsible for differences in mortality.

**Figure 5** Pro-inflammatory cytokines predominate in AM⁺ mice. Elevated lung concentrations of the pro-inflammatory mediators TNF-α, IL-1β and KC and reduced pulmonary IL-10 levels in AM mice at 20 and 44h postinfection. Data represent mean ± SEM of eight mice per time point and group; * p ≤ 0.05 AM vs AM⁺lip, * p ≤ 0.05 AM compared to AM⁺sal.

AM-deficiency impedes the clearance of apoptotic neutrophils

The deleterious outcome of AM⁺ mice with pneumonia seems to result from prolonged and exaggerated inflammation rather than impaired bacterial clearance. Apart from participating in the first line of defense, AM have been implicated in the termination and resolution of inflammation due to their ability to phagocytose and degrade PMN that are undergoing apoptosis (14, 15). Indeed, in BALF cytopsins of AM⁺ mice, i.e. mice with intact AM and moderate but persistent pulmonary inflammation 44h post inoculation, we not only found apoptotic PMN but also AM with phagocytosed apoptotic bodies (Fig. 6). Therefore, these data indicate that the depletion of AM may impair physiological repair mechanisms including elimination of apoptotic PMN thereby promoting prolonged inflammation. If this is the case, an increase of apoptotic and/or secondary necrotic PMN should be detectable in lungs of these mice. We therefore investigated the proportion of apoptotic and necrotic PMN in BALF and whole lung suspensions.
Indeed, among the elevated number of PMN we found higher proportions of apoptotic (Annexin V positive, 7-AAD negative) PMN in BALF and lung suspensions of AM mice (Fig. 7). Moreover, the percentage of dead (Annexin V and 7-AAD double-positive) PMN in BALF was significantly higher in AM mice as compared to AM+lip and AM+sal controls. This was confirmed by histological analysis of AM lungs, showing at higher magnification a lot of shrunken cells, cellular debris and pyknotic nuclei characteristic for apoptotic bodies (Fig. 8A). These findings further support the hypothesis of deleterious effects of secondary necrosis of PMN, leading to exposure of potentially harmful and pro-inflammatory cell contents to the surrounding environment thereby exacerbating local inflammation.

Caspase 3 gets activated during early stages of apoptosis and is, as an effector protease, involved in the subsequent disassembly of the cell; active caspase 3 is therefore regarded as an early marker of cells undergoing apoptosis (25). In sharp contrast to the tremendous amount of characteristic apoptotic bodies seen by conventional H&E staining in AM mice, anti-active caspase 3 immunostaining of lung specimens showed only few positive cells (Fig. 8C). Moreover, these mice displayed only slightly elevated active caspase 3 activity compared to AM control (Fig. 8B). Therefore, not enhancement of apoptosis per se, but the dysbalance between apoptosis and rapid elimination counts for the absolute increase in early and late apoptotic cells in these animals.
AM are considered major effector cells of innate immunity, capable of participating in both the initiation and resolution process of pulmonary inflammation (14). Previous publications report the importance of AM in the rapid elimination of Gram-negative pathogens like *P. aeruginosa* and *K. pneumoniae* from the respiratory tract (7, 8), whereas others emphasized the role of AM in early PMN recruitment to the alveolar space (26). Together, these reports concentrated on AM’s role in initiating and orchestrating the immediate pulmonary host defense against invading pathogens. So far, however, evidence for the equally important regulatory role of AM in the resolution process comes from *in vitro* experiments or *in vivo* studies with intact AM using oleic acid, ozone or LPS (15, 27, 28). Therefore, to examine the vital contribution of AM to resolution of pneumonia we used a direct approach and depleted mice of AM before infection with *S. pneumoniae*.

In the present study we demonstrate the *in vivo* importance of AM as major effector cells in the resolution process of pneumococcal pneumonia. The lack of AM led to prolonged and overwhelming inflammation due to insufficient clearance of apoptotic PMN, which in turn was associated with a worsened outcome. AM’s classical role as phagocytes of invading *S. pneumoniae* seems to be of minor importance as illustrated by unaltered bacterial outgrowth in AM mice.

AM+ mice exhibited a pronounced and prolonged influx of PMN within the alveoli and interstitial space - a finding consistent with previous reports on Gram-negative pneumonia, which apparently contributed this fact to increased bacterial loads in AM
animals (7, 8). We further examined these pulmonary infiltrates and disclosed a high proportion of PMN as apoptotic or dead. Apoptosis, the process of programmed cell death, is believed to play a major regulatory role in the inflammatory response as invading PMN undergo apoptosis and are readily phagocytosed by surrounding AM. This process prevents the release of potentially toxic or immunogenic intracellular contents and thereby allows an injury-limiting elimination of potentially harmful PMN (14). Thus, the higher number of apoptotic and secondary necrotic PMN we found in AM mice is very likely the result of inefficiency of the normal resolution process in the absence of AM, thereby tipping the balance toward persistent inflammation and tissue injury.

Noteworthy, apoptosis of PMN is known to be delayed in both systemic and local inflammation, including pneumonia and ARDS (29-31). This increased PMN longevity has been attributed to factors like GM-CSF, G-CSF, IFN-γ and, to some degree, to TNF-α (31-34). In accordance, despite the high number of apoptotic/necrotic PMN in lungs of AM mice, we could not find signs of accelerated PMN apoptosis itself. In apoptosis, caspase 3 is known as a key effector protease that, once activated by initiator proteases like caspase 8 or 9, irreversibly leads to cell disassembly (25, 35). Immunostaining for active caspase 3 revealed only slightly increased numbers of positive cells in AM lungs as compared to AM+ mice. This slight elevation of caspase 3 seems almost negligible compared to the enormous number of infiltrating PMN in these mice (Fig. 4).

In addition to the unquestionable importance of rapid elimination of aged PMN, it has been shown that the uptake of apoptotic PMN induces an anti-inflammatory phenotype in macrophages, as it actively inhibits the production of IL1-β, IL-8 and TNF-α by human monocyte-derived macrophages (36-38). Local cytokine production in mice with intact AM, and therefore effective elimination of senescent PMN, relatively diminished in parallel with a partial resolution of the inflammatory response. The tremendous and prolonged elevation of pro-inflammatory cytokines in AM- mice is very likely to result from ongoing inflammation fuelled by intracellular contents released from necrotic PMN. The potential cellular source of these mediators in the absence of AM can only be speculated, but bronchial epithelial cells, alveolar epithelial cells type II as well as interstitial macrophages are well known for their secretory capacity upon stimulation with pro-inflammatory cytokines, LPS or reactive oxygen intermediates (39-42).
The exposure of AM to liposome encapsulated ClβMBP leads to selective apoptosis of AM. Both in vivo and in vitro experiments have demonstrated that PMN are morphologically and functionally unaffected by this compound (16). Our observation of unaltered bacterial clearance despite prior administration of liposomal ClβMBP supports this notion of unaffected functional properties of PMN. The intra-alveolar inflammatory reaction in response to ClβMBP itself is minimal as documented by our and other laboratories (data not shown) and (7, 17, 26, 43, 44).

Liposomes were used to encapsulate ClβMBP to facilitate uptake by macrophages. As controls, we used mice pretreated with saline (AM+sal) or liposomes (AM+lip) only. Of interest, both outcome and inflammatory responses in AM+lip mice ranged in between AM+sal and AM mice, indicating that liposomes themselves somewhat impair or influence the functional properties of AM. Indeed, liposomes can reduce the phagocytic and migratory behaviour of AM (45). Similar observations have been made in earlier studies (16, 17).

Pneumonia remains a leading cause of morbidity and mortality, and S. pneumoniae is the most frequently isolated pathogen in community-acquired pneumonia. Using a well established model of murine pneumococcal pneumonia, we here demonstrate that AM phagocytose apoptotic PMN, and that the selective depletion of AM results in an accumulation of apoptotic and necrotic PMN together with an exaggerated inflammatory response and enhanced lethality. The clearance of pneumococci from the lungs was not influenced by AM depletion. These data indicate that AM play an essential role in the regulation of the lung inflammatory response during pneumococcal pneumonia.

Acknowledgment

We are grateful to Joost Daalhuisen and Ingvild Kop for expert technical assistance and to Nike Claessen for the immunohistochemical stainings.
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