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OPEN

Downregulation of Mcl-1 has anti-inflammatory pro-resolution effects and enhances bacterial clearance from the lung

CD Lucas¹, DA Dorward¹, MA Tait¹, S Fox^{1,2}, JA Marwick¹, KC Allen¹, CT Robb¹, N Hirani¹, C Haslett¹, R Duffin¹ and AG Rossi¹

Phagocytes not only coordinate acute inflammation and host defense at mucosal sites, but also contribute to tissue damage. Respiratory infection causes a globally significant disease burden and frequently progresses to acute respiratory distress syndrome, a devastating inflammatory condition characterized by neutrophil recruitment and accumulation of protein-rich edema fluid causing impaired lung function. We hypothesized that targeting the intracellular protein myeloid cell leukemia 1 (Mcl-1) by a cyclin-dependent kinase inhibitor (AT7519) or a flavone (wogonin) would accelerate neutrophil apoptosis and resolution of established inflammation, but without detriment to bacterial clearance. Mcl-1 loss induced human neutrophil apoptosis, but did not induce macrophage apoptosis nor impair phagocytosis of apoptotic neutrophils. Neutrophil-dominant inflammation was modelled in mice by either endotoxin or bacteria (*Escherichia coli*). Downregulating inflammatory cell Mcl-1 had anti-inflammatory, pro-resolution effects, shortening the resolution interval (R_i) from 19 to 7 h and improved organ dysfunction with enhanced alveolar-capillary barrier integrity. Conversely, attenuating drug-induced Mcl-1 downregulation inhibited neutrophil apoptosis and delayed resolution of endotoxin-mediated lung inflammation. Importantly, manipulating lung inflammatory cell Mcl-1 also accelerated resolution of bacterial infection (R_i ; 50 to 16 h) concurrent with enhanced bacterial clearance. Therefore, manipulating inflammatory cell Mcl-1 accelerates inflammation resolution without detriment to host defense against bacteria, and represents a target for treating infection-associated inflammation.

INTRODUCTION

Neutrophils are short-lived key innate immune defense cells that under basal conditions undergo apoptosis within hours.¹ However, following an inflammatory challenge, neutrophils are rapidly recruited to infected or damaged tissue where their lifespan is extended by host-derived factors (e.g., granulocyte-macrophage colony-stimulating factor), pathogen-derived factors (e.g., lipopolysaccharide (LPS)), local factors (e.g., tissue hypoxia), and the process of transmigration itself to allow an appropriate neutrophil response to injury.² Following successful control of tissue injury and/or infection, neutrophil function must be terminated in a timely manner to prevent neutrophil-mediated host tissue damage.^{3,4} This is achieved by the highly regulated events of neutrophil apoptosis and subsequent uptake

and removal of apoptotic neutrophils by surrounding phagocytes including tissue macrophages,^{5,6} allowing the return to tissue homeostasis. Failure of these processes has pathological consequences with deficient clearance of apoptotic neutrophils leading to deleterious secondary necrosis with consequent enhanced tissue injury.⁷ In addition, unresolved neutrophilic inflammation is a hallmark of many inflammatory conditions including sepsis, acute lung injury, and its severe counterpart acute respiratory distress syndrome.⁸ Furthermore, the delay in neutrophil apoptosis correlates with increased mortality in sepsis and sepsis-induced lung injury in humans,⁸ suggesting that manipulating neutrophil apoptosis and subsequent clearance (efferocytosis) may provide novel therapeutic strategies to enhance resolution of infection-associated lung inflammation.

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Neutrophil apoptosis can be controlled by either the extrinsic pathway upon ligation of a death receptor by its ligand (e.g., tumor necrosis factor (TNF), Fas ligand, and TNF-related apoptosis-inducing ligand (TRAIL)) or by the intrinsic pathway in response to numerous stimuli including changes in the local environment and cytokine and pharmacological manipulation.^{9–11} The intrinsic pathway is controlled by members of the B-cell lymphoma 2 (Bcl-2) family, with neutrophils expressing proapoptotic members including Bad, Bax, Bak, Bid, Bim, and Puma, as well as antiapoptotic members including Bcl-extra large (Bcl-X_L), and myeloid cell leukemia 1 (Mcl-1).^{11,12} The prosurvival member Mcl-1 is short lived with levels of Mcl-1 increased, along with inhibition of neutrophil apoptosis, by factors present at inflammatory sites including granulocyte-macrophage colony-stimulating factor and hypoxia. Furthermore, Mcl-1 expression is increased in neutrophils from patients with severe sepsis,¹³ suggesting that modulating Mcl-1 levels may be a viable therapeutic target for the treatment of neutrophil-dominant inflammation.

Neutrophil levels of Mcl-1 are amenable to pharmacological manipulation *in vitro*, with agents such as the archetypal cyclin-dependent kinase (CDK) inhibitor, R-roscovitine, downregulating Mcl-1 in tandem with the induction of neutrophil apoptosis *in vitro*.¹⁴ Our group has previously shown that R-roscovitine can reduce inflammatory cell numbers in diverse models of inflammation,¹⁴ but the consequences on organ function and host defense against invading pathogens as well as the molecular events involved *in vivo* have not been previously investigated. AT7519 is a novel CDK inhibitor drug that is currently in clinical trials for cancer and is a potent inhibitor of CDK5 and CDK9,¹⁵ both expressed at high levels in human neutrophils.¹⁶ Other pharmacological agents such as polyphenolic plant-derived flavones are also capable of downregulating neutrophil Mcl-1 levels *in vitro*, leading to neutrophil apoptosis.¹¹ Here we investigate dynamic manipulation of Mcl-1 in inflammatory cells *in vivo* to determine the resolution of established neutrophilic inflammation. Downregulation of Mcl-1 *in vitro* and *in vivo* drives neutrophil apoptosis and subsequent macrophage clearance and thereby accelerates resolution of established lung inflammation in mice, whereas stabilization of Mcl-1 delays the resolution of pulmonary inflammation. Furthermore, targeting Mcl-1 accelerates resolution of established *Escherichia coli* lung infection along with reduced bacterial lung burden, suggesting that, unlike conventional anti-inflammatory therapies, this novel therapeutic approach has anti-inflammatory effects concurrent with enhanced bacterial clearance.

RESULTS

AT7519 induces rapid concentration-, time-, and caspase-dependent human neutrophil apoptosis via downregulation of Mcl-1 and is able to override the survival effect of bacterial products

The novel CDK inhibitor AT7519 induced human neutrophil apoptosis in a concentration- and time-dependent manner

(**Figure 1a–c**). AT7519 was ~100 times more potent than the archetypal CDK inhibitor, R-roscovitine (half-maximal effective concentration of AT7519 61.1 nM at 6 h vs. 4.8 μM for R-roscovitine; **Figure 1a** and Rossi *et al.*¹⁴). Bacterial-derived products including LPS promote human neutrophil survival *in vitro*, but this could be overcome by AT7519 (**Figure 1b**), with increased numbers of apoptotic neutrophils seen in AT7519-treated groups. Similarly, AT7519 was also able to override the survival effects of the Gram-positive bacterial-derived products lipoteichoic acid (LTA) and peptidoglycan (PepG) by induction of apoptosis (**Supplementary Figure S1** online). AT7519-induced neutrophil apoptosis was caspase dependent as apoptosis was blocked by the broad-spectrum caspase inhibitors z-VAD and Q-VD (**Figure 1d**), and AT7519-induced DNA fragmentation was blocked by co-incubation with z-VAD (**Figure 1e**). Furthermore, AT7519 accelerated caspase-3 cleavage by 6 h (**Figure 1f**). Although Mcl-1 has been described as a downstream target of activated caspases.¹⁷ AT7519 downregulated Mcl-1 both before the appearance of cleaved caspase-3 (**Figure 1f**) and in the presence of caspase inhibitors (z-VAD or Q-VD; **Figure 1g**). These observations confirmed that AT7519-driven Mcl-1 downregulation was occurring independently of, and upstream to, caspase activation at these early time points. AT7519 also caused time- and caspase-dependent apoptosis of mouse bone marrow-derived neutrophils, preceded by downregulation of Mcl-1 (**Supplementary Figure S2**). Mcl-1 levels also declined in control neutrophils undergoing constitutive apoptosis (**Figure 1f**), consistent with published literature.^{11,17} To confirm that Mcl-1 downregulation was a driving factor in inducing neutrophil apoptosis, and not merely a consequence of apoptosis, we used a neutrophil precursor cell line (HL-60; human promyelocytic leukemic cell line) to examine the role of Mcl-1 in induction of apoptosis. Similar to primary human neutrophils AT7519 induced rapid time-, concentration-, and caspase-dependent apoptosis in HL-60 cells by downregulation of Mcl-1 (**Supplementary Figure S3A,B**). Small interfering RNA (siRNA) knockdown of Mcl-1 led to accelerated HL-60 cell death by apoptosis, with Mcl-1 knockdown preceding the induction of cell death (**Supplementary Figure S3C–F**). Furthermore, Mcl-1 siRNA-mediated apoptosis, but not the Mcl-1 knockdown itself, was blocked by caspase inhibition with Q-VD, further confirming both cell death by apoptosis and caspase-independent Mcl-1 downregulation (**Supplementary Figure S3G,H**).

AT7519-treated human macrophages do not undergo apoptosis despite downregulation of Mcl-1

Following neutrophil apoptosis it is essential that rapid phagocytosis ensues to prevent neutrophil progression to secondary necrosis and release of histotoxic contents into host tissues. AT7519 at concentrations that induced apoptosis of neutrophils (0.1–10 μM) did not induce apoptosis of human monocyte-derived macrophages (MDMs) (**Figure 2a**). This was despite a concentration-dependent downregulation of

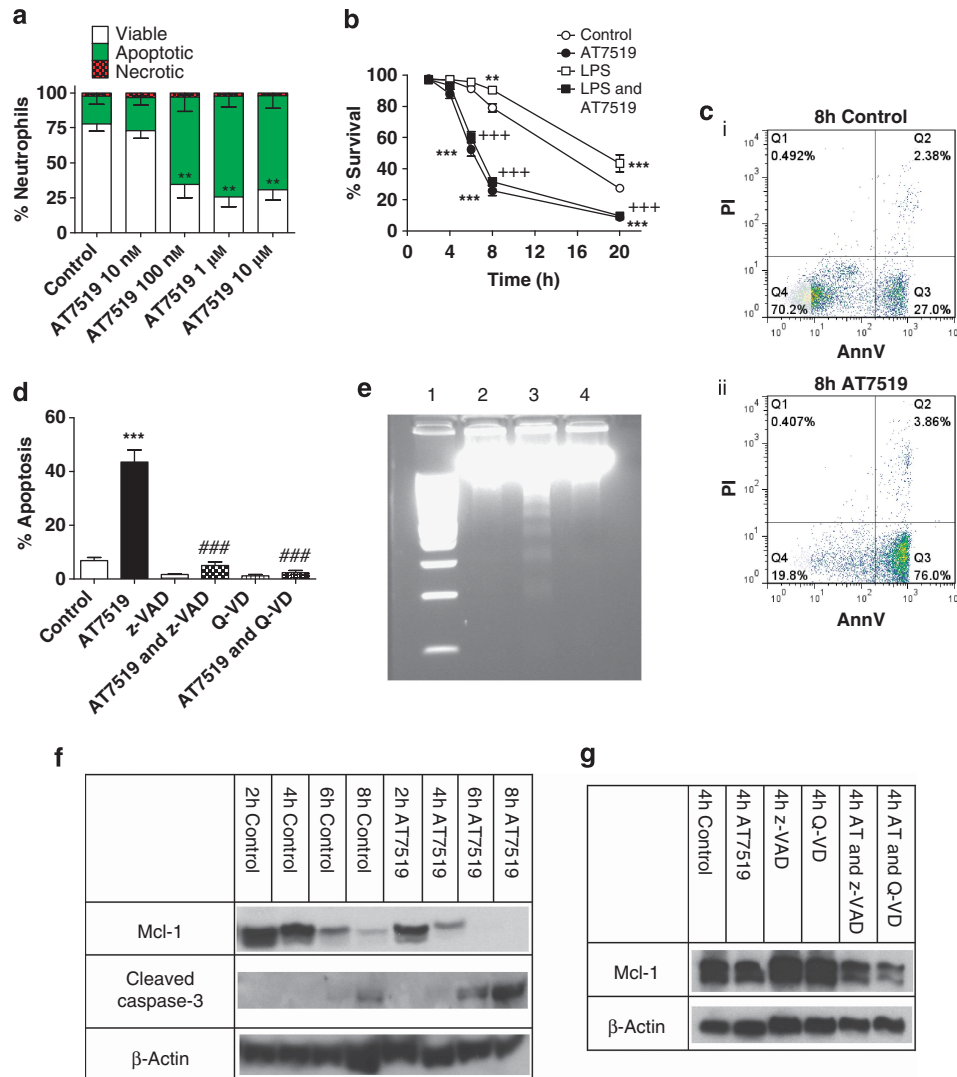


Figure 1 AT7519 induces concentration-, time-, and caspase-dependent human neutrophil apoptosis via myeloid cell leukemia 1 (Mcl-1) downregulation and overrides lipopolysaccharide (LPS)-induced survival. **(a)** Neutrophils cultured with AT7519 for 6 h before analysis of Annexin-V/propidium iodide (AnnV/PI) binding ($n=3$). **(b)** Neutrophils cultured for 2–20 h with AT7519 ($1 \mu\text{M}$), LPS (100 ng ml^{-1}), or combined AT7519 and LPS before assessment of viability (AnnV/PI binding; $n=6$). **(c)** Representative flow cytometry plots (AnnV/PI) for (i) control and (ii) AT7519-treated ($1 \mu\text{M}$) neutrophils at 8 h of culture. **(d)** Neutrophil apoptosis (AnnV/PI) assessed at 6 h after culture with AT7519 ($1 \mu\text{M}$), z-VAD ($100 \mu\text{M}$), or Q-VD ($10 \mu\text{M}$) alone or in combination ($n \geq 4$). **(e)** DNA laddering assessed after 6 h of culture; lane 1, DNA marker 180 bp; lane 2, control neutrophils; lane 3, AT7519 ($1 \mu\text{M}$); lane 4, AT7519 ($1 \mu\text{M}$) and z-VAD ($100 \mu\text{M}$). **(f)** Neutrophils aged for 2–8 h in either control media or AT7519 ($1 \mu\text{M}$) before western blotting for Mcl-1 (40 kDa), cleaved caspase-3 (17/19 kDa), and β -actin (42 kDa). **(g)** Neutrophils aged for 4 h with AT7519 ($1 \mu\text{M}$) \pm z-VAD ($100 \mu\text{M}$) or Q-VD ($10 \mu\text{M}$) before western blotting. $**P < 0.01$, $***P < 0.001$ compared with control; $+++P < 0.001$ compared with LPS alone; $###P < 0.001$ compared with AT7519 alone.

Mcl-1 by AT7519 in MDMs (**Figure 2b**). AT7519-treated MDMs were still functionally intact as they were able to phagocytose apoptotic neutrophils even after prolonged incubation (6–8 h) with AT7519 (**Figure 2c**), despite Mcl-1 loss by these times. AT7519 treatment of neutrophils enhanced their phagocytosis that was dependent on the induction of apoptosis as it was blocked by Q-VD treatment of neutrophils (**Figure 2d–f**). Phagocytosis of both control and AT7519-treated neutrophils was inhibited by preincubating MDMs for 30 min with cytochalasin D ($10 \mu\text{M}$), an inhibitor of actin polymerization (**Figure 2f**).

AT7519 enhances resolution of established neutrophilic lung inflammation

To assess the ability of AT7519 to enhance resolution of established neutrophilic inflammation *in vivo*, we used three mouse models of pulmonary inflammation. Intratracheal (i.t.) administration of LPS caused lung inflammation with peak neutrophil influx at ~ 24 h, before spontaneous resolution (**Figure 3a,b**). Administration of AT7519 24 h after LPS dramatically reduced total inflammatory cells in bronchoalveolar lavage (BAL; **Figure 3a**), mainly because of a reduction in neutrophils (**Figure 3b**). By 12 h after AT7519

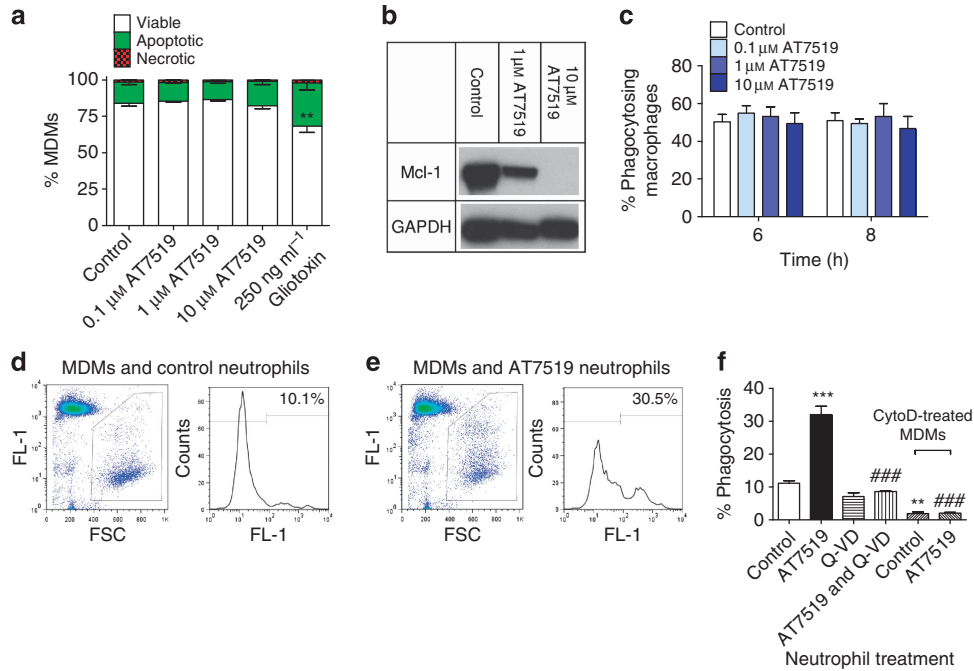


Figure 2 AT7519 does not induce death or impair phagocytosis by human monocyte-derived macrophages (MDMs) despite downregulation of myeloid cell leukemia 1 (Mcl-1), whereas AT7519-treated neutrophils are phagocytosed more readily. (a) MDMs were incubated with AT7519 or gliotoxin (250 ng ml⁻¹; included as a positive control) for 8 h before apoptosis assessment (Annexin-V/propidium iodide (AnnV/PI)). (b) MDMs were incubated with AT7519 for 4 h before western blotting for Mcl-1 (40 kDa) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 37 kDa). (c) Incubation of MDMs with AT7519 for either 6 h or 8 h before co-incubation of apoptotic neutrophils (AT7519 treated) and assessment of phagocytosis (*n* = 3). (d, e) Representative flow cytometry plots (forward scatter vs. FL1 and FL1 histogram) for (d) MDMs incubated with control aged neutrophils and (e) MDMs incubated with AT7519-treated (1 μM) neutrophils. (f) Cumulative data with neutrophils incubated in control media, AT7519 (1 μM), Q-VD (10 μM), or combinations before assessment of phagocytosis in both control and cytochalasin D (CytoD; 10 μM)-treated MDMs (*n* = 3). ***P* < 0.01, ****P* < 0.001 compared with control and ###*P* < 0.001 compared with AT7519-treated neutrophils (f).

treatment, total neutrophil numbers were less than a quarter of untreated controls. This gave a resolution interval (*R_i*; the time taken for neutrophil numbers to decline to half-maximal^{18,19}) for AT7519 of ~7 h, whereas the *R_i* for control mice was ~19 h. AT7519-induced resolution occurred in tandem with enhanced apoptosis of neutrophils (observed at 36 h; 12 h after AT7519), and enhanced macrophage clearance of apoptotic cells (Figure 3c,d), with representative cytocentrifuge preparations of BAL cells shown (Figure 3m-p). A transient decrease in BAL total monocytes/macrophages was observed at 36 h (Figure 3e) that was unchanged by 48 h. By 48 h, increasing numbers of neutrophils in the control group were undergoing apoptosis (Figure 3c), consistent with spontaneous resolution of neutrophilic inflammation. By 24 h after AT7519 administration, BAL fluid total protein and BAL immunoglobulin M (IgM), measures of alveolar-capillary barrier integrity, were significantly reduced compared with controls (Figure 3f,i). By 36 h (12 h after AT7519), histological appearances were also improved (Figure 3g,h). Analysis of inflammatory cytokines revealed that whereas TNF-α levels were unchanged with AT7519 treatment, chemokine (C-C motif) ligand 2 (CCL-2)/monocyte chemoattractant protein-1 (MCP-1) and interleukin-6 (IL-6) levels were higher in AT7519-treated animals (Figure 3j-l).

Proresolution effects of AT7519 were also investigated following i.t. administration of the Gram-positive bacterial

components LTA and PepG that signal predominantly via TLR2. Administration of AT7519 at 24 h after LTA/PepG led to a rapid reduction in total BAL cells and BAL neutrophils, with a concurrent increase in BAL apoptotic neutrophils and clearance of apoptotic cells as evidenced by macrophages containing apoptotic bodies (Supplementary Figure S4A-F). This gave a resolution interval of ~4.5 h in AT7519-treated mice vs. ~19 h in control mice. In this model, TNF-α and IL-6 levels were unaltered with AT7519 treatment, whereas CCL-2 and IL-10 were increased following AT7519 administration (Supplementary Figure S4G-J). AT7519 also improved tissue histology, and AT7519-induced neutrophil apoptosis *in vivo* was further confirmed using flow cytometric analysis of Annexin-V (AnnV) binding of neutrophils (Ly6G⁺/F4/80⁻ cells; Supplementary Figure S4K-N).

To investigate whether AT7519 could also enhance resolution in nonsterile lung inflammation, and the consequences of augmented neutrophil apoptosis on bacterial clearance, 1 × 10⁶ colony-forming units (CFUs) of live *E. coli* were administered i.t. with AT7519 given at 24 h. AT7519 was also able to enhance resolution of established *E. coli*-induced lung inflammation (Figure 4a,b) with a *R_i* for AT7519 of ~16 h, whereas the *R_i* for control mice was ~50 h. AT7519 administration also led to reduced lung interstitial neutrophils (Figure 4d) but did not

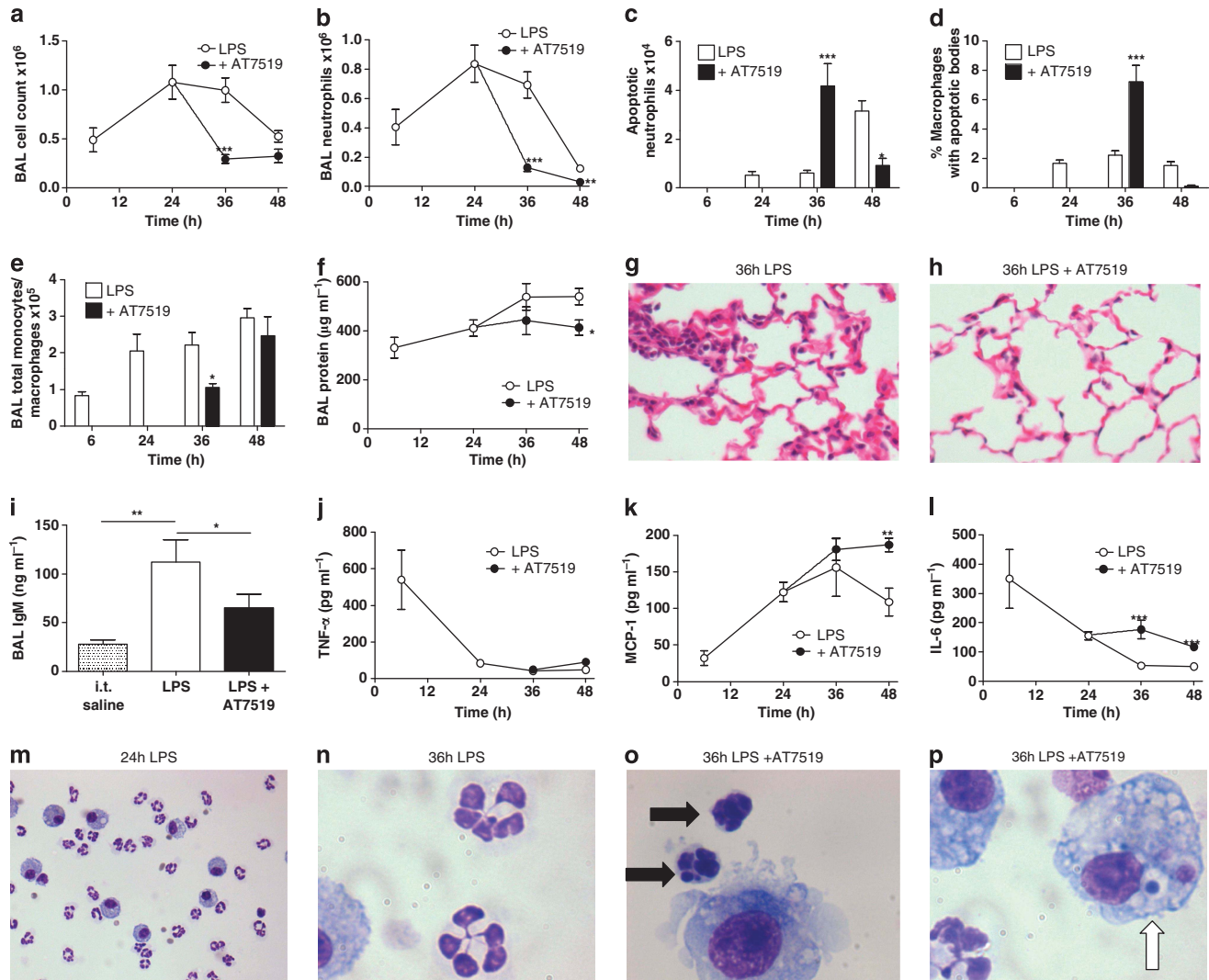


Figure 3 AT7519 accelerates resolution of established lipopolysaccharide (LPS)-mediated lung inflammation. At 24 h after intratracheal (i.t.) administration of LPS, mice received AT7519 (30 mg kg^{-1} intraperitoneal (i.p.)) or vehicle control with acquisition and analysis of bronchoalveolar lavage (BAL) fluid at indicated time points. (a) Total BAL inflammatory cells, (b) neutrophils, (c) apoptotic neutrophils, (d) macrophages containing apoptotic bodies, (e) total monocytes/macrophages, and (f) BAL protein concentration at each time point are shown. Representative tissue sections at 36 h in panel (g) LPS alone and (h) LPS- and AT7519-treated animals (original magnification $\times 200$). (i) BAL immunoglobulin M (IgM) at 48 h after LPS. BAL fluid was analyzed for the inflammatory mediators (j) tumor necrosis factor ($\text{TNF-}\alpha$), (k) chemokine (C-C motif) ligand 2 (CCL-2)/monocyte chemoattractant protein-1 (MCP-1), and (l) interleukin-6 (IL-6). Representative cytocentrifuge preparations of BAL cells are shown (m) at 24 h after LPS (original magnification $\times 200$) showing neutrophil predominant inflammation (n) at 36 h following LPS, control group (original magnification $\times 1,000$), demonstrating normal neutrophil morphology, (o, p) at 36 h after LPS, AT7519-treated group (original magnification $\times 1,000$), showing (o) neutrophils with apoptotic morphology (black arrows) with condensed nuclei and cellular shrinkage and (p) a macrophage containing two apoptotic bodies (white arrow). At each time point, $n = 5\text{--}6$ mice per group, $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ compared with control at the respective time point.

affect the numbers of BAL monocytes/macrophages (Figure 4c) or circulating neutrophils (Figure 4e). AT7519 improved histological appearances with a reduction in the predominantly perivascular inflammation seen in control animals at 48 h (Figure 4f). Importantly, lung bacterial counts were lower following AT7519 treatment, despite AT7519 having no direct effect on *E. coli* growth (Figure 4g–i). No bacteria were recovered from liver or blood at this time point in either group (data not shown). To investigate whether enhanced neutrophil apoptosis was

observed in our *E. coli* model, an earlier time point was studied (30 h; 6 h after AT7519 administration). Flow cytometric analysis of AnnV binding of BAL and interstitial neutrophils ($\text{CD45}^{+ve}/\text{Ly6G}^{+ve}$ cells) revealed increased apoptosis in AT7519-treated animals (Figure 4j–k). At this time point Mcl-1 levels were reduced in BAL cells from AT7519-treated animals, whereas levels of cleaved caspase-3 were increased (Figure 4l). Lung bacterial counts at this earlier time point were identical between control and AT7519-treated animals (130 ± 16 vs. $127 \pm 15 \text{ CFU ml}^{-1}$, $P > 0.05$).

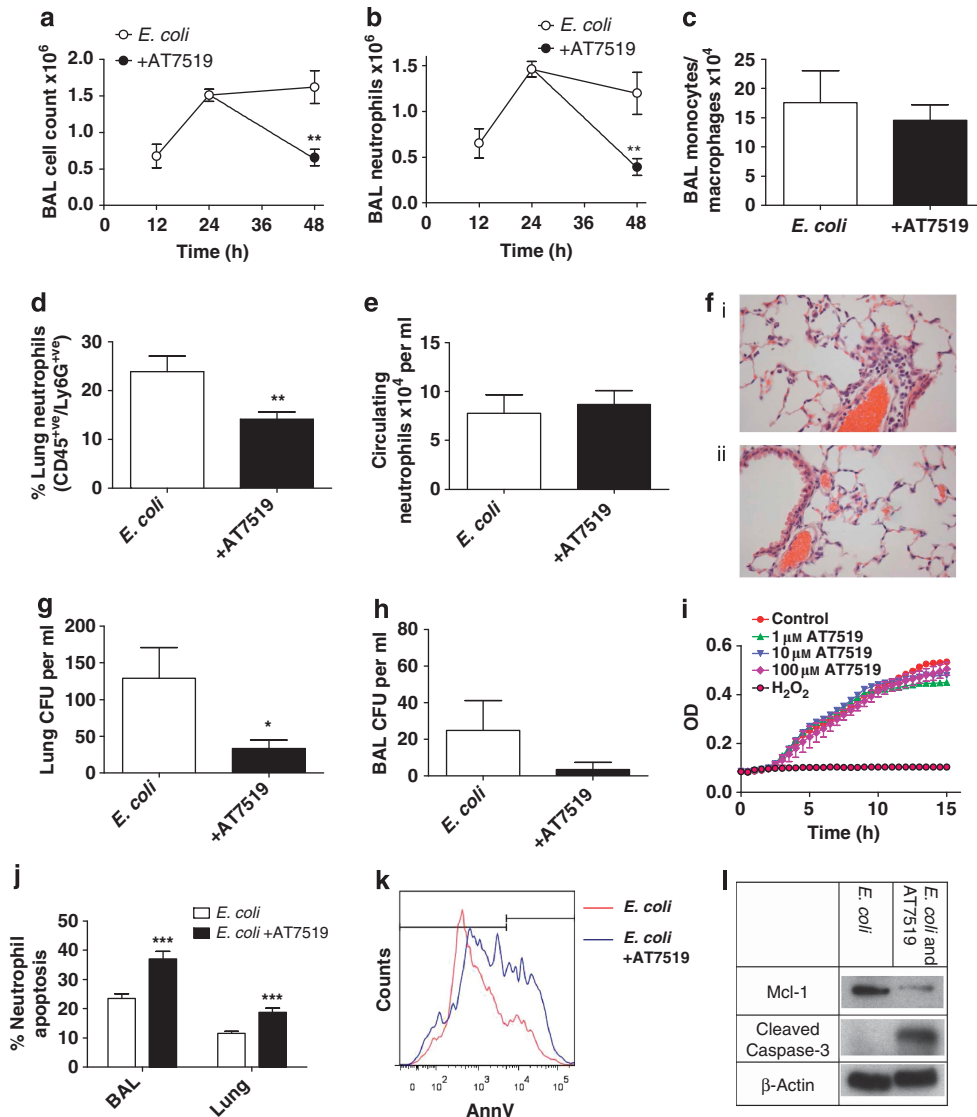


Figure 4 AT7519 accelerates resolution of established *Escherichia Coli*-induced lung inflammation and improves lung bacterial titers. At 24 h after intratracheal (i.t.) administration of 1×10^6 colony-forming units (CFUs) of *E. coli*, mice were treated with AT7519 (10 mg kg^{-1}) or vehicle control. Mice were killed at 48 h and (a) total bronchoalveolar lavage (BAL) inflammatory cells, (b) neutrophils, and (c) monocytes/macrophages determined. (d) The percentage of interstitial lung neutrophils (CD45⁺ve/Ly6G⁺ve) and (e) numbers of circulating blood neutrophils are shown. (f) Lung sections are shown in (i) *E. coli* alone and (ii) *E. coli* and AT7519-treated animals (original magnification $\times 400$). (g) Lung and (h) BAL bacterial titers were determined by overnight culture. (i) The growth kinetics of *E. coli* *in vitro* with and without AT7519 are shown, with hydrogen peroxide (H₂O₂) included as a positive control ($n = 3$). (j–l) Mice received *E. coli* alone (1×10^6 CFUs) or *E. coli* plus AT7519 (10 mg kg^{-1} at 24 h) with mice killed at 30 h (6 h after AT7519). (j) Analysis of BAL and interstitial neutrophil apoptosis was performed by Annexin-V (AnnV) binding of CD45⁺ve/Ly6G⁺ve cells, with a representative overlay histogram of AnnV binding of BAL neutrophils shown in panel k. (l) BAL fluid cells collected at 30 h (6 h after AT7519 in *E. coli* alone or *E. coli* plus AT7519) were lysed and myeloid cell leukemia 1 (Mcl-1; 40 kDa), cleaved caspase-3 (17/19 kDa), and β -actin (42 kDa) levels determined by western blotting. At each time point, $n = 8$ –9 mice per group were used, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with control.

Downregulation of Mcl-1 *in vivo* enhances resolution of established neutrophilic inflammation

To further investigate whether Mcl-1 downregulation *in vivo* was involved in the proresolution effects of AT7519, we used a pharmacological inhibitor of the proteasome (bortezomib)²⁰ to reduce Mcl-1 degradation. Co-treatment with bortezomib caused a concentration-dependent inhibition of AT7519-induced human neutrophil apoptosis *in vitro* secondary to maintenance of Mcl-1 levels (Figure 5a–d). Maintenance of Mcl-1 levels also prevented the appearance of cleaved caspase-9

and caspase-3 (Figure 5d). Bortezomib also delayed spontaneous neutrophil apoptosis consistent with previous observations.¹¹ *In vivo* treatment with bortezomib inhibited the proresolution effects of AT7519 as it prevented AT7519-mediated reductions in total BAL cells and neutrophils, and prevented increases in neutrophil apoptosis and macrophage clearance (Figure 5e–h). Bortezomib also blocked AT7519-induced improvements in lung histology (Figure 5i–l). BAL inflammatory cell Mcl-1 levels were reduced *in vivo* by AT7519 treatment, an effect attenuated by concurrent proteasomal

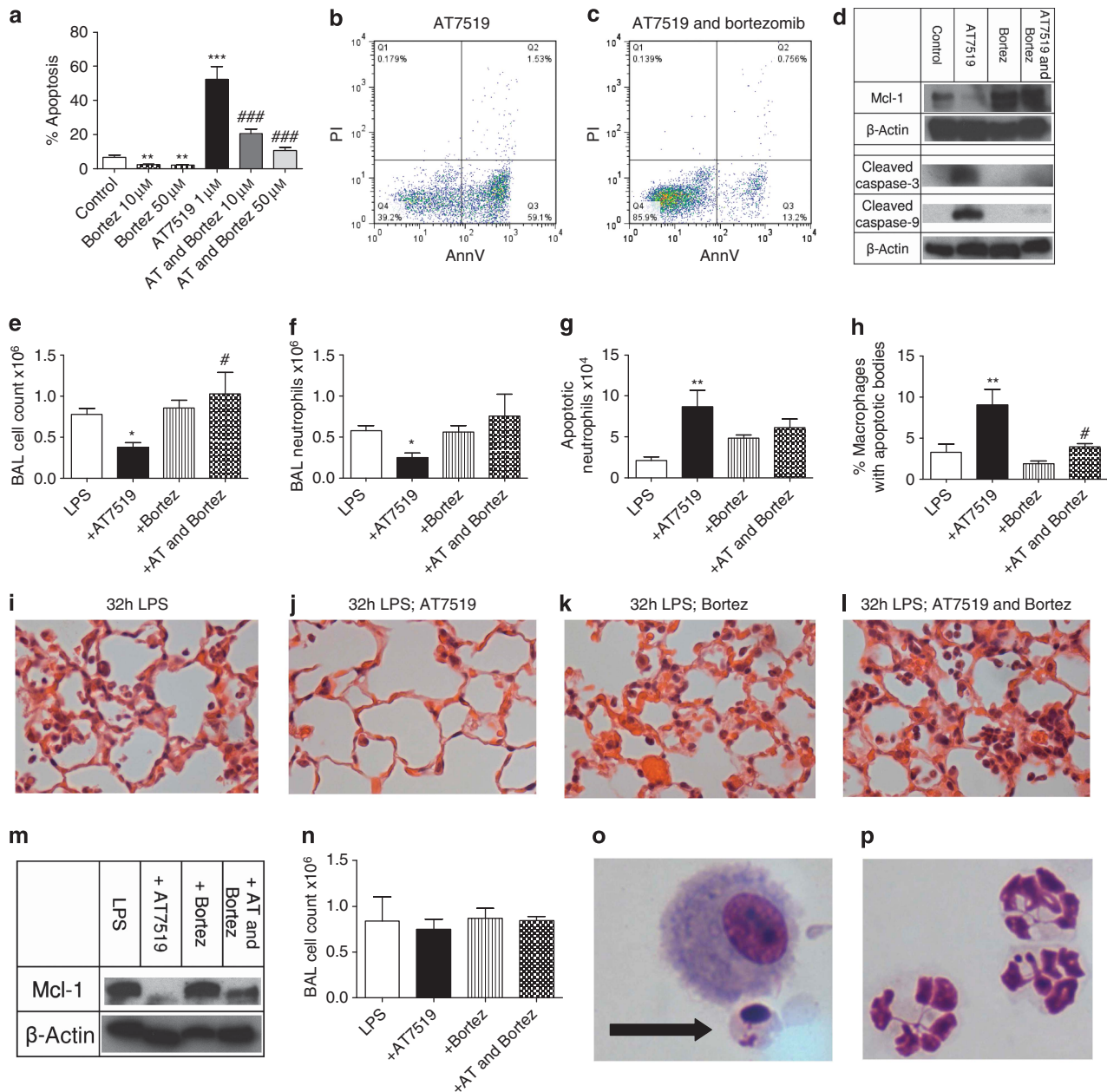


Figure 5 Proteasomal inhibition attenuates AT7519-induced myeloid cell leukemia 1 (Mcl-1) downregulation, neutrophil apoptosis, and resolution of acute lung injury (ALI). **(a)** Neutrophils cultured *in vitro* with bortezomib (Bortez; 10–50 μM), AT7519 (AT; 1 μM), or combinations for 6 h before determination of apoptosis ($n \geq 4$). **(b, c)** Flow plots for **(b)** AT7519 (1 μM) and **(c)** combined AT7519- and Bortezomib (50 μM)-treated neutrophils at 6 h. **(d)** Neutrophils were treated for 4 h with AT7519 (1 μM), bortezomib (10 μM), or AT7519 and bortezomib before western blotting for Mcl-1 (40 kDa), β-actin (42 kDa), cleaved caspase-3 (17/19 kDa), and cleaved caspase-9 (35 kDa). **(e–p)** At 24 h after intratracheal (i.t.) lipopolysaccharide (LPS), mice received AT7519 (30 mg kg⁻¹ intraperitoneal (i.p.)), bortezomib (0.4 mg kg⁻¹ i.p.), combined AT7519 and bortezomib, or vehicle control with analysis of bronchoalveolar lavage (BAL) fluid and histology at 32 h. **(e)** Total BAL inflammatory cells, **(f)** neutrophils, **(g)** apoptotic neutrophils, and **(h)** macrophages containing apoptotic bodies are shown. **(i–l)** Lung tissue sections (original magnification $\times 400$). **(m, n)** BAL fluid cells collected at 28 h after LPS (4 h after AT7519/bortezomib) in control mice and mice treated with AT7519, bortezomib, and combined AT7519 and bortezomib were lysed and **(m)** Mcl-1 levels determined by western blotting. **(n)** Total BAL cells at this earlier 28 h time point are shown. **(o, p)** BAL cells (original magnification $\times 1,000$) at 32 h showing **(o)** an apoptotic cell next to a macrophage (black arrow) in AT7519-treated mice, whereas **(p)** neutrophils from combined AT7519- and bortez-treated mice show normal nonapoptotic morphology. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with control and # $P < 0.05$, ### $P < 0.001$ compared with AT7519 alone.

inhibition (Figure 5m). Total inflammatory cells were unchanged at this early time point (Figure 5n), confirming changes in Mcl-1 levels preceded alterations in inflammatory cells.

To further investigate whether Mcl-1 downregulation was a general mechanism driving resolution of inflammation or whether this was specific to AT7519, we investigated the ability of the natural flavone wogonin, which also reduces Mcl-1 in

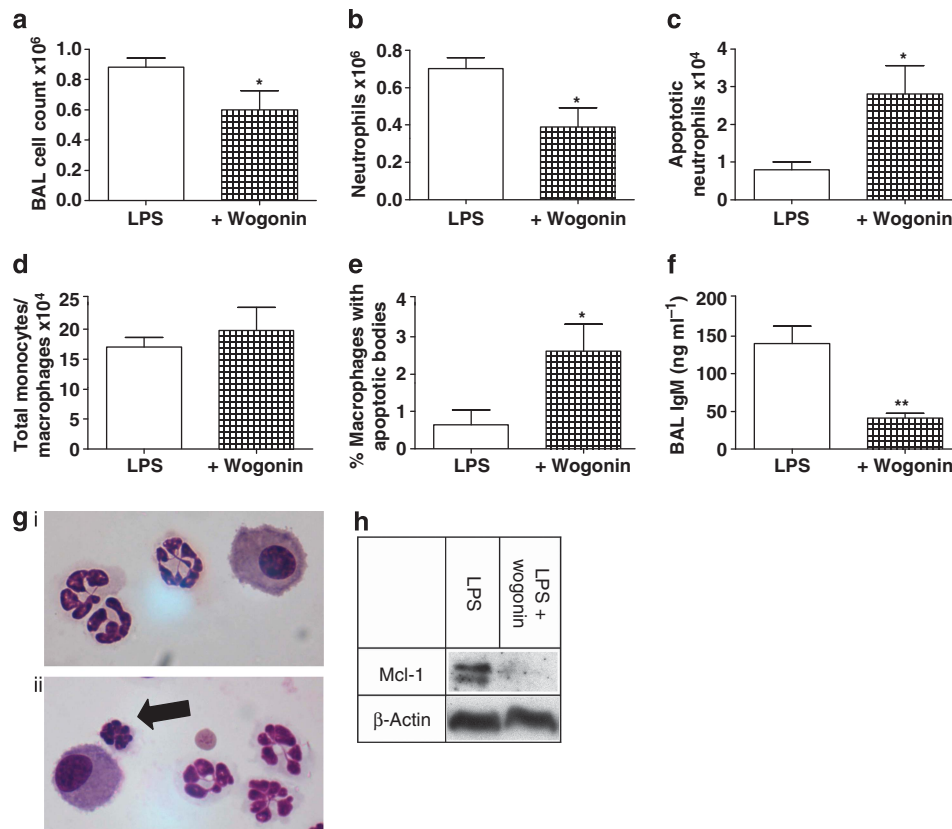


Figure 6 Downregulation of myeloid cell leukemia 1 (Mcl-1) by the flavone wogonin drives neutrophil apoptosis and clearance in established lipopolysaccharide (LPS)-mediated lung inflammation. (**a–g**) Following intratracheal (i.t.) LPS, mice received wogonin (10 mg kg^{-1} intraperitoneal (i.p.)) or vehicle control at 24 h, with acquisition and analysis of bronchoalveolar lavage (BAL) fluid at 32 h (8 h after wogonin). (**a**) Total BAL inflammatory cells, (**b**) neutrophils, (**c**) apoptotic neutrophils, (**d**) total macrophages, (**e**) macrophages containing apoptotic bodies, and (**f**) BAL immunoglobulin M (IgM) are shown. (**g**) BAL cells (original magnification $\times 1,000$) demonstrate (i) normal neutrophil morphology in the LPS-alone group and (ii) a neutrophil with apoptotic morphology (cellular shrinkage and nuclear condensation, black arrow) alongside three neutrophils with normal morphology in the wogonin-treated group. (**h**) BAL cells collected at 28 h after LPS (4 h after wogonin) were lysed before western blotting for Mcl-1 (40 kDa) and β -actin (42 kDa). * $P < 0.05$, ** $P < 0.01$ ($n = 5$ mice per group).

neutrophils *in vitro*,¹¹ to accelerate resolution of lung inflammation. Wogonin, when administered to established neutrophilic inflammation, also caused enhanced resolution of inflammatory cells in BAL, with enhanced apoptosis of neutrophils and subsequent clearance by macrophages and an improvement in alveolar–capillary barrier integrity as measured by BAL IgM (Figure 6a–g). As with AT7519, these changes were preceded by a decrease in Mcl-1 *in vivo* (Figure 6h).

DISCUSSION

Neutrophil apoptosis and subsequent clearance of apoptotic cells are key to the successful resolution of neutrophilic inflammation, with dysregulation of these processes observed in multiple human inflammatory diseases. Therefore, targeted manipulation of defective resolution pathways, by timely apoptosis of neutrophils and subsequent efferocytosis by macrophages, may provide novel therapeutic strategies to treat established mucosal inflammation. Lung injury can occur following direct pulmonary insult such as infection, or be caused by systemic insults including sepsis and trauma.

Subsequent to the inciting insult, uncontrolled inflammation occurs with recruitment and activation of leukocytes as well as increased permeability of the alveolar–capillary membrane central to disease pathogenesis.²¹ Recruited neutrophils contribute directly to the development and progression of lung injury with higher neutrophil BAL counts associated with enhanced mortality in humans,²² and neutrophil depletion or blocking recruitment being protective in animal models of acute respiratory distress syndrome.^{23–25} Here we have shown that pharmacological downregulation of the intracellular prosurvival molecule Mcl-1 drives neutrophil apoptosis and subsequent macrophage efferocytosis *in vitro*, and that Mcl-1 downregulation *in vivo* accelerates resolution of established neutrophil-mediated lung inflammation in mice.

Neutrophils are short-lived cells but their lifespan is markedly extended at sites of inflammation. Proteins from the Bcl-2 family are key controllers of the intrinsic (mitochondrial) pathway of apoptosis with neutrophils expressing both pro- and anti-apoptotic Bcl-2 family members. The key role that Bcl-2 proteins play in both neutrophil apoptosis and control of inflammation has been previously highlighted by

transgenic overexpression of Bcl-2 in mice that leads to delayed neutrophil apoptosis, increased brain edema, hemorrhage, and poorer outcomes during a model of meningitis.²⁶ Furthermore, loss of proapoptotic Puma leads to lethal sepsis in a model of *Streptococcus pneumoniae* infection.²⁷ Under pathophysiological circumstances, neutrophils can also dynamically alter Bcl-2 family member expression with cytokine-mediated decreases in proapoptotic Bax described as a way of enhancing neutrophil longevity during proinflammatory conditions.²⁸ This finding is surprising, given that proapoptotic proteins like Bax have relatively long half-lives, and that delayed neutrophil apoptosis usually requires the ongoing synthesis of new proteins.^{12,16} This suggests that synthesis of new antiapoptotic proteins may be equally important for the delayed neutrophil apoptosis seen during inflammation. Increased Mcl-1 may fit this role given that several factors that extend neutrophil lifespan (e.g., granulocyte-macrophage colony-stimulating factor and hypoxia) also increase Mcl-1 expression.^{29,30} In addition, as Mcl-1 has a short half-life ($\sim 3\text{ h}^{31}$), levels can be rapidly and dynamically regulated by both the host and pharmacological interventions.

Here we have demonstrated that the CDK inhibitor AT7519 induces rapid, caspase-dependent human and mouse neutrophil apoptosis *in vitro* by downregulation of Mcl-1, and overrides survival signals from Gram-negative (LPS) and Gram-Positive derived (LTA and PepG) bacterial products. Mcl-1 levels were reduced by AT7519 by 4 h, immediately before AT7519-induced apoptosis and the appearance of activated caspase-3. Although Mcl-1 can be downregulated by activated caspases in neutrophils, possibly acting as a positive feedback loop, AT7519 reduced Mcl-1 even in the presence of the caspase inhibitors z-VAD or Q-VD. This confirms Mcl-1 loss as an early and caspase-independent event in neutrophil apoptosis. We also observed that Mcl-1 loss induced by siRNA was by itself sufficient to drive apoptosis in a neutrophil precursor cell line.

Subsequent to induction of neutrophil apoptosis, it is essential that apoptotic neutrophils are phagocytosed to prevent progression to secondary necrosis with release of histotoxic contents and consequent enhanced tissue injury. In addition, the ingestion of apoptotic neutrophils by surrounding macrophages serves as a signal to modulate cytokine and proresolving lipid production, with alterations in lipid mediator production important in the resolution of both sterile inflammation and bacterial infection.^{19,32,33} Although treatment of MDMs with AT7519 led to reductions in Mcl-1, similar to others^{34,35} we found that this in itself did not induce MDM apoptosis. This is in contrast to previous reports where delivery of Mcl-1 antisense oligonucleotides to human macrophages has been shown to directly induce apoptosis, concurrent with a reduction in Mcl-1 levels.³⁶ In addition, Mcl-1 loss in macrophages has been associated with induction of apoptosis, although this may be also because of the observed synchronous upregulation of an Mcl-1 splice variant with proapoptotic activity.³⁷ We also observed that MDMs that had reduced Mcl-1 were still functional and able to phagocytose apoptotic neutrophils *in vitro*. However, loss of Mcl-1 enhances

macrophage apoptosis upon subsequent phagocytosis of bacteria^{34,37} that may explain the transient reduction in macrophage numbers seen *in vivo* following AT7519 treatment and phagocytosis of apoptotic neutrophils.

Although the myeloid-specific Mcl-1 knockout mouse has low levels of circulating neutrophils, demonstrating a role for Mcl-1 in neutrophil survival in noninflammatory circumstances, it is unclear whether this is because of a defect in neutrophil survival or because of a blockage in granulocyte maturation.^{34,35} Furthermore, the role of Mcl-1 in controlling the resolution of acute inflammation and infection *in vivo* has not been previously investigated. This is an important point as stimulated, inflammatory neutrophils are known to have altered control of apoptosis induction. For example, TRAIL-deficient mice show normal circulating neutrophil numbers and normal constitutive neutrophil apoptosis but demonstrate impaired apoptosis when exposed to an inflammatory environment.³⁸ Similarly, the apoptosis-inducing effect of steroids on the closely related eosinophil granulocyte is lost in the presence of factors such as IL-5 that are present at high concentrations at sites of allergic inflammation.³⁹ To investigate dynamic alterations of Mcl-1 as a potential therapeutic strategy for enhancing resolution of established inflammation *in vivo*, we utilized three separate models of neutrophil-dominant inflammation and two separate pharmacological agents. Mcl-1 downregulation, by either the CDK inhibitor AT7519 or the flavone wogonin, led to enhanced resolution of inflammation with induction of neutrophil apoptosis and subsequent efferocytosis *in vivo*. Conversely, the prevention of AT7519-induced downregulation of Mcl-1 by proteasomal inhibition attenuated both AT7519-induced neutrophil apoptosis *in vitro* and AT7519-induced resolution of inflammation *in vivo*. Although proteasomal inhibition did not itself promote inflammation and accumulation of neutrophils, this is likely to represent the early time point studied (24–32 h), as increased numbers of apoptotic neutrophils were not observed in control animals until 48 h. Importantly, the strategy of dynamically reducing Mcl-1 did not rely on either pretreatment or administration close to the onset of injury as we demonstrated proresolution effects even at the peak of inflammation, a clinically relevant time point. Although absolute changes in rates of apoptosis were low relative to changes in total neutrophil numbers, it is documented in a number of diverse experimental and clinical settings that small changes in rates of apoptosis can have dramatic changes in cellular populations over time,^{14,38} most likely because of rapid recognition and phagocytosis of apoptotic cells. In addition to changes in inflammatory cell numbers, improvements were also seen in tissue injury and markers of alveolar–capillary barrier integrity. Although improvements in BAL protein lagged behind reductions in the neutrophilic infiltrate, this is consistent with current understanding whereby successful resolution of inflammatory cell infiltrate accelerates recovery of the alveolar epithelium whose function is essential for successful removal of proteinaceous alveolar edema.²¹ Furthermore, we observed reduced bacterial lung content in our *E. coli* model despite a

reduction in inflammatory cell infiltrate and no direct effect of AT7519 on bacterial growth *in vitro*. Whether Mcl-1-driven neutrophil apoptosis and efferocytosis stimulate enhanced production of endogenous specialized proresolving mediators with antimicrobial effect¹⁹ or whether loss of Mcl-1 may enhance phagocytosis induced cell death with consequent enhanced bacterial clearance³⁷ requires further investigation. Certainly, enhanced neutrophil apoptosis was observed before changes in lung bacterial titers. Corroboration of the role of Mcl-1 in controlling resolution of inflammation by use of knockout or transgenic animals is highly desirable; although a myeloid-specific Mcl-1 knockout mouse exists,³⁵ this is essentially a neutropenic mouse and as such is not a good model system for studying neutrophilic inflammation. To the authors' knowledge, a myeloid-specific Mcl-1 transgenic mouse does not yet exist but would be ideal for future experiments.

Analysis of cytokines revealed higher IL-6 levels in the LPS model with AT7519 treatment, whereas IL-6 was unchanged in the LTA/PepG model. Although IL-6 is considered necessary for initiation of the acute-phase response, IL-6 and its receptor have pleiotropic effects with both proinflammatory and anti-inflammatory activity, with IL-6 having been shown to act in an anti-inflammatory manner in previous models of LPS-induced lung inflammation.^{40,41} Higher monocyte chemotactic protein-1 levels were also observed at later time points with AT7519 treatment, a cytokine that enhances the recruitment of monocyte/macrophage cells with an enhanced phagocytic capacity for apoptotic cells.^{42,43} In addition, IL-10 was elevated in the LTA/PepG model at 48 h, a cytokine essential for resolution of lung inflammation.⁴⁴ Whether macrophage cellular phenotype is altered *in vivo* following Mcl-1 down-regulation, with effects on subsequent tissue repair and fibrosis, requires further investigation.

In conclusion, we have demonstrated that dynamic down-regulation of the prosurvival protein Mcl-1 drives neutrophil apoptosis and subsequent macrophage clearance, and in doing so enhances the resolution of both sterile and infection-mediated established lung inflammation. As such, pharmacological agents that target reductions in inflammatory cell Mcl-1 may offer new therapeutic approaches for the treatment of infection-associated lung inflammation and other neutrophil-dominant human inflammatory diseases.

METHODS

Human neutrophil isolation. Neutrophils were isolated from the blood of healthy donors (Lothian Research Ethics Committee, 08/S1103/38) and resuspended in Iscove's modified Dulbecco's medium (PAA, Pasching, Austria) with 5% autologous serum at 5×10^6 per ml (37 °C, 5% CO₂) as previously described.¹¹ Neutrophil purity was routinely >96% with 1–3% contaminating eosinophils. Neutrophils were incubated with AT7519 (Astex Therapeutics, Astex, Cambridge, England, UK), *E. coli*-derived LPS (O127:B8, Sigma, Dorset, UK), *Staphylococcus aureus*-derived LTA (Sigma), *S. aureus*-derived PepG (Sigma), z-VAD-fmk (v-VAD; Bachem, Bubendorf, Switzerland), Q-VD-Oph (Q-VD; R&D Systems, Abingdon, UK), and bortezomib (Selleckchem, Munich, Germany) as per the figure legends.

Mouse bone marrow-derived neutrophil isolation. Bone marrow, which contains large numbers of functionally active neutrophils,⁴⁵ was removed from femurs and tibias collected from C57Bl6 mice aged 8–12 weeks. Bone marrow was disaggregated and erythrocytes lysed by hypotonic saline before granulocyte isolation via a discontinuous Percoll (GE Healthcare, Little Chalfont, UK) gradient as previously described.⁴⁶ Following isolation, neutrophils accounted for $77.7 \pm 2.1\%$ of the cellular population (determined by morphology).

Assessment of cell viability and apoptosis

Light microscopy. Cytocentrifuge preparations stained with Diff-Quick (Gamidor, Didcot, UK) were assessed for morphological changes consistent with apoptosis.

Annexin-V/propidium iodide. Viability, apoptosis, and necrosis were assessed using fluorescein isothiocyanate-labeled Annexin-V (Roche, Welwyn Garden City, UK) and propidium iodide (PI, Sigma) with samples run on a BD (Oxford, UK) FACScan or LSR Fortessa as described previously.¹¹ AnnV⁺ve/PI⁻ve cells are considered apoptotic, PI⁺ve cells necrotic, and AnnV⁻ve/PI⁻ve cells viable. For mouse BAL flow cytometry analysis of apoptosis, BAL cells were stained with Ly6G (Biolegend, Cambridge, UK) and F4/80 (AbD Serotec, Oxford, UK) with AnnV binding of Ly6G⁺ve/F4/80⁻ve cells analyzed. For MDM apoptosis, the fungal metabolite gliotoxin (Sigma) was used as a positive control.

Hypodiploid peak. Cells ($50 \mu\text{l}$ of 5×10^6) were incubated for 20 min with $250 \mu\text{l}$ of a solution containing $25 \mu\text{g}$ PI, 4.84 mg sodium citrate (Sigma), and $0.5 \mu\text{l}$ Triton X-100 (Sigma) diluted with water. Samples were run on a BD FACScan flow cytometer with apoptotic nuclei appearing as a broad hypodiploid DNA peak below the narrow peak of nuclei with normal DNA content.⁴⁷

DNA fragmentation assay. Isolated DNA from 2.5×10^6 neutrophils (Promega, Southampton, UK; genomic DNA isolation system) was run on a 1.5% agarose gel precast with $5 \mu\text{l}$ GelRed (Biotium, Hayward, CA) and visualized by UV lamp.

HL-60 cell culture and siRNA transfection. HL-60 cells (ATCC, Middlesex, UK) were cultured in RPMI-1640 (PAA) containing 10% heat-inactivated fetal calf serum, 2 mM L-glutamate, penicillin (100 U ml^{-1}), and streptomycin (100 U ml^{-1}). Cells were transfected with 200 nM Mcl-1 siRNA or scrambled sequence siRNA (Dharmacon, Rockford, IL) using an Amaxa (Cambridge, UK) Nucleofector using Nucleofector Kit V and setting T-019. HL-60 cell survival and apoptosis was assessed by changes in forward and side scatter profiles as well as assessment of hypodiploid peak.⁴⁷

Western blotting. Following incubation with compounds as described in the figure legends, cells were lysed by 0.1% Nonidet P40 in the presence of protease inhibitors as previously described.¹¹ Following BCA protein assay (Thermo Scientific, Loughborough, UK), lysates were run on a 12% sodium dodecyl sulfate gel and transferred onto polyvinylidene difluoride (Immobilon-P, Millipore, Bedford, MA), blocked with 5% wt/vol dried milk/Tris-buffered saline/0.1% Tween-20 before overnight incubation at 4 °C (or 1 h at room temperature for β -actin) with primary antibodies for Mcl-1 (1:500, Santa Cruz Biotechnology, Santa Cruz, CA), cleaved caspase-3 (1:1000, Cell Signalling, Danvers, MA), glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:10,000, Sigma), or β -actin (1:20,000, Sigma). Blots were then incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (Dako, Cambridgeshire, UK) before incubation with ECL-prime (GE Healthcare), and exposed to BioMax light-sensitive film (Kodak, Rochester, NY) and processed through an X-ray developer (Xograph Imaging Systems, Tetbury, UK).

Assessment of MDM phagocytosis of apoptotic neutrophils. The 7-day-old MDMs (differentiated in the presence of 100 nM dexamethasone⁴⁸) were used to assess phagocytosis of cell tracker green-labeled (1 µg per 10⁶ neutrophils, Invitrogen, Carlsbad, CA) apoptotic neutrophils as previously described.⁶ Neutrophils treated with 1 µM AT7519, 10 µM Q-VD, or combinations as per figure legends for 4.5 h were washed and then 7.5×10^5 co-incubated with MDMs for 1 h. After 10 min of incubation with trypsin-EDTA, resuspended cells were analyzed by flow cytometry. Cytochalasin D (Calbiochem, San Diego, CA) was used to inhibit MDM phagocytosis. In separate experiments, MDMs were incubated with AT7519 for 6–8 h before assessment of phagocytic capacity.

In vivo acute lung inflammation. Female mice (8–12-week-old C57Bl6) were injected i.t. with 1 µg *E. coli*-derived (O127:B8) LPS in 50 µl sterile saline, combined *S. aureus*-derived LTA (150 µg) and PepG (50 µg), or 1×10^6 CFUs of *E. coli* (serotype ATCC25922) grown to log phase. At 24 h after i.t. challenge, animals received either 30 mg kg⁻¹ AT7519 intraperitoneally (10 mg kg⁻¹ for *E. coli* experiments) in 200 µl sterile saline or vehicle control. For wogonin experiments animals received 10 mg kg⁻¹ wogonin intraperitoneally in dimethyl sulfoxide or vehicle control. For experiments with bortezomib, animals were pretreated for 30 min at 23.5 h with 0.4 mg kg⁻¹ intraperitoneal bortezomib in dimethyl sulfoxide or vehicle control before administration of AT7519. At indicated time points, lungs were lavaged with three aliquots of 800 µl sterile ice-cold saline. BAL fluid was centrifuged at 300 g for 5 min with supernatant from the first lavage stored for biochemical analysis. BAL total protein was measured by bicinchoninic acid assay (Pierce, Northumberland, UK), BAL IgM by enzyme-linked immunosorbent assay (ELISA; eBioscience, Hatfield, UK), and cytokines (TNF-α, IL-6, CCL-2, and IL-10) were measured by ELISA (R&D Systems). BAL fluid cells were cytocentrifuged and stained using Diff-Quick and differential counts performed (≥ 450 cells counted per slide). Separate lungs for histology were fixed with 10% formalin (Sigma) before hematoxylin and eosin staining. For analysis of interstitial lung neutrophils, mice were perfused with 20 ml phosphate-buffered saline and the right lung digested in collagenase D (Roche) before incubation with anti-CD45 (Biolegend) and anti-Ly6G (Biolegend), with flow analysis in the presence of flow-check fluorospheres (Beckman Coulter, Brea, CA). Bacterial counts were determined following overnight incubation on LB agar plates whereas *in vitro E. coli* growth in the presence of AT7519 was determined by absorbance (570 nm) every 30 min. Resolution intervals (R_i) were calculated as previously described.^{18,19} Briefly, these were calculated as $T_{50} - T_{max}$, where T_{max} is the time where neutrophil numbers were at their maximal numbers (Ψ_{max}) and T_{50} is the time after T_{max} where neutrophil numbers were half-maximal.

Statistical analysis. Results, expressed as mean \pm s.e.m., were analyzed using GraphPad Prism by two-way analysis of variance with a Bonferroni test, one-way analysis of variance with a Newman-Keuls multiple comparison test, or an unpaired *t*-test as appropriate. Significance was accepted at $P < 0.05$.

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at <http://www.nature.com/mi>

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AUTHOR CONTRIBUTIONS

C.D.L., D.A.D., M.A.T., S.F., J.A.M., K.C.A., C.R., R.D., and A.G.R. performed research; C.D.L., D.A.D., N.H., C.H., R.D., and A.G.R. designed research; C.D.L., D.A.D., and A.G.R. wrote the manuscript.

DISCLOSURE

The authors declared no conflict of interest.

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