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Alveolar Macrophage Apoptosis Contributes to Pneumococcal Clearance in a Resolving Model of Pulmonary Infection[1]

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The role of alveolar macrophages (AM) in host defense against pulmonary infection has been difficult to establish using in vivo models. This may reflect a reliance on models of fulminant infection. To establish a unique model of resolving infection, with which to address the function of AM, C57BL/6 mice received low-dose intratracheal administration of pneumococci. Administration of low doses of pneumococci produced a resolving model of pulmonary infection characterized by clearance of bacteria without features of pneumonia. AM depletion in this model significantly increased bacterial outgrowth in the lung. Interestingly, a significant increase in the number of apoptotic AM was noted with the low-dose infection as compared with mock infection. Caspase inhibition in this model decreased AM apoptosis and increased the number of bacteremic mice, indicating a novel role for caspase activation in pulmonary innate defense against pneumococci. These results suggest that AM play a key role in clearance of bacteria from the lung during subclinical infection and that induction of AM apoptosis contributes to the microbiologic host defense against pneumococci. ***The Journal of Immunology,*** **2003, 171: 5380–5388.**

Streptococcus pneumoniae, the pneumococcus, is the most frequent cause of community-acquired pneumonia (1). Colonization of the nasopharynx by pneumococci occurs in up to 40% of the population (2) and microaspiration of nasopharyngeal contents is common (3, 4). Nevertheless, the development of pneumonia is relatively rare (1, 5), which reflects the efficiency of host factors in preventing the development of pneumococcal pneumonia (6). Alveolar macrophages (AM)[3] contribute to the early stages of innate defense against bacteria in the lung by phagocytosing bacteria that reach the terminal bronchioles and alveoli (7, 8). Macrophages generate antimicrobial molecules, secrete cytokines, and present Ags, hence linking innate to adaptive immunity (9). However, the exact role of AM in host defense against pneumococci in vivo is unclear.

AM phagocytosis of pneumococci and clearance of bacteria from the lung is enhanced by opsonization of bacteria (10, 11) and is particularly reliant on the classical pathway of complement activation (12). We have previously demonstrated that phagocytosis and killing of pneumococci by macrophages in vitro is associated with apoptosis induction, suggesting a role for apoptosis in the host defense against pneumococci (13). Macrophage apoptosis was first described as a pathogen-mediated response that facilitates immune evasion by intracellular pathogens such as *Shigella flexneri* and *Salmonella typhimurium* (14–16). Host-directed macrophage apoptosis can, however, limit intracellular bacterial replication of *Mycobacterium tuberculosis* (17–19) and *Chlamydia psittaci* during chronic infection (20). Intracellular organisms inhibit macrophage apoptosis to prolong intracellular replication (21, 22). Macrophage apoptosis has not however been investigated in the context of restoration of tissue homeostasis as occurs in a resolving infection with an extracellular bacterium such as the pneumococcus.

Murine models of pneumococcal pneumonia have provided insights into critical aspects of pathogenesis but have focused on fulminant models of infection characterized by extensive polymorphonuclear cell (PMN) recruitment and death (23–26). These models are very useful for antimicrobial studies (27) and the evaluation of pneumonia pathogenesis but they provide less information concerning host defense in the early stages of infection. Recently, models have been developed to address factors associated with a favorable outcome for pulmonary pneumococcal infection (28–30).

In this study, we describe the development of a low-dose pneumococcal infection model that is characterized by the clearance of bacteria without PMN recruitment, the absence of bacteremia, and the survival of mice without development of pneumonia. In this model, AM depletion results in increased bacterial outgrowth in the lung. Furthermore, AM apoptosis is a prominent feature and inhibition of AM apoptosis using caspase inhibitors is associated with the development of bacteremia.

Materials and Methods

Materials

Clodronic acid (Cl_2MDP), Hoechst 33342, saponin, and DMSO were purchased from Sigma-Aldrich (Poole, U.K.). The pan-caspase inhibitor *N*-benzyloxycarbonyl-Val-Ala-Asp(*O*-methyl) fluoromethyl ketone (zVADfmk) and the control fluoromethyl ketone, that lacks activity as a caspase inhibitor, *N*-benzyloxycarbonyl-Phe-Ala (*O*-methyl) fluoromethyl ketone (zFAfmk) were purchased from Enzyme System Products (Livermore, CA). Benzylpenicillin was purchased from Britannia Pharmaceuticals (Redhill, U.K.) and gentamicin from Roussel Laboratories (Uxbridge, U.K.). Annexin V^{PE} or allophycocyanin, Ly-6G PE (clone IA8), streptavidin-FITC, IgG2a isotype PE, and biotinylated IgG1 were obtained from

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[3] Abbreviations used in this paper: AM, alveolar macrophage; Spn, *S. pneumoniae*; PMN, polymorphonuclear cell; BMDM, bone marrow-derived macrophage; zVADfmk, *N*-benzyloxycarbonyl-Val-Ala-Asp(*O*-methyl) fluoromethyl ketone; zFAfmk, *N*-benzyloxycarbonyl-Phe-Ala(*O*-methyl) fluoromethyl ketone; i.t., intratracheal; BAL, bronchoalveolar lavage.

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BD PharMingen (Oxford, U.K.). TUNEL reagents were obtained from Intergen (Purchase, NY). Biotinylated F4/80 was obtained from BMA Biomedicals (Rheinstrasse, Switzerland). TO-PRO-3 was purchased from Molecular Probes (Leiden, The Netherlands). Diff-Quik staining was obtained from Merck (Dorset, U.K.).

Bacteria

Type 1 *S. pneumoniae* (WHO reference laboratory strain SSISP; Statens Seruminstitut) or type 2 (D39 strain, NCTC7466) were grown to mid log phase in brain-heart infusion broth (Oxoid, Basingstoke, U.K.) with 10% FCS (Autogen Bioclear, Calne, U.K.) and stored at −70ºC. All bacteria were passaged through mice by i.p. injection of 10^7 CFU, as described previously (31). Aliquots were thawed, washed, and resuspended in PBS to give either 10^4 CFU in 20 μl (low dose) or 10^7 CFU in 20 μl (high dose).

Mice

Specific pathogen-free 8- to 12-wk-old female C57BL/6 mice (Harlan, Oxford, U.K.) weighing 16–20 g were used in all experiments. Mice were allowed access to food and water ad libitum. All experiments were conducted in accordance with the Home Office Animal (Scientific Procedures) Act 1986 and ethical review by the Animal Care and Welfare Committee of the University of Sheffield.

Instillation of pneumococci

Mice were anesthetized with ketamine (100 mg/kg i.p.; Willows Francis Veterinary, Crawley, U.K.) and acepromazine (5 mg/kg i.p.; C-Vet Veterinary Products, Lancashire, U.K.). Intratracheal (i.t.) instillation of 20-μl solutions containing pneumococci or PBS alone (mock infection) was performed as previously described (32). Mice were placed in a warmed cage until they were moving freely after anesthesia and allowed to recover with access to food and water.

AM depletion

AM were depleted with liposome-encapsulated clodronate as previously described (33, 34). Mice were sedated with ketamine (80 mg/kg i.p.) and acepromazine (4 mg/kg i.p.). One hundred microliters of liposome-encapsulated clodronate, liposome-encapsulated PBS, or PBS was delivered intranasally. The mice were infected with low-dose pneumococci 48 h later.

Caspase inhibition in vivo

The pan-caspase inhibitor zVADfmk was diluted in PBS to 1 mg/ml. zFAfmk or 2% v/v DMSO in PBS was used as controls. zVADfmk (10 mg/kg i.p.) was administered at 0 and 24 h after low-dose infection (35).

Collection of bronchoalveolar lavage (BAL), blood, and lungs

Mice were killed by an overdose of i.p. sodium pentabarbitone (Loveridge, Southampton, U.K.) and exsanguination by cardiac puncture. BAL was performed as described using 5 × 0.8-ml aliquots of ice-cold heparinized saline (10 U/ml; Leo Laboratories, Princes Risborough, U.K.) (32). The total cell counts in BAL were determined by hemocytometer and differential cell counts were calculated from these using fractions for each leukocyte population estimated by analysis of cytospin preparations (Cytospin 3; Thermo Shandon, Runcorn, U.K.) stained with Diff-Quik by blinded reviewers. Blood obtained by cardiac puncture and excised lungs were placed on ice. The lungs were homogenized in 0.5 ml of PBS and bacterial numbers in blood or lungs were determined by the surface viable count method after inoculation on blood agar and speciation (23). In some experiments, unlavaged lungs were fixed via the trachea with 10% buffered Formalin at 20 cm H_2O and paraffin-embedded sections were prepared, sectioned, stained with H&E, and evaluated by a pathologist (P.G.I.) using a Zeiss Axoplan 2E microscope (Zeiss, Oberkochen, Germany).

Bone marrow-derived macrophage isolation and in vitro killing assay

Mice were killed by an overdose of i.p. sodium pentabarbitone and femurs were removed. Bone marrow was obtained by sterile aspiration of the femurs and monolayers of bone marrow-derived macrophages (BMDM) cultured as described in 24-well plates (Costar; Corning Life Sciences, Schiphol-Rijk, The Netherlands) (36). BMDM were cultured in the absence of antibiotics for at least 1 wk before infection with nonopsonized type 1 pneumococci at a multiplicity of infection = 10. Conditions of infection, measurement of intracellular bacteria, and performance of the intracellular killing assay were as described, except that extracellular bacteria were killed with 40 megaunits benzylpenicillin and 20 μg/ml gentamicin for 30 min at 37°C and BMDM were lysed by incubation with 250 μl of 2% saponin for 12 min with addition of 750 μl of PBS and vigorous aspiration (11).

Flow cytometry

Briefly, 10^5 cells were washed, incubated in 5 μl of Annexin V^{PE} and 95 μl of annexin V binding buffer (BD PharMingen) for 30 min at 4°C, washed, and resuspended in binding buffer containing 100 nM TO-PRO-3. Cells were analyzed on a FACSCalibur flow cytometer (BD PharMingen) and 10,000 events were recorded. The annexin V-negative population was identified by incubating a negative control as above with 5 mM EDTA. Analysis was with CellQuest software (BD PharMingen) and cells that were annexin V^+/TO-PRO-3^- were recorded as apoptotic. In selected samples, staining was performed with 100 μg mouse IgG1 for 15 min at 4°C, washing, and sequentially incubating samples with 1 μg anti-Ly6G-PE, biotinylated anti-F4/80, and streptavidin-FITC secondary Ab (or appropriate isotype Abs) for 15 min at 4°C, and then annexin V-allophycocyanin and TO-PRO-3 as above. In the low-dose infection model, >95% of all BAL cells were AM ($F4/80^+$ and $Ly6G^-$). In the high-dose infection model, AM could be separated from the PMN and monocytes by Ab staining ($F4/80^+$ and $Ly6G^-$) in combination with forward/side scatter characteristics (37).

Detection of apoptosis in cytospins or lung tissue

Quantification of apoptotic cells (condensed or fragmented nuclei) on cytospins was performed as previously described by two blinded reviewers who analyzed >300 cells per cytospin (38). In selected cytospins, samples were incubated with anti-F4/80 or Ly6G as above, incubated with 2 μM Ho33342, and viewed with a confocal microscope (Leica TCS SP2 system; Leica, Deerfield, IL). Apoptosis in lung sections was determined by deparaffinizing sections as previously described and staining with TUNEL reagents as per the manufacturer's instructions (39).

Statistics

Statistical analysis was by Fisher's exact test, Mann-Whitney *U*, Friedman, or Kruskal-Wallis nonparametric analysis with Dunn's post tests as appropriate. Survival was calculated using Kaplan-Meier followed by log rank analysis. In all cases, differences were considered significant if $p < 0.05$.

Results

Establishment of a low-dose infection model

Direct instillation of pneumococci into the lung via the endotracheal route has previously been associated with delivery of >95% of the infecting inoculum into the lung (23). We confirmed these findings by demonstrating that after i.t. instillation of 10^4–10^8 CFU of pneumococci and immediate removal of the lungs, we recovered 95% (range, 90–109%) of the infecting inoculum from homogenized lungs ($n = 6$).

We therefore performed experiments using i.t. instillation of 10^4 CFU (low dose) or 10^7 CFU (high dose) of pneumococci and analyzed microbiologic outcomes in mice. As shown in Fig. 1*A*, 24 h after instillation of the low dose of type 1 pneumococci, the majority of mice had no detectable pneumococci in the lungs. In contrast after high-dose infection, the majority of mice had detectable bacteria in the lungs. Similar results were obtained with type 2 pneumococci. In addition, the majority of mice were not bacteremic 24 h after low-dose infection in contrast to the situation after high-dose infection, irrespective of the type of pneumococci administered (Fig. 1*B*). When mice were studied from 0 to 192 h after low-dose infection, we found that there was a rapid decrease in the percentage of mice with detectable bacteria in the lung from 0 to 6 h after infection (Fig. 1*C*). The median CFU detected in the lungs also decreased rapidly in this period (data not shown) and no mouse had detectable bacteria in the lung 96–192 h after low-dose infection. There was no significant difference in survival of low-dose infected mice in comparison to mock-infected mice, as shown for type 1 pneumococci in Fig. 1*D*. The majority of mice demonstrated no sign of illness following low-dose infection. In contrast, following high-dose infection with either type 1 or 2 pneumococci,

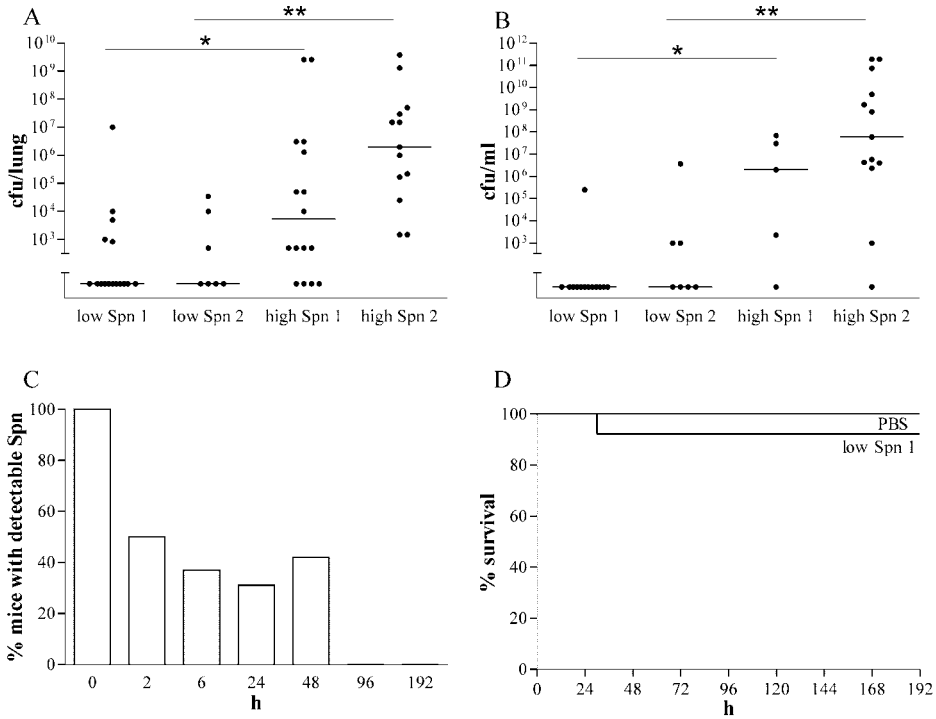

FIGURE 1. Characterization of microbiologic features of i.t. instillation of low and high inocula of pneumococci. *A*, Bacteria in lung homogenates 24 h after i.t. instillation of 10^4 CFU type 1 or 2 (low Spn 1, low Spn 2) or 10^7 CFU type 1 or 2 (high Spn 1, high Spn 2) pneumococci, low Spn 1 vs high Spn 1, $p < 0.05$ (*); low Spn 2 vs high Spn 2, $p < 0.01$ (**), Kruskal-Wallis with Dunn's post test. Bacteria were undetectable in the lungs of 69% of mice infected with low Spn 1 compared with 25% infected with high Spn 1 ($p = 0.026$) and 57% of mice infected with low Spn 2 compared with 0% infected with high Spn 2 ($p = 0.007$, Fisher's exact test). *B*, Bacteria in blood 24 h after infection, low Spn 1 vs high Spn 1, $p < 0.05$ (*); low Spn 2 vs high Spn 2, $p < 0.01$ (**), Kruskal-Wallis with Dunn's post test. Ninety-two percent of the mice were abacteremic following infection with low Spn 1 compared with 20% following high Spn 1 ($p = 0.008$) and 57% of the mice following infection with low Spn 2 compared with 8% following high Spn 2 ($p = 0.031$, Fisher's exact test). *C*, The percentage of mice with detectable bacteria in the lung 2–192 h after infection with low Spn 1 ($n = 4$–16) for each time point. *D*, Cumulative survival after instillation of low Spn 1 or mock infection (PBS; $n = 8$ in each group); low Spn 1 vs PBS ($p = 0.134$, log rank analysis).

all mice died by 3 days after infection and all were bacteremic at the time of death (data not shown).

Characterization of the inflammatory response in low- and high-dose infection models

In contrast to high dose, low-dose infection resulted in no difference in PMN numbers in the BAL fluid at 24 h as compared with mock-infected mice (Fig. 2*A*). This result was not altered if infection involved type 2 pneumococci as opposed to type 1. The low-dose infection was not characterized by any pathologic features of pneumonia (Fig. 2*B*). There were only subtle changes in comparison to mock-infected mice, with evidence of bronchial epithelial reaction and cellular hyperplasia but no PMN recruitment. We confirmed that pneumonia could be established in this mouse strain since high doses of pneumococci induced features of bronchopneumonia, with evidence of PMN accumulation in the airways and interstitial spaces. The number of PMN in the BAL was unaltered by the timing of BAL collection after low-dose infection. PMN recruitment was undetectable at any time point from 2 to 192 h after infection with low doses of pneumococci (Fig. 2*C*).

AM contribute to host defense after low-dose infection

Having established a model of resolving infection, we next examined how AM depletion altered the outcome of infection with low doses of pneumococci. Mice treated with liposome-encapsulated clodronate had a 68% reduction in AM numbers compared with PBS-treated mice 72 h after instillation ($p < 0.001$, Fig. 3*A*). Macrophage numbers were similar in mice treated with PBS or liposome-encapsulated PBS. AM depletion resulted in a significant decrease in clearance of bacteria from the lung at 24 h. The majority of infected mice that received intranasal PBS had undetectable numbers of bacteria in the lung 24 h after infection, but this was not true for mice after AM depletion (Fig. 3*B*). In keeping with the observation that liposomes decrease the efficiency of phagocytosis by AM (40), bacterial clearance was also less efficient after pretreatment with liposome-encapsulated PBS, although these mice had normal numbers of AM, but bacterial clearance was significantly greater than in the AM-depleted group. In association with decreased clearance of bacteria, AM-depleted mice recruited significantly greater numbers of PMN (Fig. 3*C*). This was neither a nonspecific effect of AM depletion (AM-depleted mice that were mock infected did not recruit significant numbers of PMN) nor of the liposomes themselves (mice treated with liposome-encapsulated PBS and infected with pneumococci did not recruit significant numbers of PMN). Failure to clear bacteria at 24 h in AM-depleted mice was not associated with decreased survival (Fig. 3*D*), as subsequent recruitment of PMN was associated with elimination of bacteria and survival of mice in the majority of infections.

Increased numbers of apoptotic cells are a feature of pneumococcal infection

To investigate whether the number of apoptotic cells was altered in the lung following low-dose pneumococcal infection, we analyzed the number of TUNEL-positive cells in lung sections 24 h after infection of mice (Fig. 4*A*). The number of TUNEL-positive cells was significantly increased in the lungs of mice infected with low-dose pneumococci (Fig. 4*B*). To further quantify the number of

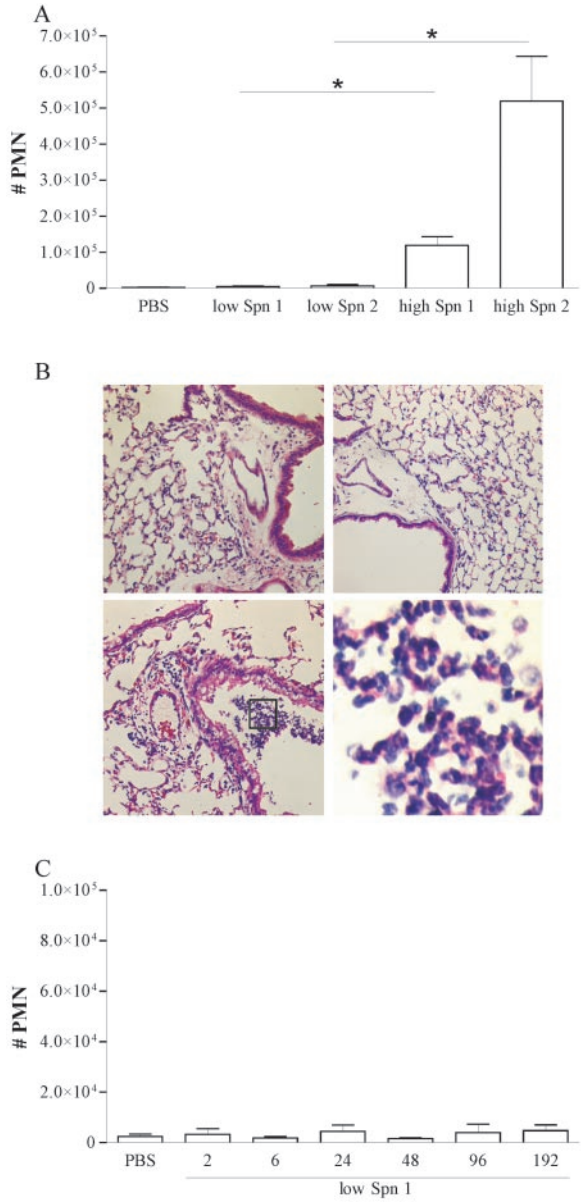

FIGURE 2. Characterization of inflammatory features in the lung of low- and high-dose pneumococcal infection. *A*, Absolute numbers of PMN (#PMN) in BAL 24 h after i.t. instillation of 10^4 CFU type 1 or 2 (low Spn 1, low Spn 2) or 10^7 CFU type 1 or 2 (high Spn 1, high Spn 2) pneumococci or mock infection (PBS, $n = 9$), mean + SEM. Low Spn 1 ($n = 7$) vs high Spn 1 ($n = 10$), $p < 0.05$ (*); low Spn 2 ($n = 7$) vs high Spn 2 ($n = 9$), $p < 0.05$ (*), Kruskal-Wallis with Dunn's post test. *B*, Representative appearances of lung sections stained with H&E from mice 24 h after i.t. instillation of PBS (*top left*), low Spn 1 (*top right*), or high Spn 2 (*bottom left*), all ×10 objective. An enlargement of the marked area of inflammation from high Spn 2 is shown *bottom right* (×40 objective) to demonstrate PMN recruitment. *C*, #PMN in BAL 2–192 h after instillation of low Spn 1 ($n = 3$–9 for each time point, mean + SEM).

apoptotic cells, cytospins obtained from BAL fluid were reviewed and apoptotic cells were recorded (Fig. 4*C*). Increased numbers of apoptotic cells were first apparent 6 h after low-dose infection and were apparent until 4 days after infection, after which numbers were similar to those of mock-infected mice (data not shown). There were significant increases in apoptotic events in the BAL fluid at 24 h after both low-dose and high-dose infection but the absolute number of apoptotic events was significantly greater following high-dose infection (Fig. 4*D*).

AM apoptosis is a feature of pneumococcal pneumonia

Apoptotic cells were detected in the alveolar units of the lung and pathologic evaluation suggested that many of these cells were AM (Fig. 5*A*). To confirm the identity of the apoptotic cells, we analyzed cytospins of BAL fluid costained with Ho33342 (to detect nuclear features of apoptosis) and anti-F4/80 (to detect a surface marker expressed by murine macrophages including AM) (37). As shown in Fig. 5*B*, fragmented apoptotic nuclei were detected in $F4/80^+$ cells. To quantify the numbers of apoptotic AM, BAL fluid was examined by flow cytometry. A combination of F4/80 and Ly6G (as a marker of PMN) staining combined with forward vs side scatter characteristics allowed us to identify the macrophage population in BAL fluid after low-dose or high-dose infection, and apoptosis was quantified in this population. As shown in Fig. 5, *C* and *D*, percentage of AM apoptosis was significantly increased following low-dose infection as compared with mock infection and following high-dose infection as compared with mock infection. This suggested increased numbers of apoptotic events were not the result of altered numbers of AM. Similar results were obtained using mice infected with type 2 pneumococci (mean percentage of apoptosis ± SEM); mock infection, 1.9 ± 0.4%; low dose, 5.1 ± 1.2%; and high dose, 17.0 ± 4.1%, low-dose type 2 vs mock infection, $p = 0.03$, and high dose vs mock infection, $p < 0.001$, Mann-Whitney *U* test ($n = 7$–11/group). Furthermore, since >95% of the cells in BAL fluid at all time points after low-dose infection were $F4/80^+$ and had the forward/side scatter characteristics of mature macrophages, these results confirm that the apoptotic cells seen in cytospins after low-dose infection represent apoptotic AM.

Caspase inhibition causes an increase in bacteremia

Increased AM apoptosis was a feature of low-dose infection, even though this model was characterized by bacterial clearance in the absence of PMN recruitment. We therefore investigated whether inhibition of apoptosis modified bacterial clearance. zVADfmk is a cell-permeable pan-caspase inhibitor that inhibits many forms of apoptosis in a variety of cell types including those of monocyte lineage (41). We have previously confirmed that it can inhibit pneumococcal-associated macrophage apoptosis in vitro (13). It inhibits apoptosis in the lung in vivo in experimental models of apoptosis in rodents (42). We confirmed that treatment with zVADfmk significantly inhibited AM apoptosis in vivo, as detected by analysis of flow cytometry of BAL fluid obtained 24 h after low-dose infection (Fig. 6*A*). These findings were replicated on cytospins (data not shown). In an intracellular killing assay, caspase inhibition decreased the killing of pneumococci by BMDM, as compared with treatment with a control zFAfmk (Fig. 6*B*), but did not alter the level of bacterial internalization (data not shown). There was no statistical difference between clearance in the zFAfmk vs the control group that received neither zVADfmk nor zFAfmk. Although caspase inhibition had only a modest effect on bacterial clearance in the lung (Fig. 6*C*), it significantly increased the percentage of bacteremic mice 24 h after low-dose infection (Fig. 6*D*). zVADfmk-treated mice showed other signs of altered course of infection. Mortality for zVADfmk-treated mice was twice that of controls, 33% vs 17%, although this did not reach statistical significance ($p = 0.13$, Fig. 6*E*). However, mild symptoms of respiratory distress, transient hunching, and piloerection were observed in surviving mice, features that were never observed in the control group.

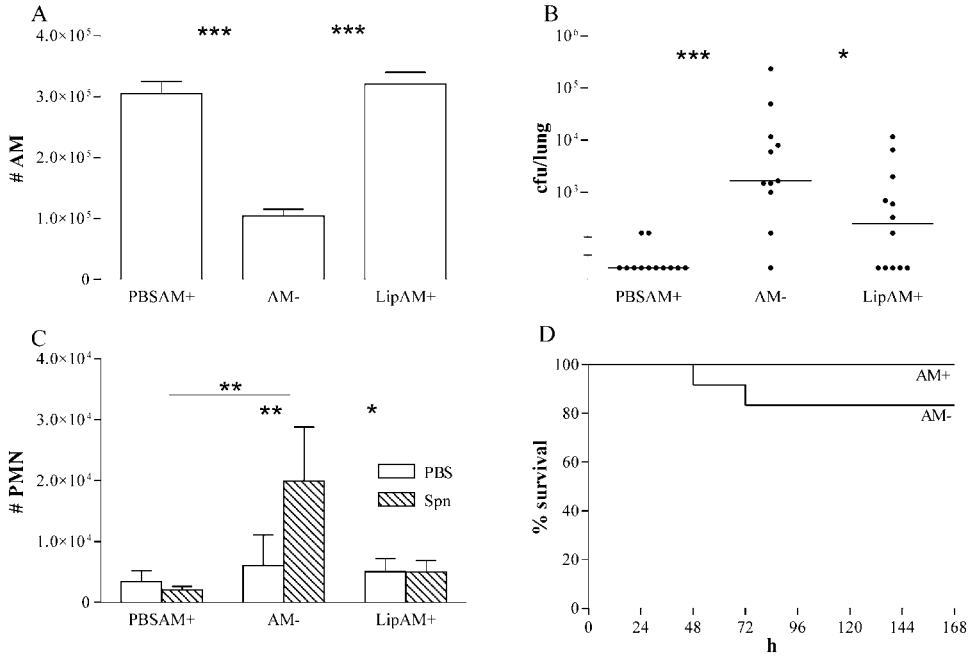

FIGURE 3. AM depletion modifies the course of low-dose pneumococcal infection in the lung. *A*, Absolute number of AM (# AM) in BAL 72 h after intranasal instillation of PBS ($PBSAM^+$), liposome-encapsulated clodronate (AM^-), or liposome-encapsulated PBS ($LipAM^+$), mean + SEM. #AM was reduced by 68% in AM^- compared with $PBSAM^+$. AM^- ($n = 19$) vs $PBSAM^+$ ($n = 19$), $p < 0.001$ (***); AM^- vs $LipAM^+$ ($n = 19$), $p < 0.001$ (***), Kruskal-Wallis with Dunn's post test. *B*, Bacteria in lung homogenates 24 h after i.t. instillation of 10^4 CFU type 1 pneumococci (Spn) in $PBSAM^+$, AM^-, or $LipAM^+$. Bacteria were undetectable in the lung of 81% of the $PBSAM^+$ group, 9% of the AM^- group, and 42% of the $LipAM^+$ group. AM^- vs $PBSAM^+$, $p < 0.001$ (***); AM^- vs $LipAM^+$, $p < 0.05$ (*), Kruskal-Wallis with Dunn's post test. *C*, Absolute numbers of PMN (# PMN) in BAL 24 h after i.t. instillation of low-dose Spn 1 (Spn) or PBS in $PBSAM^+$, AM^-, or $LipAM^+$ (mean + SEM). AM^-/Spn vs AM^-/PBS, $p < 0.01$ (**); AM^-/Spn vs $PBSAM^+$/Spn, $p < 0.01$ (**); AM^-/Spn vs $LipAM^+$/Spn, $p < 0.05$ (*), Kruskal-Wallis with Dunn's post test. *D*, Cumulative survival after infection with low-dose Spn 1 in $PBSAM^+$, AM^-, or $LipAM^+$ ($n = 12$ in each group). $PBSAM^+$ and $LipAM^+$ had identical 100% survival (AM^+), which was not significantly different from survival in the AM^- group ($p = 0.135$, log rank analysis).

Discussion

In vivo model of resolving infection

We have developed a unique murine model of resolving pneumococcal infection. In this model, we accurately instill a small dose of pneumococci mimicking the aspiration of pneumococci into the lung, an early step in pneumonia pathogenesis (5). In contrast to models of fulminant infection that induce pneumonia and death (24–26), C57BL/6 mice effectively clear this small dose of

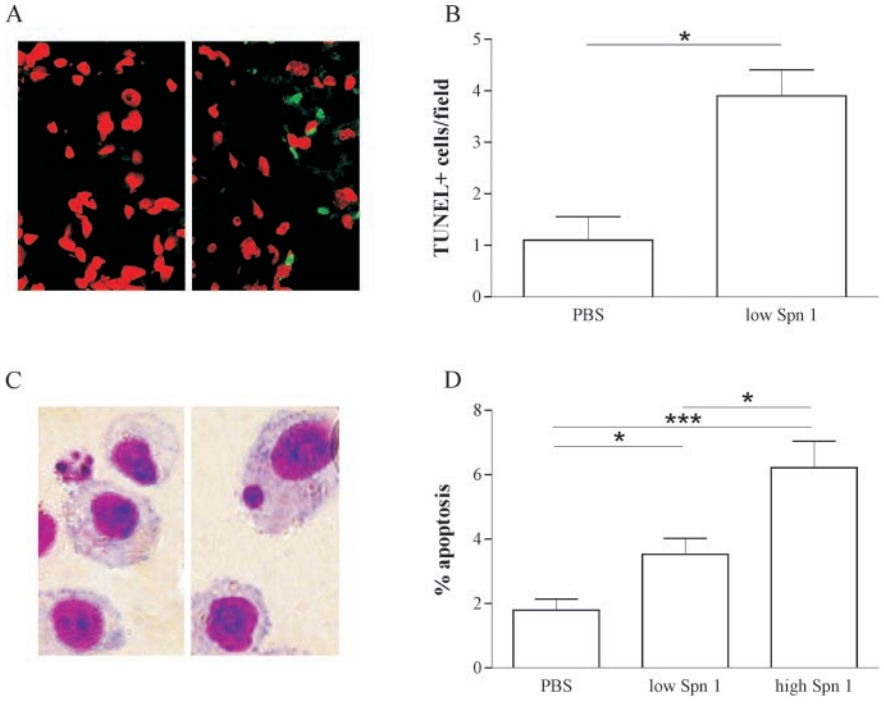

FIGURE 4. Infection with pneumococci is associated with increased numbers of apoptotic cells in the lung. *A*, Representative lung sections (×40 objective) stained by TUNEL and propidium iodide from mice 24 h after mock infection (PBS, *left panel*) or infection with 10^4 CFU type 1 pneumococci (low Spn, *right panel*); apoptotic $TUNEL^+$ cells appear green. *B*, Quantification of the number of $TUNEL^+$ cells in lung sections from low Spn 1 or PBS 24 h after infection. Mean + SEM $TUNEL^+$ cells per lung field (×100 objective), low Spn 1 vs PBS, $p < 0.05$ (*), Mann-Whitney *U* test; results are representative of three experiments. *C*, Cytospins obtained from the BAL 24 h after infection with low Spn 1 demonstrate apoptotic cells (*left panel*) and apoptotic bodies in macrophages (*right panel*). *D*, Total number of apoptotic events (apoptotic cells and bodies) in cytospins of BAL from mice 24 h after instillation of low-dose type 1 (low Spn 1, $n = 9$), high-dose type 1 (high Spn 1, $n = 6$) pneumococci or mock infection (PBS, $n = 8$), mean + SEM. PBS vs low Spn 1, $p < 0.05$ (*); PBS vs high Spn 1, $p < 0.001$ (***); low Spn 1 vs high Spn 1, $p < 0.05$ (*), Kruskal-Wallis with Dunn's post test.

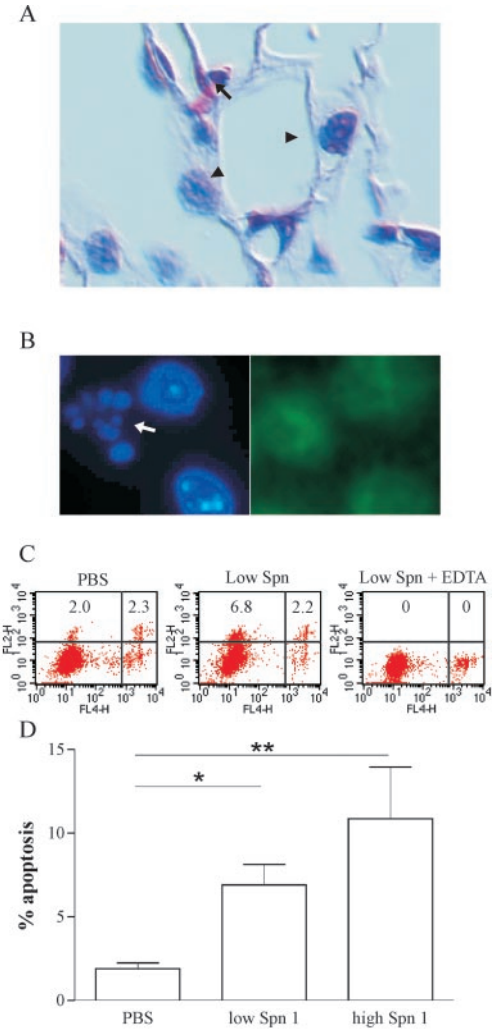

FIGURE 5. Increased numbers of apoptotic macrophages are found in the lung after infection with pneumococci. *A,* Representative appearance of lung sections stained with H&E from mice 24 h after i.t. instillation of 10^4 CFU type 1 pneumococci (low Spn 1) showing an apoptotic macrophage (arrow) and normal macrophages (arrowheads) viewed by differential interference contrast microscopy (×40 objective). *B,* Cytospin of BAL 24 h after low Spn 1 infection stained with F4/80 FITC and Ho33342. Macrophages show positive F4/80 staining (green cells), one of which demonstrates a fragmented nucleus by Ho33342 staining (arrow). *C,* Representative dot plots depicting percentage of macrophage apoptosis in BAL 24 h after i.t. instillation of PBS or infection with low-dose Spn. FL2 represents Annexin V^{PE} and FL-4 TOP-RO-3 staining. Apoptotic cells were Annexin V^{PE+}/TO-PRO-3^- and the percentage of apoptotic cells are recorded in the *upper left quadrant*. Cells treated with EDTA during staining are shown in the *right-hand panel* as a negative control for annexin V binding. *D,* Percentage of macrophage apoptosis (Annexin V^{PE+}/TO-PRO-3^-, flow cytometry) in BAL 24 h after i.t. instillation of low Spn 1 ($n = 11$), 10^7 CFU type 1 pneumococci (high Spn 1, $n = 10$), or mock infection (PBS, $n = 11$). Mean + SEM, PBS vs low Spn 1, $p < 0.05$ (*); PBS vs high Spn 1, $p < 0.01$ (**), Kruskal-Wallis with Dunn's post test.

pneumococci. C57BL/6 mice are susceptible to pneumococcal pneumonia as they develop illness when the infecting inoculum increases (29). Moreover, resolving infection is not unique to the type 1 pneumococcal strain, since type 2 pneumococci, a commonly used strain in murine pneumonia models (30, 43), produce similar results. We conclude that the innate immune response to small numbers of pneumococci in the lung involves rapid clearance of bacteria over the first 6 h after infection in the absence of PMN recruitment. We have therefore used this model of resolving infection to investigate the contribution of AM to innate defense against pneumococci.

Role of AM in host defense against pneumococci

AM are the resident phagocytes of the lung and play a critical role in the innate defense against bacteria (7, 8, 11). Previous studies have investigated the effect of AM depletion, using liposome-encapsulated clodronate, on the host response to pulmonary infections (34, 44–47). These studies have mostly focused on fulminant or chronic infections and have provided insights into the role of AM in regulating the inflammatory response in established infection. In general, they have not examined the role of AM in the prevention of pneumonia. One study has investigated depletion of AM in a low-dose infection model that involved administration of 100 CFU of *Klebsiella pneumoniae* and documented a decrease in bacterial clearance (47).

The only previous study investigating AM depletion in pneumococcal infection found AM depletion did not alter bacterial clearance (45), which is in contrast to our observations. In this study by Knapp and colleagues (45), mice developed pneumonia with extensive PMN recruitment. The authors made the novel observation that AM depletion decreased clearance of PMN and resulted in greater inflammatory lung injury and mortality. Reasons for the differences between the studies include their use of a model of fulminant as opposed to resolving infection and differences in instillation technique (intranasal vs i.t.). Furthermore, the previous study used mice from a different genetic background, BALB/c, which are relatively resistant to pneumococcal pneumonia and recruit much greater numbers of PMN to the lung than do C57BL/6 mice following pneumococcal challenge (29). BALB/c mice demonstrate a Th2 response, as opposed to the Th1 response that has been demonstrated for C57BL/6 mice, and this is associated with an M2 macrophage phenotype as opposed to an M1 phenotype in C57BL/6 mice (48). The M1 phenotype is characterized by greater NO and IFN-γ production (48) and IFN-γ is important in host defense against pneumococci in murine models (43). The C57BL/6 low-dose model is likely to be more dependent on AM function and does not require PMN recruitment to prevent development of pneumonia, whereas the BALB/c model is more dependent on PMN recruitment and may, therefore, better address the role of AM in regulating the inflammatory response to established pneumonia (45).

Treatment with PBS-liposomes decreased bacterial clearance from the lung, as compared with mice that received no liposomes. PBS-liposomes did not effect AM numbers in BAL and the nonspecific effect of liposomes on bacterial clearance is likely to reflect decreased phagocytosis by AM (40). Nevertheless, there was a significant decrease in bacterial clearance in AM-depleted mice as compared with PBS-liposome-treated mice, which confirmed the specific effect of AM depletion on bacterial clearance. In our model, AM depletion delayed bacterial clearance but did not prevent it. Although AM were not all depleted, this finding suggests that other components of the host response, including PMN, compensate for decreased AM numbers. This was the case for AM depletion in the model involving *K. pneumoniae* infection (47). At higher bacterial numbers, antibacterial AM functions are overwhelmed and PMN phagocytosis and killing of bacteria then predominates. AM depletion will effectively decrease the size of the inoculum required to overcome AM host defense, leading to recruitment of PMN. In established pneumonia, the principal role of AM is in recruitment and regulation of PMN (45).

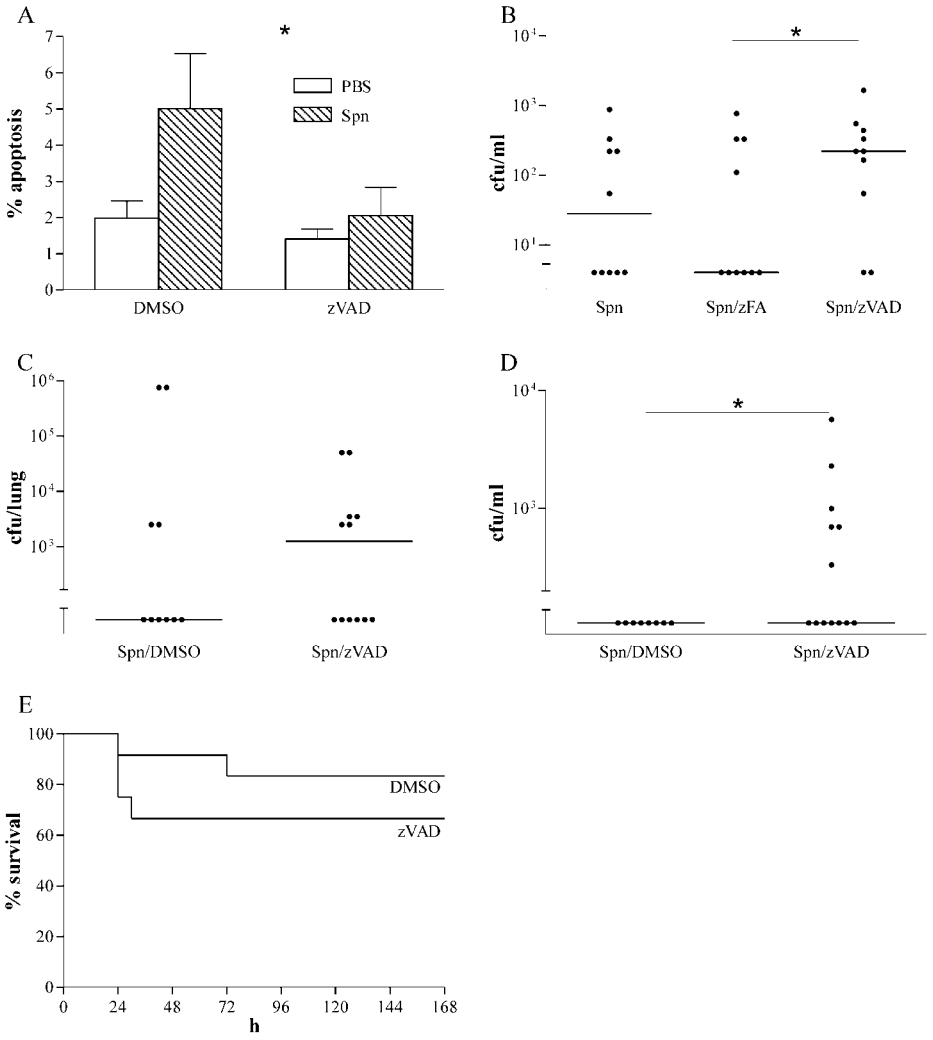

FIGURE 6. Inhibition of macrophage apoptosis is associated with increased bacteremia after low-dose pneumococcal infection. *A*, Percentage of apoptotic AM in BAL ($AnnexinV^{PE+}$/TO-PRO-3^{-}, flow cytometry) 24 h after infection with 10^4 CFU type 1 pneumococci (Spn) or mock infection (PBS) and treatment with zVADfmkfmk (zVADfmk) or vehicle control (2% DMSO, $n = 7$–9/group). Mean + SEM, Spn/DMSO vs Spn/zVADfmk, $p < 0.05$, Mann-Whitney *U* test (∗). *B*, Bacterial killing at 6 h by BMDM infected in vitro with type 1 pneumococci in the absence or presence of zVADfmk or zFAfmk (zFAfmk), zVADfmk vs zFAfmk, $p < 0.05$, Friedman test with Dunn's post test comparison (∗). *C*, Bacteria in lung homogenates 24 h after i.t. instillation of Spn and i.p. 2% DMSO or zVADfmk. *D*, Bacteria in blood 24 h after infection and i.p. 2% DMSO or zVADfmk. One hundred percent of the Spn/DMSO group were abacteremic compared with 54% of the Spn/zVADfmk group, $p = 0.046$, Fisher's exact test (∗). *E*, Cumulative survival after infection with Spn and i.p. DMSO or zVADfmk ($n = 12$ mice/group). There was no significant difference in survival, two deaths in the DMSO group compared with four in the zVADfmk group ($p = 0.13$, log rank analysis).

Macrophage apoptosis in pneumococcal infection in vivo

Although macrophage apoptosis is an in vivo feature of a number of infectious diseases (14), apoptosis of AM has not been previously described in pneumococcal pneumonia. Increased neuronal and microglial apoptosis are recognized features of pneumococcal meningitis in vivo in animal models (49, 50). Furthermore, enhanced levels of lymphocyte apoptosis have been noted in humans with pneumococcal infection (51). In addition, elevated levels of PMN and epithelial cell apoptosis have been described following in vitro pneumococcal infection (52, 53). We have previously demonstrated that macrophage apoptosis is a feature of in vitro infection with pneumococci and is associated with phagocytosis and intracellular killing of bacteria (13).

In most cases, macrophage apoptosis in association with bacterial infection has been viewed as a pathogen-mediated strategy for immune evasion (14, 15). In contrast, some intracellular bacteria gain a survival advantage by actively inhibiting macrophage apoptosis (17, 21, 22). This strategy permits more prolonged intracellular replication and bacterial persistence. It has been suggested that, to counteract this effect, the host response may include apoptosis induction to limit bacterial replication (54). Evidence for this has been provided by *M. tuberculosis* infection in which macrophage apoptosis contributes to decreased bacterial replication (18, 19). Complete eradication of bacteria is not achieved in these models and the exact role of macrophage apoptosis has been difficult to establish in view of bacterial persistence. In addition, only a minority of these studies have specifically examined AM apoptosis (19). We now present in vivo evidence of AM apoptosis in association with complete clearance of an extracellular bacterium, the pneumococcus. When considered together with evidence that macrophage apoptosis in vitro is associated with efficient intracellular killing of pneumococci (13), these data suggest a key role for AM apoptosis in the elimination of low inocula of bacteria.

Classic studies of the pathology of pneumococcal pneumonia have highlighted the importance of resolution of the acute inflammatory response in disease pathogenesis (55, 56). In particular, studies by Lord (55) outlined the importance of enzymatic factors contained in the cellular exudates to resolution of inflammation and demonstrated that this process was optimal under conditions in which bacteria were killed. PMN apoptosis is recognized as a requirement for resolution of the inflammatory response in the lung (57) and limits proinflammatory functions of PMN (58). Macrophages, unlike PMN and monocytes, are resistant to constitutive apoptosis (59) but can become apoptotic following phagocytosis of pneumococci in vitro (13) and in vivo. In addition to bacterial killing, induction of AM apoptosis may represent a mechanism by which proinflammatory signals, generated by the interaction of factors such as pneumococcal cell wall with Toll-like receptors, are down-regulated (5, 60). AM apoptosis could, therefore, also play a critical role in limiting nonspecific lung injury and resolution of the inflammatory response in vivo (54) as previously demonstrated for PMN apoptosis (57).

Effect of caspase inhibition on microbiologic outcome in vivo

To test the hypothesis that AM apoptosis contributes to host defense by enhancing clearance of pneumococci from the lung, we inhibited AM apoptosis using a caspase inhibitor (zVADfmk) that is an effective inhibitor of pneumococcal-induced macrophage apoptosis in vitro (13). Caspase inhibition markedly inhibited AM apoptosis in vivo. In addition, it inhibited in vitro killing of pneumococci by BMDM and was associated with a marked increase in the number of mice that developed bacteremia. Although clinical signs of illness occurred at greater frequency in zVADfmk-treated mice, overall survival was not adversely effected. This paradox may be explained by studies of caspase inhibitors in sepsis, where zVADfmk inhibits lymphocyte apoptosis, a feature of pneumococcal sepsis, and has been linked to improved survival (35, 51, 61). This may explain why an increased incidence of bacteremia did not result in a significant increase in mortality despite signs of illness in the mice. Although zVADfmk would also inhibit PMN and epithelial cell apoptosis, we found no evidence of significant numbers of apoptotic PMN or epithelial cells on BAL or by histology at any time point after pneumococcal infection.

This contrasts with a murine model of *Pseudomonas aeruginosa* infection in which epithelial cell apoptosis enhanced bacterial clearance, another model in which apoptosis induction improves microbiologic outcome (62). The importance of epithelial cell apoptosis in the clearance of *P. aeruginosa* has recently been further highlighted. Human bronchial epithelial cells or mice expressing mutant genes such as Δ*F508* or *G551D* for the cystic fibrosis transmembrane conductance regulator demonstrate decreased levels of epithelial cell apoptosis in response to acute *P. aeruginosa* infection, as compared with wild-type cells (63). The authors suggest decreased epithelial cell apoptosis may contribute to chronic *P. aeruginosa* infection and pulmonary inflammation in cystic fibrosis.

Caspase inhibition had no effect on PMN recruitment to the lung and no effect on phagocytosis of pneumococci by macrophages. It is also unlikely that the effects of caspase inhibition resulted from inhibition of cytokine processing. Although caspase-1 activation is implicated in IL-1β (64) and IL-18 processing (65), caspase-1 is not essential for IL-1β production in the lung following LPS challenge (38). In addition, IL-1β is not essential for host defense in murine models of pneumococcal pneumonia (66). We cannot completely exclude the possibility that caspase inhibition decreased IL-18 production, but one of the principal effects of a lack of IL-18 in murine models of pneumococcal pneumonia is enhanced PMN recruitment, which we did not observe (67). Therefore, the major effect of caspase inhibition in this model was inhibition of AM apoptosis.

In summary, we describe a murine model of resolving pneumococcal infection in which AM regulate clearance of bacteria. AM apoptosis was a prominent feature of resolving bacterial infection and was inhibited in vivo by caspase inhibition. Decreased macrophage apoptosis was associated with a predisposition to the development of bacteremia, suggesting a novel role for caspase activation and potentially AM apoptosis, in the innate immune response to pneumococcal pneumonia. This function is likely to be a critical host defense against the subclinical infection that results from aspiration of small numbers of pneumococci that commonly colonize the nasopharynx. In addition, AM apoptosis is likely to contribute to tissue homeostasis during pneumococcal infection. This stresses the importance of a more complete understanding of the pathogenesis of common human infections to improve therapeutic strategies and hence clinical outcomes in an era of increasingly limited antimicrobial options.

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