Alveolar Macrophage Apoptosis–associated Bacterial Killing Helps Prevent Murine Pneumonia

Julie A. Preston^{1,2*}, Martin A. Bewley^{1,2*}, Helen M. Marriott^{1,2*}, A. McGarry Houghton^{3,4}, Mohammed Mohasin⁵, Jamil Jubrail⁶, Lucy Morris^{1,2}, Yvonne L. Stephenson^{1,2}, Simon Cross^{1,2,7}, David R. Greaves⁸, Ruth W. Craig⁹, Nico van Rooijen¹⁰, Colin D. Bingle^{1,2}, Robert C. Read^{11,12}, Timothy J. Mitchell¹³, Moira K. B. Whyte^{6,14}, Steven D. Shapiro¹⁵, and David H. Dockrell^{6,16}

¹The Florey Institute for Host-Pathogen Interactions and ²Department of Infection, Immunity and Cardiovascular Disease, University of Sheffield Medical School, Sheffield, United Kingdom; ³Clinical Research Division, Fred Hutchinson Cancer Research Center, and ⁴Division of Pulmonary and Critical Care Medicine, University of Washington, Seattle, Washington; ⁵Department of Biochemistry and Molecular Biology, University of Dhaka, Dhaka, Bangladesh; ⁶MRC Centre for Inflammation Research, ¹⁴Department of Respiratory Medicine, and ¹⁶Infection Medicine, University of Edinburgh, Edinburgh, United Kingdom; ⁷Sheffield Teaching Hospitals, Sheffield, United Kingdom; ⁸Sir William Dunn School of Pathology, University of Oxford, Oxford, United Kingdom; ⁹Department of Pharmacology and Toxicology, Geisel School of Medicine at Dartmouth, Hanover, New Hampshire; ¹⁰Department of Molecular Cell Biology and Immunology, VU University Medical Center, Amsterdam, the Netherlands; ¹¹University of Southampton Medical School, Southampton, United Kingdom; ¹²National Institute for Health Research Southampton Biomedical Research Centre, Southampton, United Kingdom; ¹³Institute of Microbiology and Infection, School of Immunity and Infection, University of Birmingham, Birmingham, United Kingdom; and ¹⁵Division of Pulmonary, Allergy and Critical Care Medicine, University of Pittsburgh Medical Center, Pittsburgh, Pennsylvania

ORCID ID: 0000-0002-2718-4861 (D.H.D.).

Abstract

Rationale: Antimicrobial resistance challenges therapy of pneumonia. Enhancing macrophage microbicidal responses would combat this problem but is limited by our understanding of how alveolar macrophages (AMs) kill bacteria.

Objectives: To define the role and mechanism of AM apoptosis–associated bacterial killing in the lung.

Methods: We generated a unique CD68.hMcl-1 transgenic mouse with macrophage-specific overexpression of the human antiapoptotic Mcl-1 protein, a factor upregulated in AMs from patients at increased risk of community-acquired pneumonia, to address the requirement for apoptosis-associated killing.

Measurements and Main Results: Wild-type and transgenic macrophages demonstrated comparable ingestion and initial phagolysosomal killing of bacteria. Continued ingestion (for ≥ 12 h) overwhelmed initial killing, and a second, late-phase microbicidal response killed viable bacteria in wild-type macrophages, but this

response was blunted in CD68.hMcl-1 transgenic macrophages. The late phase of bacterial killing required both caspase-induced generation of mitochondrial reactive oxygen species and nitric oxide, the peak generation of which coincided with the late phase of killing. The CD68.hMcl-1 transgene prevented mitochondrial reactive oxygen species but not nitric oxide generation. Apoptosis-associated killing enhanced pulmonary clearance of *Streptococcus pneumoniae* and *Haemophilus influenzae* in wild-type mice but not CD68.hMcl-1 transgenic mice. Bacterial clearance was enhanced *in vivo* in CD68.hMcl-1 transgenic mice by reconstitution of apoptosis with BH3 mimetics or clodronate-encapsulated liposomes. Apoptosis-associated killing was not activated during *Staphylococcus aureus* lung infection.

Conclusions: Mcl-1 upregulation prevents macrophage apoptosis–associated killing and establishes that apoptosis-associated killing is required to allow AMs to clear ingested bacteria. Engagement of macrophage apoptosis should be investigated as a novel, host-based antimicrobial strategy.

Keywords: macrophage; apoptosis; pneumonia; bacteria; Mcl-1

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*These authors contributed equally to this work.

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Correspondence and requests for reprints should be addressed to David H. Dockrell, M.D., Medical Research Council/University of Edinburgh Centre for Inflammation Research, 47 Little France Crescent, Edinburgh EH16 4TJ, UK. E-mail: david.dockrell@ed.ac.uk.

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At a Glance Commentary

Scientific Knowledge on the

Subject: The exact mechanisms used by alveolar macrophages (AMs) to kill extracellular bacteria remain unclear.

What This Study Adds to the

Field: We have generated a novel transgenic mouse with AMs, which overexpresses the antiapoptotic factor Mcl-1, a molecule that is overexpressed in several patient groups at increased risk of pneumonia, which demonstrates that this transgenic mouse has a reduced capacity to clear bacteria from the lung. Apoptosis-associated killing is activated when initial phagolysosomal mechanisms are exhausted and requires a combination of reactive species, including nitric oxide and mitochondrial-derived reactive oxygen species. Reengaging apoptosis when it is deficient, using pharmacological approaches, helps prevent pneumonia in these murine models.

Community-acquired pneumonia (CAP), commonly caused by *Streptococcus pneumoniae* (the pneumococcus) and other bacteria, is a leading cause of global mortality (1). The plasticity of bacterial genomes challenges vaccination and facilitates antimicrobial resistance (2). Pathogenic bacteria frequently colonize the upper airway, but CAP is relatively uncommon, indicating efficient host responses protect most individuals.

Tissue macrophages, such as alveolar macrophages (AMs), are key effectors of antibacterial host defense (3), but the mechanisms used to kill extracellular bacteria after their internalization are incompletely defined. AMs kill

ingested bacteria in phagolysosomes, but this mechanism is less efficient than in other phagocytes. Tissue macrophages usually do not express myeloperoxidase (4) or the microbicidal serine proteases seen in neutrophils (5), and are less reliant on nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-dependent reactive oxygen species (ROS) generation (6). Nitric oxide (NO) generation in human macrophages is also less vigorous than in rodent cells or monocytes (7, 8). Moreover, pneumococci and other bacterial pathogens frequently express genes that inhibit phagolysosomal killing (9). Prolonged intracellular killing of bacteria is associated with macrophage apoptosis in human macrophages and in murine pneumonia models (3, 10). Although inhibition of apoptosis reduces bacterial killing in these murine models it has not been demonstrated if cell-autonomous macrophage apoptosis mediates pathogen clearance (3, 11). Recently, we have found that a key regulator of macrophage apoptosis during bacterial killing, the antiapoptotic protein Mcl-1 (11) is upregulated in AMs from patients at increased risk of CAP due to chronic obstructive pulmonary disease (COPD) or HIV-1 infection, where it is associated with reduced AM apoptosis and bacterial killing *ex vivo* (12, 13). Reengaging microbicidal responses downstream of apoptosis restored bacterial killing in COPD AMs (12), but whether apoptosis reconstitution in the presence of overexpression of Mcl-1 restores bacterial killing is unknown.

To test whether macrophage cell autonomous overexpression of the human Mcl-1 transgene, as observed in these patients at increased risk of CAP, modulates bacterial clearance, we generated transgenic mice that specifically express CD68.hMcl-1 in

macrophages, because the viability of these cells is closely linked to expression of this antiapoptotic protein (11, 14). We used this novel transgenic line with controlled infections and interventions to define the role, microbicidal mechanism, and potential for therapeutic reengagement of macrophage apoptosis-associated bacterial killing. Our findings show that macrophage apoptosis represents a second late phase of bacterial killing, which is activated after initial lysosome-mediated mechanisms are exhausted upon sustained bacterial uptake. Apoptosis-associated bacterial killing requires mitochondrial ROS (mROS), which act in combination with NO. This microbicidal mechanism was inhibited in the presence of CD68.hMcl-1, but was restored by BH3 mimetics or bisphosphonates.

Methods

Generation of CD68.hMcl-1 Transgenic Mice

A 1.5-kb fragment containing the cDNA sequence for human Mcl-1 (15) was cloned into a plasmid containing 2.9 kb of the CD68 promoter with the first intron enhancer IVS (16) (Figure 1A). Correct orientation and PCR mismatches were confirmed by sequencing. The transgene was isolated by restriction enzyme digestion and gel purification. The transgene was microinjected into C57Bl/6J oocytes (Washington University School of Medicine). Founders and their progeny were genotyped by PCR amplification of tail or ear biopsy DNA using the following primers: 5'-ACCATCTCCTCTCTGCCA AA-3' and 5'-GGGCTTCCATCTCCT CAA-3'. Two CD68.hMcl-1 mice transgenic lines were established. Both lines showed germline transmission and equivalent functional results. Mcl-1 transgenic mice and nontransgenic

Author Contributions: J.A.P., M.A.B., and H.M.M. contributed equally to this work and generated figures. J.A.P. made and validated the transgenic mouse and performed *in vivo* infections. M.A.B. performed killing assays and flow cytometry, collected data, and produced figures. H.M.M. performed *in vivo* experiments involving bone marrow transplantation and neutrophil depletion, and designed and conducted experiments involving therapeutic targeting. A.M.H. helped design and conduct experiments to generate the transgene construct. D.R.G. designed the CD68 construct. R.W.C. designed the Mcl-1 construct. C.D.B. helped in design of the targeting vector and experiments to evaluate its expression. M.M. designed and performed confocal microscopy experiments. J.J. performed experiments with *Staphylococcus aureus*. L.M. performed analysis of bone marrow-derived macrophage phenotype. Y.L.S. and S.C. performed histopathology. N.v.R. provided expertise in liposome experiments. R.C.R. and T.J.M. helped design infection models. J.A.P., M.A.B., H.M.M., M.K.B.W., S.D.S., and D.H.D. designed and conceived the experiments. J.A.P., M.A.B., H.M.M., M.K.B.W., D.R.G., R.W.C., and D.H.D. wrote the manuscript with input from all other authors.

Figure 1. Macrophages from CD68.hMcl-1 transgenic mice express human Mcl-1 (hMcl-1) and have selective resistance to apoptosis. (A) Schematic representation of the transgene construct. hMcl-1 expression is driven in macrophages by 2.9 kb of the CD68 promoter and Intron 1 (IVS-1). (B–D) Western blot analysis of human (h) and murine (m) Mcl-1 protein expression in bone marrow–derived macrophages (BMDMs) (B), alveolar macrophages (C), and peritoneal macrophages (D) from CD68.hMcl-1 nontransgenic (non-Tg) or transgenic (Tg) mice. (E) Peripheral blood neutrophils and splenic CD19⁺ B lymphocytes (CD19⁺) or CD3⁺ T lymphocytes (CD3⁺) were isolated from non-Tg or Tg mice. Cells were lysed and probed by Western blot for hMcl-1 protein expression. Blots are representative of three independent experiments. The positive control (+ve) is human monocyte-derived macrophage lysate. (F and G) BMDMs from non-Tg or Tg mice were irradiated with ultraviolet (UV) radiation and assessed for nuclear fragmentation at the indicated time points ($n = 4$, *** $P < 0.001$, two-way ANOVA) (F) or caspase 3/7 activation by measuring relative luminescence units (RLU) at 8 hours after UV treatment or in untreated negative controls (–ve) ($n = 4$, * $P < 0.05$, two-way ANOVA) (G). Data are represented as mean $\pm$ SEM. (H) Alveolar macrophages (AMs) from non-Tg or Tg mice were left untreated (negative) or UV treated. At 8 hours after UV exposure, apoptosis was assessed by nuclear fragmentation ($n = 4$ –5, *** $P < 0.001$, two-way ANOVA). Data are represented as mean $\pm$ SEM. See also Figure E1.

littermates, from the same cages, were inoculated with bacteria and samples collected as described previously (3) and in the online supplement.

Bacteria

Details on the bacteria and culture conditions are provided in the online supplement.

Isolation and Culture of Macrophages and Other Leukocytes

Bone marrow–derived macrophages (BMDMs), resident AMs, peritoneal macrophages, peripheral blood neutrophils, B cells, and T cells were obtained from

C57Bl/6J mice (17, 18). Human monocyte-derived macrophages (MDMs) were from whole blood donated by healthy volunteers (10). Further details are available in the online supplement.

Flow Cytometry and Confocal Microscopy

Further details on flow cytometry and confocal microscopy experiments are available in the online supplement.

Intracellular Killing Assay

Assessment of intracellular bacterial viability was performed as previously

described (19), and as outlined in the online supplement.

Reconstitution of Apoptosis

Apoptosis was reconstituted *in vitro* with clodronate-encapsulated liposomes or the indicated BH3 mimetics and *in vivo* AM apoptosis was reconstituted in transgenic mice using clodronate containing liposomes or the indicated BH3 mimetics (further information in the online supplement), with instillation of bacteria at the same time to ensure induction of early-stage apoptosis, but not macrophage depletion (20).

Ethics

Animal experiments were conducted in accordance with the Home Office Animals (Scientific Procedures) Act of 1986, authorized under a U.K. Home Office License 40/3251 with approval of the Sheffield Ethical Review Committee. MDMs were isolated from healthy volunteers with written informed consent and approval from the South Sheffield Regional Ethics Committee.

Statistical Analysis

Results are recorded as mean and SEM. Sample sizes were informed by SE obtained from similar assays in prior publications (10, 11). D'Agostino-Pearson normality tests guided test selection. Comparisons between two conditions were performed using a paired or unpaired *t* test for parametric data, or a Mann-Whitney *U* test or Wilcoxon signed rank test for nonparametric data using Prism 6.0 software (GraphPad Inc.). Comparisons between three or more conditions were performed using a normal or repeated measures one-way ANOVA with Bonferroni *post hoc* test for parametric data, or a Friedman test with Dunn's multiple comparison *post hoc* test for nonparametric data. When two or more conditions were assessed in two experimental groups, data were analyzed by two-way ANOVA with Bonferroni's *post hoc* test. Significance was defined as *P* less than 0.05.

Results

CD68.hMcl-1 Transgenic Mice Demonstrate Reduced Macrophage Apoptosis

A CD68 promoter construct (21) ensured macrophage-specific human Mcl-1 (hMcl-1) expression, to generate a transgenic mouse with selective apoptosis resistance in macrophages (Figure 1A). Equivalent functional results were generated from both founder lines. Macrophage-specific hMcl-1 expression was documented in AMs and other macrophage lineages in CD68.hMcl-1 transgenic mice (Figures 1B–1D), but not neutrophils or lymphocytes (Figure 1E). CD68.hMcl-1 transgenic mice lacked a gross developmental phenotype or loss of fertility and showed normal survival. CD68.hMcl-1 mice had normal numbers of leukocyte subsets in blood and of

macrophages in tissues, whereas splenic lymphoid tissue and lung parenchyma showed no histological abnormalities (Figures E1A–E1F in the online supplement).

Importantly, the transgene reduced susceptibility to apoptosis in BMDMs (CD68.hMcl-1⁺ BMDMs), AMs, and peritoneal macrophages (Figures 1F–1H and E1G). In contrast, CD68.hMcl-1⁺ BMDMs demonstrated no decrease in binding or ingestion of latex beads or in the numbers of viable intracellular bacteria present after 4 hours of exposure to *S. pneumoniae* (a marker of early ingestion and killing [10]; Figures E1H and E1I). The generation of ROS and NO was also unaltered by the transgene at 4 hours (Figures E1J and E1K). Mcl-1 thus did not alter initial innate immune responses.

Mcl-1 Expression Regulates Apoptosis-associated Killing When Phagolysosomal Bacterial Killing Is Exhausted

Exposure of macrophages to pneumococci for 16–20 hours results in apoptosis without a loss of membrane integrity (22). Importantly, this was inhibited in the presence of the CD68.hMcl-1 transgene, which acted at the level of the mitochondrial execution of the program of apoptosis (Figures E2A–E2D). The transgene also increased survival of ingested bacteria (Figure 2A), although it had no effect on lysosomal acidification, lysosomal membrane permeabilization, or activation of the lysosomal protease cathepsin D, which occurs upstream of the mitochondrial apoptotic program after pneumococcal challenge (11, 14) (Figures E2E–E2G). To prove that reduction of macrophage apoptosis was the mechanism by which Mcl-1 inhibited bacterial killing, apoptosis was reconstituted with the BH3 mimetic, ABT-737. Although ABT-737 cannot reverse the antiapoptotic effect of Mcl-1 directly, it interacts with Bcl-2/Bcl-X_L, displacing proapoptotic Bcl-2 proteins to stimulate apoptosis (23). ABT-737 increased the number of cells with loss of inner mitochondrial transmembrane potential and nuclear fragmentation in CD68.hMcl-1⁺ BMDMs exposed to pneumococci (Figures E3A and E3B), and restored apoptosis-associated intracellular bacterial killing (Figure 2B), whereas additional BH3 mimetics also increased bacterial killing at

20 hours, but not at 4 hours (Figures E3C and E3D).

To dissect the role of apoptosis-associated killing in host defense, BMDMs were “pulsed” with bacteria and the kinetics of killing of internalized bacteria were measured after antimicrobial “chase” to remove extracellular bacteria. The chase does not itself alter internalized bacteria, because the cell membrane remains intact at this early stage of apoptosis (22), and ensures that changes in viable bacteria are the result of intracellular killing, but not continued phagocytosis. We identified two phases of intracellular killing. An initial phase, occurring immediately after bacterial ingestion (Figure 2C), was consistent with phagocytosis-associated phagolysosomal killing (24), and was similar in transgenic and nontransgenic BMDMs. A late phase of intracellular killing occurred at 16–20 hours in nontransgenic BMDMs, but was blunted in CD68.hMcl-1⁺ BMDMs. This late phase coincided with the onset of apoptosis (Figure 2D), but occurred before any reduction in macrophage cell numbers (Figure 2E). By varying the duration of the pulse, we confirmed that the initial phase of bacterial killing was sustained for up to 12 hours, and that, after it ceased, the late phase of killing cleared viable internalized bacteria (Figure 2F). Overall, the CD68.hMcl-1 transgene inhibited the late phase of bacterial killing without any impact on early ingestion or killing.

We tested whether bacterial ingestion was compromised after 12-hour or greater exposure to bacteria, as this would remove the stimulus for phagolysosomal killing. We investigated this using a second pulse with a distinct strain of bacteria (Figure 2G). Bacterial internalization occurred at 12 hours or greater in the presence or absence of the Mcl-1 transgene, but was accompanied by continued intracellular killing only in CD68.hMcl-1[−] BMDMs. Sustained bacterial ingestion activated a late phase of killing, requiring apoptosis induction, killing viable internalized bacteria.

mROS Is Required for Apoptosis-associated Bacterial Killing

An inhibitor of NOS2 reduced both the late phase of bacterial killing and apoptosis in nontransgenic BMDMs (Figures 3A and 3B). This suggested that NO generation

Figure 2. Apoptosis-associated killing ensures intracellular bacterial clearance when canonical phagolysosomal killing is exhausted. (A) Bone marrow-derived macrophages (BMDMs) from CD68.hMcl-1 nontransgenic (non-Tg) or transgenic (Tg) mice were mock infected or challenged with serotype 2 *Streptococcus pneumoniae* (Spn) at a multiplicity of infection (MOI) of 10 and intracellular colony-forming units assessed 20 hours after infection, $n = 18$, $**P < 0.01$, unpaired Student's t test. (B) Non-Tg or Tg BMDMs were challenged with Spn in the presence (+) or absence (-) of ABT-737. At 20 hours after challenge, cells were assessed for intracellular colony-forming units ($n = 9$, $***P < 0.001$, two-way ANOVA). (C) Non-Tg or Tg BMDMs were challenged with Spn at an MOI of 10 for 4 hours and extracellular bacteria removed ("pulse-chase" design). BMDMs were lysed for initial assessment of colony-forming units or incubated in vancomycin until the designated time point when colony-forming units were also determined ($n = 4$, $**P < 0.01$, two-way ANOVA). (D and E) BMDMs were challenged with Spn at an MOI of 10. At the designated time after challenge, levels of apoptosis (D)

contributed to bacterial killing, but occurred upstream of apoptosis, consistent with its known role in sensitizing mitochondria to apoptosis induction (19). An antioxidant also inhibited late phase bacterial killing, although it did not affect apoptosis induction. This suggested that ROS are also required for this phase, but if related to apoptosis are a consequence rather than a cause. Because NADPH oxidase does not contribute to macrophage apoptosis-associated killing during pneumococcal infection (25), we addressed another source of antimicrobial ROS, mROS (26).

Increased mROS were apparent after 16–20 hours of bacterial exposure, but were reduced by the Mcl-1 transgene (Figure 3C). Peak generation of mROS coincided with peak NO production in human MDM (Figure E4A) and mROS/NO colocalized with bacteria (Figures E4–E6). Because mROS generation was a late response contemporaneous with apoptosis onset, we tested whether caspase 3 activation, which inhibits mitochondrial electron transport complex I and II, contributed to mROS generation (27). Caspase activation increased mROS production, and, consistent with the role of Mcl-1 in limiting caspase activation, the caspase 3/7⁺ population was expanded in nontransgenic versus transgenic BMDMs and produced significantly more mROS after 20 hours of exposure to bacteria (Figure 3D). Comparable findings were observed in human MDM (Figure 3E). Crucially, an inhibitor of mROS, mitoTEMPO, blocked the late phase of pneumococcal killing in CD68.hMcl-1⁺ (but not CD68.hMcl-1⁻) BMDMs (Figure 3F) and also in human MDM (Figure 3G). Inhibition of mROS did not modify BMDMs apoptosis induction or Mcl-1 expression (Figures 3H and 3I), consistent with mROS acting downstream of apoptosis in bacterial killing. Because mROS also activates proinflammatory cytokine expression (28), we confirmed that differential cytokine expression was not contributing to differences in late

microbicidal responses (Figures E7A–E7C). Because pneumococci intrinsically resist oxidative stress (29), our results suggest that apoptosis-associated killing requires caspase-dependent mROS generation, combined with NO, to mediate bacterial killing.

Apoptosis-associated Killing Is Required for Bacterial Clearance *In Vivo*

We next addressed the role of apoptosis-associated bacterial killing by AMs *in vivo*. Initially we used a low dose of pneumococci, which AMs clear efficiently, an intermediate dose, which represents the “tipping point” at which AMs start to fail to control infection and where any increase in dose or perturbation of macrophage function results in development of pneumonia, and a high dose, where AMs are overwhelmed and mice develop systemic infection (3, 30). CD68.hMcl-1⁺ transgenic mice failed to clear the low dose of pneumococci (10⁴ cfu) by 24 hours, whereas nontransgenic mice cleared all bacteria (Figure 4A) (3). Only transgenic mice developed bacteremia at the low dose (Figure 4B). At intermediate doses, CD68.hMcl-1⁺ transgenic mice also exhibited increased bacterial colony-forming units in lungs and blood compared with nontransgenic mice. Crucially, the transgenic mice had significant neutrophil recruitment in BAL at this intermediate dose, a feature of pneumonia, whereas the nontransgenic animals had no neutrophil recruitment (Figure 4C). AM numbers were not altered by low-dose infection or transgene expression, and were only reduced in the high-dose infection in transgenic mice in association with high levels of inflammatory cell recruitment (Figure 4D). The bacterial clearance that occurred in nontransgenic animals was completely overwhelmed, as expected, at an inoculum of 10⁷ cfu macrophages (3). Along with reduced bacterial clearance and an increased requirement for neutrophil recruitment, CD68.hMcl-1⁺ transgenic mice exhibited reduced AM apoptosis in

BAL after bacterial challenge (Figure 4E). To exclude any role for low numbers of lung neutrophils in the differential clearance of bacteria, we repeated low-dose bacterial challenge after neutrophil depletion and again confirmed reduced bacterial clearance in the transgenic mice (Figure E8). Overall, this proved that the transgene reduced bacterial clearance and also the threshold at which neutrophils were recruited to control bacteria in the lung, but only during the specific stages where AMs are the major effector of bacterial clearance in the lung.

CD68.hMcl-1⁺ transgenic mice also exhibited impaired clearance of low doses of *Haemophilus influenzae*, another respiratory pathogen (31). At high doses, infection progressed to pneumonia with pulmonary neutrophil recruitment in all mice, because AM clearance capacity was overwhelmed (Figures 4F and 4G). Reduced bacterial clearance at low doses was also associated with reduced macrophage apoptosis in the BAL (Figure 4H). Apoptosis-associated killing likewise contributed to bacterial clearance at extrapulmonary sites: CD68.hMcl-1⁺ transgenic mice given a low peritoneal dose of *S. pneumoniae* showed impaired peritoneal clearance of bacteria, increased numbers of bacteria in blood, enhanced neutrophil numbers, and reduced macrophage apoptosis (Figures 4I–4L).

Reengagement of Macrophage Apoptosis Enhances Pulmonary Bacterial Clearance *In Vivo*

To confirm that the differential levels of apoptosis explained the transgene effect *in vivo*, we reconstituted AM apoptosis in the CD68.hMcl-1⁺ transgenic mice using liposomes containing clodronate (20). Liposomes ensure macrophage targeting through phagocytic uptake, whereas clodronate induces a mitochondrial pathway of apoptosis with loss of mitochondrial transmembrane potential providing an alternative route of engagement of the mitochondrial apoptosis

Figure 2. (Continued). and the average number of cells per field (E) were measured ($n = 4$, $*P < 0.05$, two-way ANOVA). (F) Non-Tg or Tg BMDMs were challenged with Spn at an MOI of 10 for varying times before extracellular bacteria were killed and intracellular colony-forming units estimated immediately or after a further 2-hour incubation to measure intracellular killing capacity ($n = 4$, $**P < 0.01$, two-way ANOVA). (G) Non-Tg or Tg BMDMs were challenged with Spn at an MOI of 10 for varying intervals before extracellular bacteria were killed and cultures split, and one group was “pulsed” with streptomycin-resistant Spn (FP58) for 2 hours, after which the intracellular colony-forming units of FP58 were estimated as a marker of recent ingestion ($n = 5$). Data are presented as mean $\pm$ SEM. See also Figures E2 and E3.

Figure 3. Mcl-1 regulates caspase-induced late-phase mitochondrial reactive oxygen species (mROS) production. (A and B) Bone marrow-derived macrophages (BMDMs) from CD68.hMcl-1 nontransgenic (non-Tg) or transgenic (Tg) mice were challenged with serotype 2 *Streptococcus pneumoniae* (Spn) at a multiplicity of infection (MOI) of 10 in the presence (+) or absence of the antioxidant Trolox, or an inducible nitric oxide synthase inhibitor (1400W). (A) Intracellular bacterial colony-forming units were estimated 16 hours after challenge ($n = 5$), or (B) nuclear fragmentation was recorded 20 hours after challenge ($n = 6$, $*P < 0.05$ and $**P < 0.01$, one-way or two-way ANOVA for analyses within or between groups, respectively). (C) Non-Tg or Tg BMDMs were mock infected (MI) or challenged with Spn at an MOI of 10. At the indicated times after challenge, cells were stained with MitoSOX and analyzed by flow cytometry ($n = 3$, $*P < 0.05$, two-way ANOVA with indicated *post hoc* test). (D) Non-Tg or Tg BMDMs were MI or challenged with Spn at an MOI of 10. At the designated time points,

Figure 3. (Continued). cells were stained for mROS and caspase 3/7 activity by flow cytometry. Cells were selected by forward/side scatter (gray contour plots) before being designated as caspase negative or positive (green contour plots). MitoSOx red staining was assessed for each caspase subpopulation and cell populations as a whole (histograms). Representative plots are shown, with collated data in the graph below ($n = 3$, $^*P < 0.05$, two-way ANOVA). (E) Experiments in D were repeated in human monocyte-derived macrophages (MDMs; $n = 4$, $^{**}P < 0.01$, one-way ANOVA). (F and G) Non-Tg or Tg BMDMs (F) or human MDMs (G) were challenged with Spn at an MOI of 10 in the presence or absence (vehicle) of mitoTEMPO (mT), 1400W, or a combination of both (Combo). At 16 hours after challenge, intracellular colony-forming units were assessed ($n = 5$ for F and $n = 8$ for G; $^*P < 0.05$ and $^{**}P < 0.01$, repeated measures one-way ANOVA for F or Friedman test for G). (H and I) BMDMs from non-Tg or Tg mice were MI or challenged with Spn at an MOI of 10 in the presence (+) or absence (-) of mT. At 24 hours after challenge, cells were assessed by nuclear morphology (H) ($n = 3$, $^*P < 0.05$ and $^{**}P < 0.01$, two-way ANOVA), or (I) lysates were probed for human (h) and murine (m) Mcl-1 expression by Western blot and densitometry performed on three independent experiments. Data are presented as mean $\pm$ SEM. See also Figures E4–E7. FSC = forward scatter; MFI = median fluorescence intensity; ns = not significant; SSC = side scatter.

pathway in the absence of Mcl-1 downregulation (32, 33). Liposome dosing was adjusted to induce apoptosis in CD68.hMcl-1⁺ BMDMs exposed to bacteria (Figure 5A), without altering bacterial internalization (Figure 5B). *In vivo*, we administered liposomes at the same time as bacteria, to ensure that the early stages of liposome-induced AM apoptosis occurred together with the initiation of the antibacterial apoptotic program and before AM depletion reduced AM numbers (Figures 5C and 5D). Reconstitution of AM apoptosis in CD68.hMcl-1⁺ AMs increased bacterial

clearance from the lung, reduced levels of bacteria in the blood, and reduced neutrophil numbers in BAL (Figures 5E–5G). Similar results were obtained with BH3 mimetics (ABT-263, an oral derivative of ABT-737, and sabutoclax, a pan Bcl-2 family inhibitor [34]). These BH3 mimetics increased bacterial clearance from lung and blood (Figures 5H and 5I) and AM apoptosis, but not AM numbers, in infections inducing minimal neutrophil recruitment (Figure E9). They also reduced viable bacteria in MDMs at late, but not early, time points (Figures E9D and E9E).

Adoptive transfer of bone marrow between nontransgenic and transgenic mice confirmed that our results reflected macrophage expression of the transgene. Bone marrow transplantation reduces the ability of mice to clear the low dose of pneumococci (14, 35), and bacteria were not completely cleared in either group of mice (Figures E10A and E10B). However, the recipients of transgenic bone marrow exhibited reduced AM apoptosis and greater numbers of macrophage-associated bacteria (consistent with reduced intracellular clearance), while recruiting significantly more neutrophils (Figures

Figure 4. Apoptosis-associated killing mediates bacterial clearance *in vivo*. (A–E) CD68.hMcl-1 nontransgenic (non-Tg) or transgenic (Tg) mice were challenged with the designated dose of serotype 1 *Streptococcus pneumoniae* (Spn). At 24 hours after instillation, bacterial colony-forming units in the lung homogenate (A) or blood (B), the number of polymorphonuclear leukocytes (PMNs) (C), the number of alveolar macrophages (AMs) (D), or the percentage of apoptotic AMs (E) in the BAL were measured ($n = 4$ –11 mice per group from three independent experiments, $*P < 0.05$ and $**P < 0.01$, two-way ANOVA). (F–H) Non-Tg and Tg mice were challenged with *Haemophilus influenzae* type b (Hib) at the designated dose. At 24 hours after instillation, colony-forming units in the lung homogenate (F), PMN numbers in the BAL (G), and AM apoptosis (H) were measured ($n = 4$ –13 mice per group from two independent experiments, $***P < 0.001$, two-way ANOVA). (I–L) Non-Tg or Tg mice were challenged with the designated dose of Spn intraperitoneally. At 24 hours after challenge, the bacterial colony-forming units in the peritoneal lavage (PL; $n = 7$; I) or blood ($n = 7$; J) were determined, and PMN numbers ($n = 7$; K) and levels of peritoneal macrophage (PM) apoptosis ($n = 9$; L) in the PL were assessed by microscopy. In all experiments, $*P < 0.05$ and $**P < 0.01$, two-way ANOVA. See also Figure E8.

E10C–E10E). This suggested that there was less effective macrophage killing in transgenic mice.

Staphylococcus aureus Infection Does Not Activate Apoptosis-associated Killing

Staphylococcus aureus upregulates Mcl-1 in macrophages (36). We wondered whether this would phenocopy the effects seen with the CD68.hMcl-1 transgene.

Induction of macrophage apoptosis requires downregulation of Mcl-1 to allow mitochondrial outer membrane permeabilization and the execution phase of apoptosis (11, 14). *S. aureus* failed to induce the anticipated Mcl-1 downregulation after sustained bacterial ingestion (Figure E11A). Moreover, it was not associated with apoptosis or late-phase bacterial killing (Figures 6A and 6B), despite exhaustion of the

initial phase of killing in the setting of sustained ingestion of bacteria (Figures 6C and 6D). Exposure *in vivo* to a range of bacterial doses (from doses AMs can control to 100-fold higher) did not reveal any differences in bacterial clearance, neutrophil recruitment, or AM apoptosis, irrespective of transgene expression (Figures 6E–6G). In contrast to pneumococcal infection, ABT-737 failed

Figure 5. Apoptosis-associated killing can be reconstituted after challenge with *Streptococcus pneumoniae* (Spn). (A) Bone marrow-derived macrophages (BMDMs) from CD68.hMcl-1 nontransgenic (non-Tg) or transgenic (Tg) mice were challenged with liposomes containing PBS (LIPO-PBS) or clodronate (LIPO-CLOD). At the designated time point, cells were fixed and analyzed for nuclear fragmentation ($n = 3$). (B) Wild-type (non-Tg) or Tg BMDMs were challenged with serotype 2 Spn D39 at a multiplicity of infection of 10 in the presence of LIPO-PBS or LIPO-CLOD. At 4 hours after challenge, numbers of intracellular bacterial colony-forming units were assessed ($n = 3$). (C and D) Non-Tg or Tg mice were infected with 10^5 cfu serotype 1 Spn in the presence of LIPO-PBS or LIPO-CLOD. Alveolar macrophage (AM) numbers in BAL (C) and AM apoptosis in BAL (D) were measured by microscopy 24 hours after challenge (both $n = 4$, $*P < 0.05$, two-way ANOVA). (E–G) Non-Tg and Tg mice were challenged with 10^5 cfu of serotype 1 Spn and LIPO-PBS or LIPO-CLOD. At 24 hours after challenge, colony-forming units in the lung (E) and blood (F), and total polymorphonuclear leukocyte (PMN) numbers in the BAL (G) were measured ($n = 6$ –13 mice per group from three independent experiments). (H and I) Tg or non-Tg were instilled intranasally with 10^5 cfu of serotype 2 Spn then immediately treated with ABT-263 or sabutoclox. At 24 hours after challenge, colony-forming units in the lung (H) and blood (I) were measured (median + interquartile range; $n = 8$ –10, $*P < 0.05$, unpaired Student's t test, or two-way ANOVA) for analyses within or between groups, respectively. See also Figures E9 and E10.

to enhance bacterial killing or to reconstitute apoptosis at the dose that induced apoptosis in transgenic BMDMs after pneumococcal challenge (Figures E11B and E11C). ABT-737 was used to reconstitute apoptosis, because it does not alter uptake, in contrast to liposomes

(3), in which altered phagocytosis can confound interpretation during high-uptake phagocytosis, as seen with *S. aureus*. These findings highlight the importance of apoptosis-associated killing as a mechanism subverted by some pathogens.

Discussion

Development of a macrophage-specific CD68.hMcl-1 transgenic mouse provided a unique means of examining the role and mechanism of macrophage apoptosis-associated bacterial killing in the

Figure 6. *Staphylococcus aureus* infection does not trigger apoptosis-associated killing. (A) Bone marrow–derived macrophages (BMDMs) from CD68.hMcl-1 nontransgenic (non-Tg) or transgenic (Tg) mice were challenged with *S. aureus* (Sa) at a multiplicity of infection of 5. BMDMs were lysed at varying time points during a “pulse–chase” to allow detection of intracellular bacterial colony-forming units ($n = 3$). (B) BMDM apoptosis in the same experiments ($n = 3$). (C) BMDMs were lysed for initial assessment of colony-forming units or incubated in lysostaphin for 2 hours before colony-forming unit estimation to assess bacterial killing between the indicated time points ($n = 3$). (D) BMDMs were challenged with Sa at a multiplicity of infection of 5 for varying time periods, extracellular bacteria killed and BMDMs incubated with kanamycin and kanamycin-resistant Sa before extracellular bacteria were killed with lysostaphin, and intracellular colony-forming units measured at the designated time points. The graph shows intracellular colony-forming units, cultured in the presence of kanamycin to measure kanamycin-resistant (recently ingested) bacteria over each time increment ($n = 3$). (E–G) Non-Tg or Tg mice were challenged with Sa at the designated dose. At 24 hours after challenge, bacterial colony-forming units in the lung homogenate (E), PMN numbers in the BAL (F), and alveolar macrophage (AM) apoptosis (G), were measured ($n = 4$ –9 mice per group from three independent experiments). See also Figure E11.

lung. Use of this model identified a novel paradigm, whereby macrophage apoptosis kills internalized bacteria that remain viable after initial phagolysosomal killing is exhausted. During apoptosis

induction, caspase-dependent mROS production combines with NO to achieve a second late phase of bacterial killing. Inhibition of macrophage apoptosis by Mcl-1 increases susceptibility to

bacterial infection, but can be modulated pharmacologically to enhance pulmonary bacterial clearance.

Macrophages' avid phagocytic capacity ensures intracellular loading with ingested

bacteria (37, 38). Phagocytosis activates an initial phase of bacterial killing, consistent with observations describing temporal association of the NOX2 complex with neutrophil phagocytosis (24), but sustained phagocytosis overwhelms initial microbicidal responses. A late-phase microbicidal response, during the initial stages of apoptosis, clears remaining viable internalized bacteria. Macrophage apoptosis occurs during *Mycobacterium tuberculosis* infection (39), but also with other pulmonary micro-organisms, such as pneumococci, that are unable to persist intracellularly, suggesting that it limits intracellular persistence (11, 14).

Our approach, using macrophage-specific transgene expression (16), allowed selective modulation of the early stages of apoptosis via Mcl-1, with relative resistance to apoptosis, which regulates macrophage survival after pneumococcal infection (11). Mcl-1 is unique among antiapoptotic Bcl-2 proteins, because it is an early response gene with rapid induction and turnover (40). Mcl-1 transgene expression in myeloid cells prolongs macrophage survival, but ensures sensitivity to physiological constraints on viability and that cell numbers remain within the normal range (15).

Emerging data in patients at risk of CAP show that Mcl-1 upregulation in AMs is associated with reduced bacterial killing (12, 13). During HIV-1 infection, gp120 inhibits Mcl-1 ubiquitination and proteasomal degradation, whereas, in COPD, transcriptional upregulation is associated with antioxidant responses during oxidative stress (12, 13). In our murine model, overexpression of Mcl-1 using the human transgene converted low-dose lung infections, which macrophages normally control (3), into established infections inducing neutrophilic inflammation, phenocopying the susceptibility of patient AMs *ex vivo*. In comparison with *S. pneumoniae* and *H. influenzae*, *S. aureus* is less readily killed by differentiated macrophages (41), and internalized bacteria remain viable for several days (42). *S. aureus*-containing phagosomes fail to mature appropriately, decreasing cathepsin D activation required for Mcl-1 proteasomal degradation (14, 43). We show that *S. aureus* prevents apoptosis-associated killing; however, unlike pneumococcal infection, we could not reconstitute apoptosis-associated killing after *S. aureus* infection. A potential

explanation for this finding could be that altered endosomal trafficking of *S. aureus* needs to be corrected (43) to allow induction of apoptosis and to allow colocalization with mitochondria to mediate microbicidal killing (26). Thus, HIV, COPD, and *S. aureus* infection all inhibit bacterial killing by upregulating Mcl-1, similar to the overexpression of the human Mcl-1 transgene in our murine model.

The relevance of animal models to human disease merits careful scrutiny. Murine pneumonia models confirm roles for the key innate cell populations contributing to pathogenesis in CAP, and reprise the susceptibility of key, single-gene defects or polymorphisms identified in humans, despite some differences in specific innate responses (e.g., extent of reliance on NOS2 [inducible NO synthase] in macrophages, α -defensin expression in neutrophils, or activation patterns after specific Toll-like receptor ligands) (44). The C57BL/6 strain has intermediate susceptibility to pneumococci (45), and inocula can be adapted to favor AM-dependent clearance or sequential requirement of T cells and recruited neutrophils in this model (30). They also show evidence of AM apoptosis (3) and increased susceptibility to pneumococcal disease after Mcl-1 overexpression (11). A murine model allowed us to test the impact of genetic modulation of Mcl-1 *in vivo* in the context of early-stage subclinical infection, something not possible in patients who present at the later stage of established disease. Impaired clearance of pneumococci in association with Mcl-1 upregulation in patient groups at increased risk of CAP suggests that these findings are relevant to human disease. Moreover, we demonstrated key mechanistic requirements for caspase-dependent mROS, combined with NO, in apoptosis-associated killing in human MDMs, as well as increased bacterial clearance with BH3 mimetics.

Apoptosis-associated bacterial killing requires mROS, a recently identified microbicidal (26). During apoptosis execution, caspase 3 inhibits mitochondrial electron transport complexes I and II (27), resulting in generation of superoxide (46). SOD2 (superoxide dismutase 2) is a mitochondrial protein that is upregulated during pneumococcal infection, preventing necroptosis (47).

This ensures that mitochondrial permeabilization is limited in extent, a specific feature of apoptosis (11, 14, 48, 49). However, antioxidant protection does not extend to the immediate environment of the phagolysosome, permitting microbial killing (26). Pneumococcal antioxidant systems protect against NADPH-dependent ROS generation in neutrophils (9). Our results, however, suggest that peak mROS and NO coexist, consistent with the role of NO in the late microbicidal macrophage response (19, 25). Potential sources of NO include NOS2, but also NOS3 (endothelial NOS), which contributes to AM microbicidal responses to pneumococci (50) and mitochondria, which generate NO through NOS-independent and NOS-dependent mechanisms (including the debated existence of an inner membrane-associated or matrix isoform) (51). Cross-reactivity of inhibitors between isoforms and residual controversies concerning NOS2 in humans means the source of NO requires further clarification. We propose a model where mROS and NO generation is temporally and spatially linked, and colocalizes with bacteria containing phagolysosomes, as previously shown (19), allowing generation of reactive nitrogen species. We did not identify NO regulation by mROS, because mROS occurred downstream of Mcl-1-mediated apoptosis regulation, whereas NO production was upstream and unaltered by the Mcl-1 transgene. NO and reactive nitrogen species can, however, enhance mROS generation (51). We found no evidence that the role of mROS in killing was mediated by differential cytokine expression. Although mROS can induce proinflammatory cytokine expression (28), during the early stages of bacterial-associated apoptosis induction, protein translation is reduced, limiting this possibility (36).

Because we demonstrate a critical role for apoptosis-associated killing in mediating bacterial clearance by macrophages, it follows that upregulation of Mcl-1 influences susceptibility to bacterial pneumonia. The use of a murine model in which we could alter Mcl-1 expression through transgene expression and deliver controlled infections and pharmacological interventions allowed us to confirm the role and mechanisms of this process to an extent not possible with our prior studies in patients (12, 13).

Our data suggest that susceptibility to bacterial infection can be reversed through therapeutic targeting of the mitochondrial-microbicidal axis or modulation of Mcl-1. Several classes of therapeutics, including bisphosphonates and BH3 mimetics, target these pathways (20, 23). As proof of concept of this repurposing approach, ABT737 has recently demonstrated utility in preventing intracellular replication of *Legionella*

pneumophila in AMs through induction of apoptosis (52). We also demonstrated that Bcl-2-specific and pan-Bcl-2 inhibitors enhanced pneumococcal clearance, with more significant results demonstrated for sabutoclax, an agent that inhibits Mcl-1 (53). However, for some pathogens, such as *S. aureus*, the strategy may need to be adapted to reverse altered endosomal trafficking (43). In view of the ongoing therapeutic

challenge of antimicrobial resistance, reengaging this fundamental microbicidal mechanism in AMs merits further evaluation. ■

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References

- O'Brien KL, Wolfson LJ, Watt JP, Henkle E, Deloria-Knoll M, McCall N, et al.; Hib and Pneumococcal Global Burden of Disease Study Team. Burden of disease caused by *Streptococcus pneumoniae* in children younger than 5 years: global estimates. *Lancet* 2009;374:893–902.
- Croucher NJ, Harris SR, Fraser C, Quail MA, Burton J, van der Linden M, et al. Rapid pneumococcal evolution in response to clinical interventions. *Science* 2011;331:430–434.
- Dockrell DH, Marriott HM, Prince LR, Ridger VC, Ince PG, Hellewell PG, et al. Alveolar macrophage apoptosis contributes to pneumococcal clearance in a resolving model of pulmonary infection. *J Immunol* 2003;171:5380–5388.
- Cohen AB, Cline MJ. The human alveolar macrophage: isolation, cultivation *in vitro*, and studies of morphologic and functional characteristics. *J Clin Invest* 1971;50:1390–1398.
- Jin M, Opalek JM, Marsh CB, Wu HM. Proteome comparison of alveolar macrophages with monocytes reveals distinct protein characteristics. *Am J Respir Cell Mol Biol* 2004;31:322–329.
- Kobzik L, Godleski JJ, Brain JD. Oxidative metabolism in the alveolar macrophage: analysis by flow cytometry. *J Leukoc Biol* 1990;47:295–303.
- Jesch NK, Dörger M, Enders G, Rieder G, Vogelmeier C, Messmer K, et al. Expression of inducible nitric oxide synthase and formation of nitric oxide by alveolar macrophages: an interspecies comparison. *Environ Health Perspect* 1997;105:1297–1300.
- Daigneault M, Preston JA, Marriott HM, Whyte MK, Dockrell DH. The identification of markers of macrophage differentiation in PMA-stimulated THP-1 cells and monocyte-derived macrophages. *PLoS One* 2010;5:e8668.
- Aberdein JD, Cole J, Bewley MA, Marriott HM, Dockrell DH. Alveolar macrophages in pulmonary host defence—the unrecognized role of apoptosis as a mechanism of intracellular bacterial killing. *Clin Exp Immunol* 2013;174:193–202.
- Dockrell DH, Lee M, Lynch DH, Read RC. Immune-mediated phagocytosis and killing of *Streptococcus pneumoniae* are associated with direct and bystander macrophage apoptosis. *J Infect Dis* 2001;184:713–722.
- Marriott HM, Bingle CD, Read RC, Braley KE, Kroemer G, Hellewell PG, et al. Dynamic changes in Mcl-1 expression regulate macrophage viability or commitment to apoptosis during bacterial clearance. *J Clin Invest* 2005;115:359–368.
- Bewley MA, Preston JA, Mohasin M, Marriott HM, Budd RC, Swales J, et al. Impaired mitochondrial microbicidal responses in chronic obstructive pulmonary disease macrophages. *Am J Respir Crit Care Med* 2017;196:845–855.
- Collini PJ, Bewley MA, Mohasin M, Marriott HM, Miller RF, Geretti AM, et al. HIV gp120 in the lungs of antiretroviral therapy-treated individuals impairs alveolar macrophage responses to pneumococci. *Am J Respir Crit Care Med* 2018;197:1604–1615.
- Bewley MA, Marriott HM, Tulone C, Francis SE, Mitchell TJ, Read RC, et al. A cardinal role for cathepsin D in co-ordinating the host-mediated apoptosis of macrophages and killing of pneumococci. *PLoS Pathog* 2011;7:e1001262.
- Zhou P, Qian L, Bieszczyk CK, Noelle R, Binder M, Levy NB, et al. Mcl-1 in transgenic mice promotes survival in a spectrum of hematopoietic cell types and immortalization in the myeloid lineage. *Blood* 1998;92:3226–3239.
- Gough PJ, Gordon S, Greaves DR. The use of human CD68 transcriptional regulatory sequences to direct high-level expression of class A scavenger receptor in macrophages *in vitro* and *in vivo*. *Immunology* 2001;103:351–361.
- Shipley JM, Wesselschmidt RL, Kobayashi DK, Ley TJ, Shapiro SD. Metalloelastase is required for macrophage-mediated proteolysis and matrix invasion in mice. *Proc Natl Acad Sci U S A* 1996;93:3942–3946.
- Cotter MJ, Norman KE, Hellewell PG, Ridger VC. A novel method for isolation of neutrophils from murine blood using negative immunomagnetic separation. *Am J Pathol* 2001;159:473–481.
- Marriott HM, Ali F, Read RC, Mitchell TJ, Whyte MK, Dockrell DH. Nitric oxide levels regulate macrophage commitment to apoptosis or necrosis during pneumococcal infection. *FASEB J* 2004;18:1126–1128.
- Van Rooijen N, Sanders A. Liposome mediated depletion of macrophages: mechanism of action, preparation of liposomes and applications. *J Immunol Methods* 1994;174:83–93.
- Greaves DR, Quinn CM, Seldin MF, Gordon S. Functional comparison of the murine macrophage and human CD68 promoters in macrophage and nonmacrophage cell lines. *Genomics* 1998;54:165–168.
- Bewley MA, Naughton M, Preston J, Mitchell A, Holmes A, Marriott HM, et al. Pneumolysin activates macrophage lysosomal membrane permeabilization and executes apoptosis by distinct mechanisms without membrane pore formation. *MBio* 2014;5:e01710–e01714.
- van Delft MF, Huang DC. How the Bcl-2 family of proteins interact to regulate apoptosis. *Cell Res* 2006;16:203–213.
- DeLeo FR, Allen LA, Apicella M, Nauseef WM. NADPH oxidase activation and assembly during phagocytosis. *J Immunol* 1999;163:6732–6740.
- Marriott HM, Hellewell PG, Whyte MK, Dockrell DH. Contrasting roles for reactive oxygen species and nitric oxide in the innate response to pulmonary infection with *Streptococcus pneumoniae*. *Vaccine* 2007;25:2485–2490.
- West AP, Brodsky IE, Rahner C, Woo DK, Erdjument-Bromage H, Tempst P, et al. TLR signalling augments macrophage bactericidal activity through mitochondrial ROS. *Nature* 2011;472:476–480.
- Ricci JE, Gottlieb RA, Green DR. Caspase-mediated loss of mitochondrial function and generation of reactive oxygen species during apoptosis. *J Cell Biol* 2003;160:65–75.
- Bulua AC, Simon A, Maddipati R, Pelletier M, Park H, Kim KY, et al. Mitochondrial reactive oxygen species promote production of proinflammatory cytokines and are elevated in TNFR1-associated periodic syndrome (TRAPS). *J Exp Med* 2011;208:519–533.
- Yesilkaya H, Andisi VF, Andrew PW, Bijlsma JJ. *Streptococcus pneumoniae* and reactive oxygen species: an unusual approach to living with radicals. *Trends Microbiol* 2013;21:187–195.
- Marriott HM, Daigneault M, Thompson AA, Walmsley SR, Gill SK, Witcher DR, et al. A decoy receptor 3 analogue reduces localised defects in phagocyte function in pneumococcal pneumonia. *Thorax* 2012;67:985–992.
- King P. *Haemophilus influenzae* and the lung (*Haemophilus* and the lung). *Clin Transl Med* 2012;1:10.

32. Lehenkari PP, Kellinsalmi M, Näpänkangas JP, Ylitalo KV, Mönkkönen J, Rogers MJ, *et al.* Further insight into mechanism of action of clodronate: inhibition of mitochondrial ADP/ATP translocase by a nonhydrolyzable, adenine-containing metabolite. *Mol Pharmacol* 2002;61:1255–1262.
33. van Rooijen N, Sanders A, van den Berg TK. Apoptosis of macrophages induced by liposome-mediated intracellular delivery of clodronate and propamidine. *J Immunol Methods* 1996;193:93–99.
34. Bajwa N, Liao C, Nikolovska-Coleska Z. Inhibitors of the anti-apoptotic Bcl-2 proteins: a patent review. *Expert Opin Ther Pat* 2012;22:37–55.
35. Ojielo CI, Cooke K, Mancuso P, Standiford TJ, Olkiewicz KM, Clouthier S, *et al.* Defective phagocytosis and clearance of *Pseudomonas aeruginosa* in the lung following bone marrow transplantation. *J Immunol* 2003;171:4416–4424.
36. Koziel J, Maciag-Gudowska A, Mikolajczyk T, Bzowska M, Sturdevant DE, Whitney AR, *et al.* Phagocytosis of *Staphylococcus aureus* by macrophages exerts cytoprotective effects manifested by the upregulation of antiapoptotic factors. *PLoS One* 2009;4:e5210.
37. Steinman RM, Mellman IS, Muller WA, Cohn ZA. Endocytosis and the recycling of plasma membrane. *J Cell Biol* 1983;96:1–27.
38. Cohn ZA, Fedorko ME, Hirsch JG. The *in vitro* differentiation of mononuclear phagocytes: V. The formation of macrophage lysosomes. *J Exp Med* 1966;123:757–766.
39. Keane J, Balcewicz-Sablinska MK, Remold HG, Chupp GL, Meek BB, Fenton MJ, *et al.* Infection by *Mycobacterium tuberculosis* promotes human alveolar macrophage apoptosis. *Infect Immun* 1997;65:298–304.
40. Yang T, Kozopas KM, Craig RW. The intracellular distribution and pattern of expression of Mcl-1 overlap with, but are not identical to, those of Bcl-2. *J Cell Biol* 1995;128:1173–1184.
41. Jonsson S, Musher DM, Chapman A, Goree A, Lawrence EC. Phagocytosis and killing of common bacterial pathogens of the lung by human alveolar macrophages. *J Infect Dis* 1985;152:4–13.
42. Kubica M, Guzik K, Koziel J, Zarebski M, Richter W, Gajkowska B, *et al.* A potential new pathway for *Staphylococcus aureus* dissemination: the silent survival of *S. aureus* phagocytosed by human monocyte-derived macrophages. *PLoS One* 2008;3:e1409.
43. Jubrail J, Morris P, Bewley MA, Stoneham S, Johnston SA, Foster SJ, *et al.* Inability to sustain intraphagolysosomal killing of *Staphylococcus aureus* predisposes to bacterial persistence in macrophages. *Cell Microbiol* 2016;18:80–96.
44. Mizgerd JP, Skerrett SJ. Animal models of human pneumonia. *Am J Physiol Lung Cell Mol Physiol* 2008;294:L387–L398.
45. Gingles NA, Alexander JE, Kadioglu A, Andrew PW, Kerr A, Mitchell TJ, *et al.* Role of genetic resistance in invasive pneumococcal infection: identification and study of susceptibility and resistance in inbred mouse strains. *Infect Immun* 2001;69:426–434.
46. Cai J, Jones DP. Superoxide in apoptosis: mitochondrial generation triggered by cytochrome c loss. *J Biol Chem* 1998;273:11401–11404.
47. Galluzzi L, Kroemer G. Necroptosis: a specialized pathway of programmed necrosis. *Cell* 2008;135:1161–1163.
48. Bewley MA, Pham TK, Marriott HM, Noirel J, Chu HP, Ow SY, *et al.* Proteomic evaluation and validation of cathepsin D regulated proteins in macrophages exposed to *Streptococcus pneumoniae*. *Mol Cell Proteomics* 2011;10:M111.008193.
49. Golstein P, Kroemer G. Cell death by necrosis: towards a molecular definition. *Trends Biochem Sci* 2007;32:37–43.
50. Yang Z, Huang YC, Koziel H, de Crom R, Ruetten H, Wohlfart P, *et al.* Female resistance to pneumonia identifies lung macrophage nitric oxide synthase-3 as a therapeutic target. *Elife* 2014;3:e03711.
51. Lacza Z, Pankotai E, Busija DW. Mitochondrial nitric oxide synthase: current concepts and controversies. *Front Biosci* 2009;14:4436–4443.
52. Speir M, Lawlor KE, Glaser SP, Abraham G, Chow S, Vogrin A, *et al.* Eliminating *Legionella* by inhibiting BCL-XL to induce macrophage apoptosis. *Nat Microbiol* 2016;1:15034.
53. Dash R, Azab B, Quinn BA, Shen X, Wang XY, Das SK, *et al.* Apogossypol derivative BI-97C1 (Sabutoclax) targeting Mcl-1 sensitizes prostate cancer cells to mda-7/IL-24-mediated toxicity. *Proc Natl Acad Sci U S A* 2011;108:8785–8790. [Published erratum appears in *Proc Natl Acad Sci U S A* 114:E4522–E4523.]