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Azithromycin Increases Survival and Reduces Lung Inflammation in Cystic Fibrosis Mice

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Abstract

Objective and Design—Azithromycin (AZM) has been used as an anti-inflammatory agent in the treatment of cystic fibrosis (CF), particularly those with chronic infection with *P. aeruginosa* (PA). To investigate mechanisms associated with the beneficial effects of AZM in CF, we examined bacterial load, cytokine levels, and clearance of inflammatory cells in CF mice infected with mucoid PA and treated with AZM.

Methods—Gut-corrected *Cfr^{tm1Unc}-TgN(FABPCFTR)*#Jaw CF mice infected with an alginate-overproducing PA CF-isolate were treated with AZM or saline and examined for survival of animals, lung bacterial load, inflammation, cytokine levels and apoptotic cells up to 5 days post-infection.

Results—Administration of AZM (20 mg/kg) 24 h after the infection improved 5-day survival to 95% compared to treatment with saline (56%). AZM administration was associated with significant reductions in bacterial load, decreased lung inflammation and increased levels of IFN- γ . AZM increased macrophage clearance of apoptotic neutrophils from the lung.

Conclusion—AZM enhances bacterial clearance and reduces lung inflammation by improving innate immune defense mechanisms in CF mice.

Keywords

P. aeruginosa; lung infection; macrolide; macrophage; innate immunity

Introduction

The inflammatory response in cystic fibrosis (CF) is characterized by the excess expression of inflammatory cytokines coupled with chronic endobronchial neutrophilia, leading to bronchiectasis and a progressive decline in pulmonary function [1]. *Pseudomonas aeruginosa* (PA) causes persistent infection in CF patients. The ability of this organism to convert into a mucoid phenotype and form biofilm is an important feature in the

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pathogenesis of lung disease in CF patients. Mucoid PA (MPA) resists detection and phagocytosis by immune cells [2-4] and has increased tolerance to antibiotics [5]. PA also impairs neutrophil bactericidal function, accelerates neutrophil death and inhibits the clearance of apoptotic neutrophils by macrophages [6]. This results in decreased pool of viable neutrophils which can phagocytose and kill bacteria. Release of elastase from necrotic neutrophils can cause further tissue damage and recruit more neutrophils to airways [6, 7].

Macrolides have been used successfully in the treatment of diffuse pan-bronchiolitis, a lung condition associated with chronic endobronchial infection with PA and other bacteria. Macrolide therapy significantly improves lung function in CF patients [8, 9]. In azithromycin (AZM) clinical trials, CF patients receiving AZM showed improved viscoelasticity of sputum [10], decreased sputum PA load, fewer pulmonary exacerbations and improved lung function [11-13]. However, the precise mechanisms by which AZM improves pulmonary status in patients with CF are unclear.

In vitro studies demonstrate that macrolides modulate pro-inflammatory responses from bronchial epithelial cells and macrophage function. Clarithromycin suppresses early phase IL-8 production from normal airway epithelial cells in response to PA flagella [14]. AZM selectively reduces TNF- α expression in CF airway epithelial cells [15]. AZM treatment increases the phagocytosis of apoptotic cells by macrophages, implying that this macrolide may augment clearance of apoptotic cells from the lung [16]. In addition to these anti-inflammatory effects, AZM inhibits transcription of PA genes responsible for quorum-sensing, which in turn controls biofilm formation and regulation of virulence factors [17]. AZM also inhibits assembly of type IV pili, thus restricting twitching motility required for biofilm formation, and increasing susceptibility of bacteria to phagocytosis [18].

Macrolide therapy decreases bronchoalveolar lavage neutrophils in patients with chronic airways disease with PA infection [19]. Using a mouse model of chronic endobronchial PA infection, we have found that treatment with AZM attenuates neutrophil recruitment in the lung [20]. Macrolide treatment of mice chronically infected with PA also normalizes lymphocyte counts and CD4⁺/CD8⁺ ratio, while reducing lung levels of IL-1 β , IL-2, IL-4, IL-5, IFN- γ , and TNF- α [21, 22]. Recently, AZM was shown to reduce spontaneous and LPS-induced acute inflammation in CF mice [23] and decrease bacterial load, neutrophil infiltration and lung pathology in PA-infected, gut-corrected CF mice by blocking quorum-sensing and biofilm formation [24]. The latter study employed a novel model of chronic endobronchial infection in which mice are infected with PA suspended in alginate [25]. This model offers an advantage over previous endobronchial infection models, which employ agarose beads to harbor bacteria, thereby preventing clearance of PA from the airways. In the present study, we used this model of PA infection to examine the effect of AZM on the pulmonary inflammatory response in CF mice. We hypothesized that AZM attenuates the inflammatory response to MPA infection in CF mice by augmenting the clearance of bacteria and infiltrating neutrophils from the lungs.

Materials and Methods

Bacteria and growth conditions

NH57388A, a stable MPA isolate from a CF patient, was kindly provided by Dr. N. Hoffman (University of Copenhagen, Denmark) [25]. Bacteria were maintained as a glycerol stock at -80°C and subcultured on *Pseudomonas* isolation agar (Difco Laboratories, Detroit, MI). For infection, bacteria were grown in tryptic soy broth overnight, harvested by centrifugation and finally suspended in purified alginate to a required concentration based on OD₆₀₀ (1 O.D. unit is equivalent to 1×10^9 CFU/ml). The actual concentration of bacteria in a suspension was determined by plating.

Isolation and purification of alginate

PA alginate was isolated as described previously [26, 27] with some modifications. Briefly, the MPA isolate NH57388A was grown in 200 ml of *Pseudomonas* isolation media containing 1% glycerol for 36 h. Culture supernatant was harvested by centrifugation at $25,000 \times g$ for 30 min at 4°C and incubated at 80°C for 30 min. Alginate was precipitated by adding three volumes of ethanol, spooled with a sterile glass rod, washed three times with alcohol and dried under a vacuum. Alginate was dissolved in sterile PBS at 5 mg/ml, treated with proteinase K for 4 h at 56°C, and incubated at 80°C for 30 min to inactivate the enzyme. Alginate was precipitated and washed again with ethanol as described above and lyophilized. This partially purified alginate was redissolved in sterile PBS to 30 mg/ml by dry weight. For infection of mice, bacteria were suspended in 10 mg/ml alginate.

Animals

Ten to twelve week-old female homozygous CF mice, strain *Cfr^{tm1/Unc}*-TgN(FABPCFTR)Jaw/J, were purchased from Case Western Reserve University Animal Resource Center (Cleveland, OH). Mice were maintained in a specific pathogen-free barrier facility in microisolator cages throughout the experiment. Mice were acclimatized for 2-3 weeks before infection. All procedures were approved by the Animal Ethics Committee of the University of Michigan.

Infection and treatment of animals

Mice were anesthetized with a mixture of ketamine (50 mg/kg) and xylazine (1 mg/kg) and infected with 50 µl of alginate alone (sham) or alginate containing MPA (5×10^6 CFU) by the intratracheal route. After 24 hours, MPA-infected animals were treated with 100 µl of saline or saline containing AZM (20 mg/kg). The AZM dose was chosen based on our previous pharmacokinetics study, which showed plasma concentration of AZM equivalent to that found in humans treated with AZM [20]. Mice were sacrificed at 3 or 5 days post-infection by intraperitoneal injection of 0.3 ml of 30% pentobarbital. Lung homogenates from 7 to 9 mice per group were used to determine lung bacterial load, myeloperoxidase activity and cytokine levels. BAL fluid was obtained from 5 animals per group. Lungs from 3 mice per group were processed for histology.

Lung bacterial load

Lungs were harvested aseptically and homogenized in sterile PBS. A ten-fold serial dilution of lung homogenates was plated on *Pseudomonas* isolation agar.

Analysis of lung cytokines and myeloperoxidase activity

Lung homogenates were prepared in the presence of protease inhibitors and centrifuged. Supernatant cytokines were analyzed by bioplex immune assay (BioRad, Hercules, CA). Lung myeloperoxidase activity was quantified by a method described previously [28].

Bronchoalveolar lavage (BAL)

Total and differential cell counts in BAL were determined by staining cytopins with Diff Quick (Dade Behring, Newark, DE), as described previously [29, 30].

Histopathology

Lungs were inflation fixed via the trachea with 10% formalin overnight and embedded in paraffin. Lung sections (5 μ m thick) were stained with hematoxylin and eosin or periodic acid Schiff's reagent.

Apoptotic cells in lungs

Paraffin sections of lungs were deparaffinized, rehydrated and stained for apoptotic cells by using a DeadEnd Fluorometric TUNEL assay kit (Promega, Madison, WI) following the manufacturer's instructions. Briefly, sections were treated with proteinase K to permeabilize the cells, and the fragmented DNA in apoptotic cells was labeled by catalytically incorporating fluorescein-12-dUTP at the 3'-hydroxyl ends of fragmented DNA using the enzyme terminal deoxynucleotidyl transferase. Sections were counterstained with DAPI nuclear stain. The fluorescein-dUTP-labeled DNA was then visualized by confocal microscopy. To quantify the phagocytic capacity of macrophages, BAL cells were stained with DeadEnd Fluorometric TUNEL assay as above. Apoptotic cells were quantified by flow cytometry and expressed as the percentage of cells with apoptotic nuclei. Cytopins of stained cells were prepared and first 200 macrophages were counted for the presence of intracellular apoptotic bodies.

Killing of biofilm bacteria by neutrophils

An enriched population of neutrophils was obtained by lavaging the peritoneal cavity with PBS, 3 h after intraperitoneal injection of thiglycollate [31]. After lysis of erythrocytes in ACK lysis buffer (Invitrogen), white blood cells were washed once with PBS, suspended in RPMI, and purity of the neutrophil population determined by staining cytopins with Diff-quick. Neutrophil purity was > 90%. Mucoid PA was grown as a biofilm in 96 well plates, as described [32]. Planktonic bacteria were removed by washing once with sterile PBS and incubated with AZM (0 to 1000 ng/ml) in the presence or absence of 1×10^5 neutrophils suspended in RPMI for 3 h at 37°C. Media from the wells was collected for determination of density of planktonic bacteria, and biofilm mass was determined by crystal violet staining [32].

Statistical analysis

Because sample sizes were not similar between groups (due to decreased survival in an experimental group infected with MPA and treated with PBS), results are expressed as geometric means $\pm$ SEM. Data were analyzed by using SigmaStat statistical software (Systat Software, San Jose, CA). ANOVA with Student's *t* test or Tukey-Kramer post-hoc analysis was performed as appropriate to compare groups and a *P* value <0.05 was considered significant.

Results

Survival of MPA-infected CF mice

To determine the effect of AZM on the survival of MPA-infected CF mice, mice were infected with MPA, treated with either saline or AZM daily starting at 24 h post-infection, and monitored for mortality until day 5. Around 4% of the infected animals died 24 h post-infection, *i.e.*, before starting treatment. In MPA/saline group, mortality reached 54% by 5 days after infection (Figure 1A). The surviving mice in this group appeared very ill with ruffled skin, lethargy and weight loss. In contrast, animals in MPA/AZM group showed improvement in their condition with time, and only 5% of the mice died. Animals treated with saline or alginate showed 100% survival.

Bacterial burden in the lungs

Mice infected with alginate alone or MPA were sacrificed after 24 hours to determine initial bacterial load. As expected, sham-infected mice did not show bacteria in their lungs, whereas MPA-infected mice showed $9.6 \times 10^6 \pm 1.3 \times 10^6$ CFU/lung, indicating persistence of bacteria in these mice at day 1 post-infection. Animals in MPA/saline group showed a significantly higher bacterial load in the lungs at both 3 and 5 days than the MPA/AZM group (Figure 1B). In contrast, 9 out of 11 mice in the AZM-treated group cleared bacteria from their lungs 5 days post infection. These results indicate that AZM augments bacterial clearance.

Inflammatory cells in BAL fluid

BAL was performed on sham- or MPA-infected groups at 24 h post-infection, and on MPA-infected mice treated with saline or AZM at 3 or 5 days post-infection. At 24 hours post-infection, MPA-infected mice showed significantly more neutrophils than the sham-infected animals (Figure 2A). Saline treatment of MPA-infected mice did not affect neutrophil number at either 3 or 5 days post-infection. In contrast, AZM treatment decreased the number of neutrophils at both 3 and 5 days post-infection compared to MPA/saline group. The difference was statistically significant at 5 days post-infection. We also observed a small but significant increase in number of lymphocytes in the MPA/AZM group at both 3 and 5 days post-infection compared to the MPA/saline group.

MPO activity was significantly increased in mice infected with MPA compared to sham-infected animals 24 h post-infection (Figure 2B). Treatment of MPA-infected animals with saline did not significantly reduce lung MPO activity. In contrast, AZM treatment

significantly reduced MPO activity at 3 d, and levels returned to normal by 5 days, consistent with neutrophil counts from the BAL fluid.

Cytokine analysis

Pro-inflammatory cytokines were measured in lung homogenates by multiplex immune assay. Twenty-four h after infection with MPA, mice showed significant increases in the proinflammatory cytokines IL-1 β , IL-6, IL-17 and TNF- α , as well as the chemokines mouse growth related oncogene - α (KC) and monocyte chemoattractant protein (MCP)-1, compared to sham-infected control mice. Mice treated with AZM following MPA infection showed significantly lower levels of IL-1 β , IL-6, IL-10, IL-17, TNF- α , MCP-1 and KC 3 and 5 days post-infection compared to mice treated with saline (Figure 3). In contrast, AZM-treated mice showed increased levels of IFN- γ at both time points. These results are consistent with the decrease in neutrophil infiltration in the AZM-treated group.

Histology and detection of apoptotic cells

Histological evaluation of H & E stained sections revealed normal morphology in sham-infected mice (Figure 4A). Animals in the MPA/saline group at 5 days post infection showed infiltration of neutrophils in airway lumen, peribronchiolar and perivascular areas (Figure 4B to 4D). These mice also showed pneumonic consolidation in isolated areas of parenchyma (Figure 4E) with neutrophils being dominant cell type (Figure 4F). We observed very few activated macrophages in these mice (Figure 4G). In contrast, animals in MPA/AZM group showed mild inflammation in peribronchiolar and perivascular areas with mononuclear cells being predominant cell type (Figure 5A and 5B). Unlike animals in MPA/saline group, these mice did not show pneumonic consolidation in the parenchyma. However, we noticed many activated macrophages in the peribronchiolar, perivascular and alveolar air spaces (Figure 5C). Some of the macrophages appear to have phagocytosed neutrophils within them.

We next examined the abundance of apoptotic cells and the association of apoptotic nuclei with macrophages to determine the effect of AZM on macrophage function. Animals in MPA/saline group showed a large number of apoptotic cells in the airway lumen (Figure 6A) and peribronchiolar and perivascular areas (Figure 6B), with no obvious apoptotic bodies associated with macrophages (Figure 6C). On the other hand, animals in MPA/AZM group showed a few apoptotic cells in the peribronchiolar area (Figure 6D). Higher magnification revealed the presence of apoptotic bodies within the macrophages (Figures 6D and 6E). The number of BAL macrophages containing apoptotic bodies was quantitated by microscopy (Figure 6F). Animals in MPA/saline group showed only 1-2% of macrophages positive for apoptotic bodies, whereas in MPA/AZM group 14% and 7.5% of macrophages were positive for apoptotic bodies at 3 and 5 days post-infection, respectively. These results imply that AZM improves the phagocytic uptake and clearance of apoptotic neutrophils by alveolar macrophages.

Killing of biofilm bacteria by neutrophils

MPA grown as a biofilm was incubated with neutrophils in the presence of AZM (0 to 1000 ng/ml), and biofilm mass and density of planktonic bacteria determined (Figure 7). AZM by

itself decreased the biofilm mass in a dose-dependent manner. The presence of neutrophils further reduced the mass of biofilm, particularly in the presence of higher doses of AZM. In the presence of AZM alone, the density of planktonic bacteria did not change significantly irrespective of the concentration of AZM used. In contrast, neutrophils decreased the number of planktonic bacteria significantly in the presence of AZM, compared to no AZM control and AZM alone. These results indicate that AZM disperses bacteria from the biofilm mass and renders them susceptible to killing by neutrophils.

Discussion

Pulmonary inflammation due to chronic infection with MPA is a major cause of morbidity and mortality in CF. Despite the availability of antibiotics, it is a challenge to eradicate PA infection in CF. This is probably due to an exaggerated host response to infection, combined with the immunomodulatory action of PA virulence factors and emergence of multi-drug resistant PA variants. This results in persistent neutrophilic inflammation which ultimately contributes to destruction of surrounding lung tissue. In this study, using a novel mouse model of endobronchial PA infection [25], we show that low (sub-bactericidal) dose of AZM (20mg/kg) significantly reduces inflammation and augments bacterial clearance in CF mice. This effect was associated with significant reduction in the lung cytokines IL-1 β , IL-6, IL-17, TNF- α , MCP-1 and KC, and an increase in IFN- γ . In addition, AZM-treated mice showed improvement in clearance of apoptotic cells by macrophages. AZM also increased the bacterial killing of biofilm bacteria by neutrophils *ex vivo*.

In recent years, AZM has been used in the treatment of CF lung disease because of its anti-inflammatory and immunomodulatory actions. However, the precise mechanisms by which AZM improves pulmonary status in CF are unclear. In our previous studies, using an agar bead model of infection in normal mice, we demonstrated that subcutaneous administration of AZM once a day reduced inflammatory cell infiltration in the lungs without substantial changes in the bacterial load [20]. We also demonstrated that AZM attenuated neutrophil accumulation in the lungs partly by reducing the expression of neutrophil-attracting chemokines. Similarly, it has recently been demonstrated that AZM reduced both spontaneous and LPS-induced pulmonary inflammation in CF mice by inhibiting TNF- α and MIP-2 production. [23]. To examine if AZM exerts a similar anti-inflammatory effect in MPA-infected CFTR knockout mice, we infected animals with MPA and treated with AZM subcutaneously starting from 1 day after infection. Treatment of CF mice with AZM prior to infection killed all the animals, indicating that these mice differ from normal mice in their innate defense mechanisms. However, treatment with AZM after the infection improved the survival rate by 95% compared to saline. As recently reported, we also observed a reduction in lung bacterial load in AZM-treated mice [24]. Since AZM does not have a bactericidal effect on gram-negative bacteria, we speculated that AZM aids the clearance of bacteria by indirect mechanisms. MPA often exists in biofilm matrix in the lungs, and are protected by phagocytes [2-4]. In this study, we demonstrated that AZM significantly decreased biofilm mass and increased the killing of planktonic bacteria by neutrophils *ex vivo*. This may explain the observed bacterial clearance in MPA-infected mice treated with AZM

Reduced bacterial load in the lungs of AZM treated mice was associated with significant reduction in neutrophil numbers and levels of the pro-inflammatory cytokines IL-1 β , IL-6, IL-17 and TNF- α , as well as the chemokines KC and MCP-1. Since a neutrophilic inflammatory response is needed for clearance of PA from the lungs, it may seem paradoxical that AZM reduces both lung inflammation and bacterial load. This point is exemplified by the high mortality in mice pre-treated with AZM prior to PA infection (data not shown). However, once infection is established, release of elastase from necrotic neutrophils causes further tissue damage and recruits more neutrophils to airways [6, 7]. Administration of AZM apparently increases survival in part by reducing lung inflammation. Of particular interest is IL-17, which is exclusively produced by Th₁₇ lymphocytes. IL-17 is increased during pulmonary exacerbations in CF, induces expression of neutrophil-attracting chemokines from airway epithelial cells, and plays a regulatory role in neutrophil recruitment in a murine model of *P. aeruginosa* infection [33, 34]. AZM has been demonstrated to effectively inhibit the IL-17-stimulated IL-8 production in human airway smooth muscle cells [35]. Thus, AZM may exert its anti-inflammatory effect by inhibiting IL-17 expression, which in turn attenuates airway epithelial cell chemokine expression. AZM also has direct inhibitory effects on airway epithelial cell IL-8 expression [36].

We also found increased lung levels of IFN- γ and decreased IL-10 in AZM-treated mice. IFN- γ improves phagocytic killing of MPA protected in alginate biofilm by mononuclear cells [2]. *P. aeruginosa* induces a Th2-type response in mouse models of CF, and the observed shift to a Th1 response, including increased production of IFN- γ and decreased IL-10, may decrease lung bacterial load and inflammation while improving survival [37, 38]. Consistent with this, CF patients with chronic infection with MPA show decreased levels of lung IFN- γ , and growing evidence suggests that increasing IFN- γ production may improve the prognosis in these patients [37, 39, 40]. In contrast to our findings, Sugiyama *et al.* [41] found that AZM increased IL-10 and decreased IFN- γ production from bone marrow-derived dendritic cells and spleen-derived T cells in response to LPS stimulation *in vitro*. This discrepancy may be due to dissimilar experimental conditions employed.

AZM also directly promotes the phagocytic function of macrophages *in vitro* [16]. Macrophages play a pivotal role in clearing inflammatory cells that enter the lungs in response to infection and undergo apoptosis. Failed or reduced clearance of these apoptotic cells results in accumulation of inflammatory cells which eventually undergo necrosis and release tissue-damaging proteases.[42]. This scenario is frequently observed in CF lungs [43, 44]. Indeed, it was recently demonstrated that low dose AZM improved phagocytic capacity of alveolar macrophages *in vitro* and in patients with COPD [16, 45]. In the present study, we observed that, at 3 and 5 days post-infection, mice treated with AZM showed fewer lung neutrophils and an overall reduction in the abundance of apoptotic cells compared to mice treated with PBS. Microscopic examination revealed a significant increase in number of macrophages with intracellular apoptotic bodies. These results imply that AZM promote clearance of apoptotic cells by improving the phagocytic function of macrophages.

In summary, we have demonstrated that AZM effectively reduces PA-induced inflammation by reducing bacterial load, decreasing elaboration of pro-inflammatory cytokines, increasing IFN- γ and improving clearance of apoptotic cells by macrophages.

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Abbreviations used

CF	Cystic fibrosis
PA	<i>P. aeruginosa</i>
MPA	mucoid <i>P. aeruginosa</i>
AZM	Azithromycin
KC	mouse growth related oncogene – α
MCP-1	monocyte chemoattractant protein-1

Figure 1.

AZM increases survival of mice infected with MPA and reduces pulmonary bacterial load. CF mice infected with MPA were treated subcutaneously with either saline or AZM. A. Percent survival of mice was calculated based on two independent experiments with 12 mice per group and counting number of animals that died each day. B. Mice were sacrificed 1, 3 or 5 days post-infection. Bacterial burden was determined in whole lung homogenates. Data points represent individual mouse and the geometric mean calculated from three

independent experiments with a total of 9-11 mice per group (*different from saline treated mice, $p < 0.05$, ANOVA).

Figure 2.

AZM treatment decreases BAL neutrophil counts and lung MPO activity in MPA-infected mice. Mice were infected and treated with AZM or saline as previously described. A. Total neutrophil counts in BAL. B. Total lymphocyte counts. C. MPO activity in lung homogenates. Data represents geometric mean and SEM calculated from three independent experiments with a total of 5-8 animals per group (*different from sham-infected mice; † different from saline-treated mice, $p < 0.05$, ANOVA).

Figure 3.

AZM treatment modulates cytokine expression in PA-infected mice. Supernatant of lung homogenates were used to determine cytokine levels by bioplex immune assay. Data represent geometric mean and SEM calculated from three independent experiments with a total of 9-11 mice per group (*different from sham infected mice; †different from saline treated groups, $p < 0.05$, ANOVA).

Figure 4.

PA-infected, saline-treated mice show severe lung inflammation. Animals were sacrificed 5 days after infection and the lung tissue examined for pathologic changes. A. Sham-treated animal with normal histology; B-G mouse infected with MPA-and treated with saline and sacrificed 5 days post-infection and represents peribronchiolar and perivascular inflammation (B), peribronchiolar neutrophils (C), inflammatory cells in the airway lumen (D), parenchymal consolidation (E), neutrophils in the consolidated parenchyma (F) and neutrophils in the alveolar air space (G). Images are representative of 3 animals per group.

Figure 5. MPA-infected, AZM-treated mice show reduced lung inflammation. A. Peribronchiolar area B. Enlarged view of an inflammatory area showing neutrophils (arrow). C. Enlarged view of an alveolar airspace showing the accumulation of macrophages with phagocytosed neutrophils in cytoplasm (arrow). Images are representative of 3 animals per group.

Figure 6.

AZM treatment reduces apoptotic cells in the lungs. Paraffin lung sections from mice infected with MPA and treated with either saline or AZM and sacrificed 5 days post infection were used to detect apoptotic cells by TUNEL assay. A and B represent apoptotic cells in airway lumen and peribronchiolar area respectively in MPA-infected/saline-treated animal. C, enlarged view of peribronchiolar area and arrow points to apoptotic neutrophils. D and E represent apoptotic cells in the peribronchiolar area and macrophages containing apoptotic bodies in animal infected with MPA and treated with AZM. Arrows in panel E

points to macrophage containing apoptotic bodies. Green represents normal nuclei; red and yellow represent apoptotic nuclei. Images are representative of 3 animals per group. (F and G). BAL cells were isolated at 3 and 5 days post-infection and stained by TUNEL assay. The percentage of apoptotic cells was determined by flow cytometry (F) and percentage of macrophages positive for intracellular apoptotic bodies was determined by fluorescence microscopy (G). Data represent mean and standard deviation calculated from 6-9 animals per group (*different from saline-treated group, $p < 0.05$, ANOVA).

Figure 7.

AZM decreases MPA biofilm mass and increases killing of bacteria by neutrophils. MPA grown as biofilm in a 96 well plate was treated with AZM (0, 1, 10, 50, 100, 500 or 1000 ng/ml) in the presence or absence of added neutrophils for 3 h. Biofilm mass (A) and density of planktonic bacteria (B) were determined by crystal violet staining and plating, respectively. Data represent mean and standard deviation calculated from 3 independent experiments carried out in quadruplicate (*different from AZM alone at respective concentrations; †different from control group with no added AZM or neutrophils, $p < 0.05$, ANOVA).