Azithromycin Alters Macrophage Phenotype and Pulmonary Compartmentalization during Lung Infection with Pseudomonas

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Infection with mucoid strains of Pseudomonas aeruginosa in chronic inflammatory diseases of the airway is difficult to eradicate and can cause excessive inflammation. The roles of alternatively activated and regulatory subsets of macrophages in this pathophysiological process are not well characterized. We previously demonstrated that azithromycin induces an alternatively activated macrophage-like phenotype in vitro. In the present study, we tested whether azithromycin affects the macrophage activation status and migration in the lungs of P. aeruginosa-infected mice. C57BL/6 mice received daily doses of oral azithromycin and were infected intratracheally with a mucoid strain of P. aeruginosa. The properties of macrophage activation, immune cell infiltration, and markers of pulmonary inflammation in the lung interstitial and alveolar compartments were evaluated postinfection. Markers of alternative macrophage activation were induced by azithromycin treatment, including the surface expression of the mannose receptor, the upregulation of arginase 1, and a decrease in the production of proinflammatory cytokines. Additionally, azithromycin increased the number of CD11b+ monocytes and CD4+ T cells that infiltrated the alveolar compartment. A predominant subset of CD11b+ cells was Gr-1 positive (Gr-1*), indicative of a subset of cells that has been shown to be immunoregulatory. These differences corresponded to decreases in neutrophil influx into the lung parenchyma and alteration of the characteristics of peribronchiolar inflammation without any change in the clearance of the organism. These results suggest that the immunomodulatory effects of azithromycin are associated with the induction of alternative and regulatory macrophage activation characteristics and alteration of cellular compartmentalization during infection.

Chronic inflammation of the airways is observed in asthma, cystic fibrosis (CF), interstitial lung diseases, and chronic obstructive pulmonary disease (COPD). The lungs of patients with pulmonary disorders are often colonized by Pseudomonas aeruginosa, which induces a cycle of inflammation, airway damage, remodeling, and functional decline (3, 34, 49). Pseudomonas subacutely infects the lungs of up to 80 to 90% of patients in the end stages of CF and through periodic exacerbations accelerates the pulmonary disease pathology (16, 34). The repeated activation of immune cells in this setting contributes to progressive lung destruction, increased exacerbations of disease, and increased mortality (28, 33, 44). The continuous influx of neutrophils into the airways is primarily responsible (43), with cytokines and chemokines secreted by both epithelial cells and macrophages driving the response (5, 9).

Macrophage activity has been defined along a spectrum of functional activity (18). While the function of classically activated macrophages (CAMs) is well-defined, alternatively activated macrophages (AAMs) also play a distinct role in both host defense and the maintenance of homeostasis within the lung. In response to infection, CAMs phagocytose and kill bacteria, initiate inflammation through cytokine and chemokine release, and are induced to full function in Th1 types of responses by T-cell secretion of gamma interferon (IFN-γ). Conversely, alternative activation occurs through the induction of a distinct set of genes upon the binding of interleukin-4 (IL-4) and/or IL-13, primarily produced by Th2-type CD4+ T lymphocytes. These cells express high levels of mannose receptor (MR) and produce a distinct cytokine pattern (29, 37, 47). The main roles of AAMs are to scavenge debris, phagocytose apoptotic cells after inflammatory injury, and orchestrate tissue remodeling and repair through the production of extracellular matrix proteins. The function of AAMs has mainly been evaluated in disease processes that illicit Th2-type immune responses, including asthma and a variety of infectious etiologies (23, 48, 55). These studies demonstrate the ability of AAMs to suppress effector functions of CAMs, which include inducible nitric oxide synthetase (iNOS) upregulation, inflammatory cytokine and chemokine secretion, and the induction of neutrophil infiltration (26, 27, 58, 61). AAMs inhibit CAM-driven inflammatory processes in part through the upregulation of arginase 1, which competes with iNOS for l-arginine, thereby decreasing reactive oxygen species formation and increasing extracellular matrix protein production (36, 38). The properties of classical macrophage activation in response to bacterial infection have been shown to potentially contribute...
to the pathophysiology of CF, including increased proinflammatory cytokine secretion (6, 7), decreased IL-10 production (52), and altered phagosome acidification (11). Whether the induction of alternative macrophage activation during a Gram-negative bacterial infection could beneficially alter the immune response is the focus of our work. In addition, various groups have identified a third population of macrophages that has a more regulatory phenotype. This subset is anti-inflammatory and is hallmarked by high levels of IL-10 production and high levels of expression of antigen presentation molecules (41, 51). A comparison of these macrophage subsets is given in Table 1.

Long-term anti-inflammatory therapy with the azalide antimicrobial agent azithromycin (AZM) has been established as a beneficial intervention in the treatment of patients with CF. Three randomized, placebo-controlled trials have demonstrated an improvement in lung function with 3 to 6 months of treatment in patients with CF who are colonized with *P. aeruginosa* (13, 50, 62). In addition, AZM has been shown to improve macrophage function in subjects with COPD (21) and also to be beneficial in patients with bronchiolitis obliterans syndrome following lung transplantation (19). To date, the precise means by which AZM favorably affects inflammatory lung conditions is unknown. In vitro studies suggest that AZM inhibits proinflammatory cytokine secretion (24) and decreases the levels of chemotaxis, oxidative burst, and cellular adhesion of neutrophils (24, 31). The mechanism of immunomodulation by AZM is attributed by the drug’s ability to inhibit NF-κB activation (1, 10).

We have recently demonstrated that AZM polarizes cells from murine macrophage line J774 to an alternatively activated-like phenotype in vitro (44). This occurs despite the stimulation of these cells with the classical activation signal of IFN-γ followed by the binding of toll-like receptor (TLR) 4 with the bacterial constituent lipopolysaccharide (LPS). On the basis of these observations, we hypothesized that AZM would induce the polarization of macrophages to an alternatively activated-like phenotype during an acute infection with *Pseudomonas* in both the alveolar space and the lung parenchyma. We predict that by controlling the macrophage activation status, it is possible to blunt the inflammation and tissue damage that occurs during bacterial pneumonia. A murine model of *P. aeruginosa* lung infection was utilized to evaluate AZM’s effect on the distribution of inflammatory cells between the alveolar space and parenchyma and to delineate the functional status of these cells. In the work described here, we demonstrated that steady-state exposure to AZM induces not only an AAM response postinfection but also that of a regulatory macrophage phenotype that has been shown to alter T-cell responses in several infection models.

### MATERIALS AND METHODS

#### Mice and drug administration.
C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and were utilized for all experiments. All animal studies were approved by the University of Kentucky Institutional Animal Care and Use Committee. The mice were housed under conditions of pathogen-free isolation and were transferred to a biosafety level 2 housing unit after infection. All mice were within 5 to 7 weeks of age at the time of infection. Tablets of AZM (Pliva Inc., Zagreb, Croatia) were triturated and the powder was suspended in 2% methylcellulose. Beginning 4 days prior to infection, the mice were given AZM at 0.16 g/kg of body weight in 150 μl or vehicle only (control) via oral gavage in order to reach a steady-state concentration of drug exposure at the time of infection. This design was used to mimic the prolonged clinical use of AZM in CF and other disease states. We dosed AZM in excess to increase the likelihood of producing the phenotypic changes observed within 4 days and because the interspecies differences in intracellular AZM concentrations are unknown. Administration of drug or vehicle was continued daily until the time that the mice were killed. Groups of three to six mice per time point were used, and all experiments were performed at least thrice to confirm the results.

#### Infection protocol.
Clinical mucoid strain *P. aeruginosa* M57-15, a kind gift from Anna Van Heeckeren, Case Western University, was utilized in these experiments. The bacteria were grown in Trypticase soy broth (TSB) to late log phase or early stationary phase. The method for incorporation of the bacteria into agarose beads, essential for the induction of prolonged infection in these mice, was adapted from previously described methods (45, 59). Utilizing growth curve information and the optical density of the culture, we established the appropriate starting number of bacteria required to produce a bead preparation that would result in an inoculum that fell within the desired range at the time of infection. The beads were immediately homogenized, and the number of CFU was determined after overnight growth on Trypticase soy agar. The *P. aeruginosa*-laden agarose beads were then diluted to achieve the desired inoculum of 10,000 to 10^7 CFU in 100 μl, which represents approximately the 10% lethal dose (LD_{10}). One exception to this target inoculum was used to generate survival data, in which the LD_{50} was targeted. The beads were instilled intratracheally by using a blunted 24-gauge curved inoculation needle while the animals were under isoflurane anesthesia. To confirm that the mice received the desired inoculum, an aliquot of the bead preparation used was homogenized and plated on *Pseudomonas* selection agar immediately after infection and the numbers of CFU were counted after overnight incubation.

The mice were humanely killed on day 0 and on postinfection days 3, 7, and 14; and bronchoalveolar lavage fluid was obtained as a representative of the airway compartment. The lungs were lavaged with 5 ml of buffered solution containing 0.3 mM EDTA in 1-ml aliquots. The first 1 ml, deemed the “first wash,” was collected separately and centrifuged, and the supernatant was removed and frozen at −80°C for cytokine measurements. Pelleted cells from the first washes were then added back to the remainder of each lavage fluid sample. The lungs were removed and digested in RPMI medium containing 5% heat-inactivated fetal calf serum with 1 mg/ml collagenase A and 50 U/ml DNase for 1 h at 37°C.

#### In vitro studies suggest that AZM inhibits proinflammatory cytokine secretion (24) and decreases the levels of chemotaxis, oxidative burst, and cellular adhesion of neutrophils (24, 31). The mechanism of immunomodulation by AZM is attributed by the drug’s ability to inhibit NF-κB activation (1, 10).

### Table 1. Properties of macrophage activation states

<table>
<thead>
<tr>
<th>Property</th>
<th>Classical</th>
<th>Alternative</th>
<th>Regulatory</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surface expression level</td>
<td>High</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>CD80/86</td>
<td>High</td>
<td>Low</td>
<td>High</td>
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<tr>
<td>MR</td>
<td>Low</td>
<td>High</td>
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<tr>
<td>CCR7</td>
<td>High</td>
<td>Low</td>
<td>Low</td>
</tr>
<tr>
<td>Effector molecules</td>
<td>Nitric oxide</td>
<td>Arginase, ECM(^b)</td>
<td>Arginase, nitric oxide IL-10</td>
</tr>
<tr>
<td>Cytokines</td>
<td>IL-12, TNF-α, IL-6</td>
<td>Th2 responses, remodeling, fibrosis</td>
<td>Anti-inflammatory, suppression of T cells</td>
</tr>
<tr>
<td>Function</td>
<td>Kill microorganisms, initiate inflammation</td>
<td></td>
<td></td>
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\(^a\) See Edwards et al. (12), Gordon (18), and Mosser and Zhang (42).
\(^b\) ECM, extracellular matrix.
\(^c\) TGF-β, transforming growth factor beta.

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### References

The lung fragments were then filtered, and the red blood cells were lysed in a hypotonic solution and analyzed as the lung interstitium. An aliquot was plated to assess the bacterial burden by manual counting of the numbers of CFU on *Pseudomonas* selection agar. This agar was utilized to avoid contamination from the upper airway flora. We have verified that the lavage procedure does not significantly affect the bacterial counts in homogenized lung tissue.

**Flow cytometry.** Surface protein expression and intracellular cytokine secretion were characterized by flow cytometry. Lavage and digest cell aliquots were incubated with combination panels of fluorescently labeled antibodies (Abs) specific for CD11b, Gr-1 (R6-8C5 monoclonal antibody, including specificity for Ly6G and Ly6C epitopes), CD80, CD4, major histocompatibility complex (MHC) class II (MHC-II; I-α), and CD19 (BD Biosciences) and MR (Serotec). For intracellular cytokine staining, 2 × 10^6 cells from each sample were cultured for 4 h with 50 ng/ml phorbol myristate acetate and 500 ng/ml ionomycin in the presence of 10 μg/ml brefeldin A. The cells were surface stained for CD11b and CD11c and fixed in 4% formalin. The cells were then permeabilized in 0.05% saponin, exposed to anti-CD16/32 Ab to block Fc binding, and incubated with anti-tumor necrosis factor alpha (TNF-α) and anti-IL-10 Abs. The cells were washed thoroughly and resuspended in phosphate-buffered saline for analysis. The labeled cells were analyzed with an LSRII flow cytometer system (BD Biosciences), and greater than 50,000 events were routinely acquired per sample. In figures in which cell numbers are given, the percentage of the gated cell subset was multiplied by the number of cells manually counted in each sample. FlowJo software (Tree Star) was used to analyze the data.

**Cytokine concentrations.** The levels of the following cytokines were measured in the first wash of the lavage fluid samples by using cytometric bead array assay kits (BD Biosciences): IL-12p70, chemokines (C-C motif) ligand 2 (CCL2), TNF-α, IL-10, IFN-γ, and IL-6. Bead populations of distinct sizes that were coated with capture antibodies specific for each cytokine were incubated with a 5-fold dilution of each sample for 3 h at room temperature along with a phycoerythrin-conjugated detection antibody. The beads were then washed, and the fluorescence intensity was assayed by flow cytometry, as outlined above. Intensities were then compared to those on a standard curve generated for each cytokine to determine the concentration in each sample.

**Arginase quantification.** Cell aliquots were pelleted and lysed with buffer containing 10 mM Tris-HCl, 0.4% (wt/vol) Triton X-100, and Roche minitablet cytokine to determine the concentration in each sample. The total amount of protein in the cell lysates was quantified containing 10 mM Tris-HCl, 0.4% (wt/vol) Triton X-100, and Roche minitablet cytokine to determine the concentration in each sample. The levels of the following cytokines were measured in the first wash of the lavage fluid samples by using cytometric bead array assay kits (BD Biosciences): IL-12p70, chemokines (C-C motif) ligand 2 (CCL2), TNF-α, IL-10, IFN-γ, and IL-6. Bead populations of distinct sizes that were coated with capture antibodies specific for each cytokine were incubated with a 5-fold dilution of each sample for 3 h at room temperature along with a phycoerythrin-conjugated detection antibody. The beads were then washed, and the fluorescence intensity was assayed by flow cytometry, as outlined above. Intensities were then compared to those on a standard curve generated for each cytokine to determine the concentration in each sample.

**Histopathology.** The mouse lung tissue was excised and immediately fixed in 10% buffered formalin. After an overnight incubation at 4°C, sections were washed three times with 0.1 M phosphate buffer and were then transferred into 2 ml sterile 20% sucrose (pH 7.2) and stored at 4°C. The samples were then transferred into optimal cutting temperature (OCT) embedding compound and incubated at 4°C for 24 h. The fixed cryoprotected tissues were sectioned at 10 μm (Thermo Fisher Scientific cryostat) and mounted on slides to dry overnight. The slides were stained with hematoxylin–eosin (H&E) by a standardized protocol, and photographs were taken with an AxioCam HRc camera mounted on a Zeiss Imager.Z1 microscope.

**Statistical analyses.** Measurements were determined for each individual mouse, and the means and standard deviations (SDs) were generated for each treatment group. Survival data were analyzed by the log-rank method. For all other data, one- or two-way analysis of variance (ANOVA) was used to compare the characteristics among the postinfection time points between groups of mice. One-way ANOVA on ranks was used for nonparametric data. The Student New- man-Keuls post hoc test was used to discriminate the differences between individual groups at each time point when they were detected. All data were analyzed by using the SigmaStat statistical software package (Systat, San Jose, CA).

### RESULTS

**Azithromycin did not alter weight loss or bacterial clearance.** Infected mice consistently lost weight over the first 3 days, after which time they regained weight over the remainder of the study period (Fig. 1A). Statistically significant weight loss was achieved in both infected groups of mice compared to the weight of a sham infection control group on days 1 through 3. No statistically significant difference in the weights between the groups of infected animals was observed as a result of AZM treatment. To analyze the impact of AZM on the survival of the mice, in one experiment the mice received 1.3 × 10^6 CFU (1 × LD<sub>50</sub>) of *P. aeruginosa* M57-15 (Fig. 1B). Although the percentage of mice that survived was higher in the group receiving AZM, the difference between that group and the control group of infected mice was not significant (*P* = 0.583). Importantly, the bacterial burden did not differ at any time point examined during any experiment performed, indicating that AZM did not have an effect on bacterial clearance. The *P. aeruginosa* lung burden from an experimental replicate in which mice received 1.1 × 10^5 CFU of *P. aeruginosa* is depicted in Fig. 1C.

**Azithromycin increased monocyte influx and decreased neutrophil influx.** The percentages and absolute numbers of im-
mune cells in various subsets in lung digest and lung lavage fluid samples, determined on the basis of surface receptor expression analysis, are depicted over time. The total number of cells in the lung digest approximately doubled in the control group by day 7, while the total number of cells in the AZM group stayed relatively constant. By day 14, we consistently observed higher numbers of total cells in the lung digest (Fig. 2A) in the mice receiving AZM, although not to a statistically significant degree ($P = 0.067$ for the representative experiment). Expression of the CD11b protein was evaluated as a marker of cells that were elicited into the lungs postinfection. While the number of CD11b-positive (CD11b$^+$) cells in the lung digest did not differ between the treatment groups, a higher number infiltrated the alveolar lavage fluid (Fig. 2B) in the AZM group on day 7 postinfection ($P = 0.006$). The trend in the difference among all time points combined also reached statistical significance here ($P = 0.024$). The infiltration of the cell subset defined here as neutrophils (CD11b negative [CD11b$^-$] and Gr-1 positive [Gr-1$^+$]) was observed by day 3 postinfection but was significantly blunted in the AZM group by day 7 compared to that in the mice not receiving the drug (Fig. 2A) ($P = 0.002$). Likewise, we consistently observed lower numbers of neutrophils in the alveolar spaces, although statistical significance was not reached. The number of CD4$^+$ T cells in the lung parenchyma increased similarly through day 7, but by day 14 there was a significantly higher number of CD4$^+$ T cells in the mice treated with AZM ($P < 0.001$). Similarly, the number of CD4$^+$ T cells that infiltrated the alveolar space was higher in mice treated with AZM on day 7 postinfection ($P = 0.004$).

**Azithromycin increased CD11b$^+$ cell influx into the airway compartment.** To further investigate the cellular partitioning into the alveolar spaces, we compared the characteristics of the inflammatory cell populations that penetrated the airways over time. Figure 3 depicts the dynamics of the nonlymphocytic cellular makeup in both the lung digest (Fig. 3A) and lavage fluid (Fig. 3B) after *P. aeruginosa* infection. After lymphocytes and dead cells were eliminated from the analysis, the populations were categorized as infiltrating monocytes (a high level of CD11b expression and a low level of CD11c expression) and resident alveolar macrophages (a high level of CD11c expression and a low level of CD11b expression), with significant differences between groups being denoted by the boldface gates in Fig. 3A and B. It is important to note that these definitions are not absolute, as infiltrating cells take on a more resident phenotype over time and shift their surface expression of these proteins accordingly. On day 7 postinfection, there was a significantly higher percentage of cells in the lung digest that were CD11b$^+$ CD11c$^+$ (CD11c$^+$) versus CD11b$^+$ CD11c$^-$ (CD11c$^-$) ($P = 0.001$), with this subtype making up 17.10% ± 8.30% of the nonlymphocytic cells in the AZM-treated group but only 5.16% ± 1.09% in the control group. None of the differences in the proportions of other subsets reached statistical significance between treatment groups. The percentages of the individual cell subsets are listed in Table 2.

The percentage of CD11b$^+$ cells in the lung lavage fluid was increased by AZM, and the corresponding percentage of CD11c$^+$ resident cells was lower in this group. While we consistently observed an earlier influx of CD11b$^+$ cells in the lung lavage fluid of mice in the control group by day 3 postinfection, this difference never reached statistical significance. However, by day 7 there was a dramatic increase in the CD11b$^+$ subsets that were both CD11c$^+$ and CD11c negative (CD11c$^-$) in the lavage fluid of animals treated with AZM ($P < 0.001$ for both subsets). The percentages of all cell subsets are given in Table 2.

In addition, we calculated the percentage of each cell type measured that migrated into the alveolar space in relation to the total number of cells in the lungs exhibiting the respective phenotype. Figure 4 shows that AZM significantly increased the overall percentage of CD11b$^+$ cells ($P < 0.001$), as well as the subset of CD11b$^-$ cells that were also Gr-1$^-$ ($P = 0.001$), in the alveolar space on day 7 postinfection. No differences in
the proportion of the neutrophil (CD11b<sup>−</sup> Gr-1<sup>+</sup>), CD11c<sup>+</sup>, or CD4<sup>+</sup> T-cell subsets were observed in the alveoli.

**Azithromycin induced alternative, regulatory macrophage activation characteristics.** Because CD11b<sup>+</sup> cells displayed differences in migration characteristics with AZM exposure, we analyzed the activation characteristics of these cells. As shown in Fig. 5A, when the surface receptor expression of cells in the lung digest that were CD11b<sup>+</sup> Gr-1 negative (Gr-1<sup>−</sup>) (monocytes) was examined, MR expression was consistently increased in the AZM group on day 7 postinfection (P < 0.003). Conversely, the classical activation marker CD80 was upregulated on a significantly lower number of cells in the lung digest in mice receiving AZM (P = 0.002). Other surface receptors, including CD23, CCR7, and MHC-II, were upregulated postinfection on CD11b<sup>+</sup> Gr-1<sup>−</sup> cells in a similar manner between the treatment groups. In addition, the concentration of the AAM effector molecule arginase was assessed in the lung digest of each mouse. A significant increase in arginase activity was found in the AZM-treated group on day 7 postinfection (P < 0.001) (Fig. 5B).

Next, we examined the activation characteristics of the CD11b<sup>+</sup> Gr-1<sup>−</sup> group of cells (Fig. 6). While neutrophils can also express these two surface proteins, the majority of the CD11b<sup>+</sup> Gr-1<sup>−</sup> cells were large and granular. This expression pattern is typical of a regulatory subset of myeloid-derived suppressor cells, mostly made up of immature macrophages but also consisting of a small number of immature neutrophils (15). Interestingly, AZM induced increases in the levels of MHC-II, CD80, and MR surface receptor expression on this subset, most markedly on day 7 postinfection (Fig. 6A). Increased numbers of CD11b<sup>+</sup> Gr-1<sup>−</sup> cells expressing these receptors were observed in the alveolar space on day 7 postinfection.
compartments were phenotyped by flow cytometry on the basis of size, granularity, and surface receptor expression. Mean values less than 0.05 (*P*) were deemed statistically significant. For the levels of participation of both AAMs and CD11b+ Gr-1+ cells in regulating the immune response to *P. aeruginosa*, patients with chronic inflammatory lung conditions who are infected with *P. aeruginosa* are susceptible to the development...
of inflammation and fibrosis surrounding the small airways. To determine the extent to which AZM affected the damage to the lung parenchyma, histology sections were prepared from lungs isolated at each time point. Evaluation by the use of H&E staining revealed inflammatory changes in both the AZM-treated and the untreated groups at days 3 and 7 postinfection. Sections from AZM-treated animals obtained on day 7 were suggestive, however, of a decreased amount of interstitial inflammation surrounding diseased bronchioles (Fig. 7). In addition, on day 7 postinfection, a systematic examination of the sections showed that the inflammatory cells surrounding the bronchioles mainly consisted of monocytic cells in mice that received AZM, whereas in the control group, neutrophils dominated the infiltrates (Fig. 7). This finding corresponds to the flow cytometry data presented and, in addition, confirms that a majority of the CD11b+/Gr-1+ cells induced by AZM treatment were macrophages.

**Azithromycin decreased proinflammatory cytokine production.** We determined the impact of AZM upon the production of cytokines specifically from CD11b+ cells in the lung digest. Figure 8A depicts the intracellular cytokine staining results for CD11b+ cells in the lung digest over time postinfection. The number of CD11b+ cells producing TNF-α was significantly decreased by AZM treatment (P = 0.027), while the number of cells producing IL-10 was not different between the two groups (P = 0.272). Additionally, the cytokine concentrations in the lung lavage fluid were measured, as shown in Fig. 8B. The concentrations of the inflammatory proteins TNF-α, IL-6, and CCL2 were decreased in the animals receiving AZM compared to the concentrations in the control group (P < 0.001 for each). The concentrations of IL-10, IFN-γ, and IL-12p70 were not significantly different in the lavage fluid between the treatment groups at any time point.

**DISCUSSION**

Macrophages in various activation states work in concert with other cell types during infection to both initiate and terminate the inflammatory response and to repair and remodel tissues after the event. The study described here demonstrates that during *Pseudomonas* pneumonia, AZM exposure alters the distribution of inflammatory cells between the alveolar space and the lung interstitium. In addition, we found that AZM enhanced the characteristics of AAMs in the lungs of mice infected with *P. aeruginosa*. Specifically, AZM increased the number of CD4+, CD11b+, and CD11b+ Gr-1+ cells present after infection in the alveolar space interstitium. We have previously indicated that AZM increases the levels of MR expression and arginase activity in an *in vitro* setting when macrophages are stimulated with classical activation signals in the presence of the drug (44). In the present study we show a similar shift in the levels of MR expression and arginase activity in an *in vivo* setting when macrophages are stimulated with classical activation signals in the presence of the drug (44). In the present study we show a similar shift in the levels of MR expression and arginase activity in an *in vitro* setting when macrophages are stimulated with classical activation signals in the presence of the drug (44). In the present study we show a similar shift in the levels of MR expression and arginase activity in an *in vitro* setting when macrophages are stimulated with classical activation signals in the presence of the drug (44). In the present study we show a similar shift in the levels of MR expression and arginase activity in an *in vitro* setting when macrophages are stimulated with classical activation signals in the presence of the drug (44).
AAMs have been shown to inhibit CAM proliferation and function, as well as to suppress Th1-type cell-mediated immune responses through a characteristic pattern of gene up-regulation that includes the production of type II cytokines and arginase 1 (26, 27, 36, 38, 58, 61). Our data demonstrate that the underlying mechanism of the immunomodulatory effects of AZM may be due to its ability to alter the activation state of macrophages in inflammatory tissues and to affect the recruitment of immunomodulatory cells to areas of infection. At no time did the 4 days of AZM exposure prior to infection alter any of the endpoints measured. This supports the findings of our in vitro work published previously, suggesting that AZM has no effect on macrophage protein expression unless the cells are stimulated (44).

A study described in a paper recently published by Tsai et al. examined the effect of AZM on inflammation and survival in a Pseudomonas infection model in CF mice (56). The mortality rate for the CF mouse strain was higher than that which we observed among our mice, but AZM improved survivability through day 5 to a significant degree, even with the use of a dose of AZM lower than that used in our investigation. AZM altered the cytokine profile and the influx of inflammatory cells into the lungs in a fashion similar to that observed in our work. While that group did not examine macrophages in terms of activation status or compartmentalization, their results suggest that alternative macrophage activation was induced by AZM in the CF mouse strain, on the basis of the cytokine profile and increased efferocytosis that, in part, characterizes the AAMs (39). This, in conjunction with our data presented here, supports the hypothesis that the benefit of AZM may be in part due to the alteration of macrophage programming.

In contrast, while Tsai et al. (56) found that the IFN-γ concentrations in the lung homogenates were increased by AZM, we did not see a similar effect. This could be due to differences in T-lymphocyte function between these two models, as Th2-type responses have been shown to predominate in patients with CF who are infected with Pseudomonas (20). While we found IFN-γ in the mice treated with AZM, it may be that macrophages are more refractory to the effects of the cytokine in the presence of the drug. This is consistent with the findings of our previous in vitro study (44). The suppressive effect of AZM upon TNF-α, IL-6, and CCL2 was similar between the studies performed Tsai et al. (56) and by our group.

We demonstrate a dramatic reduction in the CCL2 concen-
trations within the lung lavage fluid, which is consistent with previous results that have been reported from studies of AZM treatment of CF mice (56) and patients with CF (40). Downregulation of the production of CCL2 is consistent with increased alternative macrophage activation, as gene expression studies have demonstrated that while *P. aeruginosa* infection increases the level of CCL2 transcription, mouse models that induce alternative macrophage activation do not (32). Furthermore, this effect on CCL2 may help to explain some of the compartmental differences that were observed between the lung interstitium and the airways. This chemokine is essential for monocyte trafficking under inflammatory conditions, and it has been shown to increase the levels of paracellular permeability and disruption of the tight junctions in the vascular endothelium (30).

We also demonstrate differences in the relative distribution of T cells, CD11b^+^ and CD11b^-c^- monocytes, and CD11b^-Gr-1^- cells between the alveolar space and the interstitium with AZM treatment. AZM is known to inhibit the expression of several adhesion molecules, including intracellular adhesion molecule 1 and several soluble selectins (53). In addition, interesting work has demonstrated that AZM alters the localization of several proteins important in tight junction formation between airway epithelial cells (2). This effect of the drug could be the cause of alterations in the compartmentalization of cells infiltrating the lung. Increases in the numbers of lymphocytes and macrophages in lavage fluid have been observed to be induced by AZM in other studies with mice and humans, and the effect on decreasing the number of neutrophils is typical of the drug (14, 56, 57).

There is growing evidence that the CD11b^-Gr-1^- subset of myeloid cells is an important population of cells that is described to have a deactivating function in several infection models (8, 54, 60). These cells have been described as myeloid-derived suppressor cells, a heterogeneous population with a myeloid origin consisting of immature macrophages, immature neutrophils, and immature dendritic cells (15). These cells have been shown to possess regulatory characteristics, including the deactivation of Th1-type T cells, the induction of Th2-associated T-cell characteristics, and the production of a cytokine profile similar to that produced by AAMs (8, 54, 60). Our

**FIG. 7.** Lung sections from day 7 postinfection with *P. aeruginosa* of mice receiving AZM or vehicle only (control). The mice were humanely killed after infection, and lung tissue sections were examined for inflammatory changes. Hematoxylin-eosin staining of the lung sections from day 7 postinfection from AZM-treated (A) and control (B) mice are shown. As the magnification increases from top to bottom, the scale bars represent distances of 50, 25, and 12.5 μm, respectively. Representative pictures show a predominantly monocytic cellular infiltration into the lung parenchyma in the AZM-treated animals (triangular pointers), while the inflammatory response in the control mice on day 7 postinfection is dominated by neutrophils (arrows). Br, bronchiole; V, venule.
data that demonstrate an increase in CD80 and MHC-II expression on these cells further confirm that the subset induced by AZM primarily comprises macrophages and is similar to that identified by other groups (41, 51). In other mouse models, CD11b+ Gr-1+ cells have been shown to suppress T-cell function in an arginase-mediated manner (35). Due to these effects, Makarenkova et al. proposed that this subtype of cells is responsible for a transient immune hyporesponsiveness that is part of the pathophysiology of infection with these organisms (35). In our study, this subpopulation of regulatory cells was expanded in the alveolar space of mice treated with AZM at the peak of the infection with P. aeruginosa, as the drug specifically increased the migration of the subset of CD11b+ cells that were Gr-1+. The presence of increased numbers of cells of this phenotype in mice treated with AZM may contribute to the mechanism by which the drug affects inflammation and the resultant fibrosis associated with bacterial infection.

Macrophage activation is overwhelmingly shifted toward a classical activation set of genes in response to a broad range of bacteria (4, 46). While shifting of the predominant macrophage response to a more deactivating phenotype could be beneficial, especially in patients with dysregulated immune responses, such as those observed in CF, a concern is that bacterial clearance could be compromised. While AAMs have an enhanced phagocytosis ability, they have a decreased ability to kill pathogens (39). Our data indicate that there is no difference in the clearance of P. aeruginosa in AZM-treated and untreated animals. Importantly, the drug has been shown to actually enhance the clearance of P. aeruginosa in a CF mouse model (56). As a limitation to our findings, AZM could have altered the pathogenicity of bacteria, as has been reported previously (17, 22, 25), thereby contributing to the changes in the immune response observed. We believe, however, that these indirect effects are minimal, due to our previous in vitro work demonstrating a direct effect of AZM on cells stimulated with LPS (44).

The clinical significance of this aspect of AZM’s effects cannot be definitively assessed by this work. However, defining the role of regulatory and alternatively activated macrophage subsets in bacterial pneumonia could lead to the development of therapeutic modalities that disrupt the cycle of inflammation and remodeling, which could slow the progression of fibrotic changes in the lungs without altering the clearance of pathogens. Future work will help determine both the mechanism by which AZM alters macrophage programming and the influence that both AAMs and CD11b+ Gr-1+ cells have on other immune subtypes in this infection model.

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