Azithromycin alters macrophage phenotype

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Objectives: To investigate the in vitro effects of azithromycin on macrophage phenotype. Utilizing a mouse macrophage cell line (J774), we examined the effect of azithromycin on the properties that define classical macrophage activation (M1) and alternative macrophage activation (M2).

Methods: J774 cells were cultured in the presence of azithromycin and stimulated with classical activation [interferon-γ (IFN-γ)] and alternative activation [interleukin (IL)-4 and IL-13] cytokines along with lipopolysaccharide (LPS). Macrophages were analysed for inflammatory cytokine production, surface receptor expression, inducible nitric oxide synthase (iNOS) protein expression and arginase activity.

Results: Azithromycin altered the overall macrophage phenotype. Azithromycin-treated J774 macrophages demonstrated a significantly reduced production of the pro-inflammatory cytokines IL-12 and IL-6, increased production of the anti-inflammatory cytokine IL-10 and decreased the ratio of IL-12 to IL-10 by 60%. Receptor expression indicative of the M2 phenotype (mannose receptor and CD23) was increased, and receptor expression typically up-regulated in M1 cells (CCR7) was inhibited. The presence of azithromycin increased arginase (M2 effector molecule) activity 10-fold in cells stimulated with IFN-γ and LPS, and iNOS protein (M1 effector molecule) concentrations were attenuated by the drug.

Conclusions: These data provide evidence that azithromycin affects the inflammatory process at the level of the macrophage and shifts macrophage polarization towards the alternatively activated phenotype. This recently defined M2 phenotype has been described in conditions in which pulmonary inflammation and fibrosis are major determinants of clinical outcome, but the concept of antibiotics altering macrophage phenotype has not yet been critically evaluated.

Keywords: macrolides, inflammation, cystic fibrosis

Introduction

Azithromycin has been shown in clinical trials to have anti-inflammatory properties. This effect has been associated with improved lung function in cystic fibrosis (CF) patients infected with Pseudomonas aeruginosa.1–3 The drug has been proposed to counteract the effects of inflammatory cytokines and to alter gene expression and virulence of P. aeruginosa.4–8 Recently, three randomized, placebo-controlled trials have shown an improvement in lung function in CF patients with chronic P. aeruginosa infection treated with 3–6 months of azithromycin as the forced expiratory volume in 1 s (FEV1), per cent predicted improved between 3.2% and 6.2%.1–3

Evidence suggests several potential mechanisms for azithromycin including modulation of the host inflammatory response to infection4,5,8,10 and suppression of P. aeruginosa virulence factors (including biofilm formation).6,7,11–13 While macrolides appear to act at multiple points in the inflammatory cascade,4,9 most published reports have demonstrated this anti-inflammatory effects on neutrophils. It has been shown that macrolides not only attenuate pro-inflammatory cytokine production by neutrophils, but also by monocytes and bronchial epithelial cells.
presumptively via inhibition of the NF-κB pathways.\textsuperscript{14,15} However, the effect of azithromycin on the alteration of macrophage phenotype and function has not been investigated.

Different macrophage phenotypes have been described and may exist at various stages of disease progression in a CF patient (from non-infected to chronic infection with acute exacerbations). Classical activation of macrophages (M1) by interferon-γ (IFNγ) creates a cell that actively secretes inflammatory cytokines and chemokines, phagocytoses and kills invading microorganisms, and initiates adaptive immune responses.\textsuperscript{1,6,11} In contrast, macrophages stimulated by interleukin (IL)-4/IL-13 (M2) display a distinct alternative pattern of activation.\textsuperscript{18–21} M2 cells play a role in directing Th2 humoral responses, allergic and parasitic responses and the coordination of repair following an inflammatory reaction. These functional differences are reflected in the expression levels of surface proteins, the inflammatory cytokine production profile and the expression of the two opposing effector molecules, inducible nitric oxide synthase (iNOS) and arginase. The contribution of M2 cells to the pathophysiology of CF is unknown. However, in light of their role in inflammation and airway remodelling,\textsuperscript{22} they may contribute to the fibrotic changes that occur in the CF lung.

How azithromycin affects macrophage phenotype may be an important component in understanding the mechanism of macrolides in the anti-inflammatory context.\textsuperscript{20,21,23} We studied the ability of azithromycin to alter macrophage surface receptor expression and characterized the effect of azithromycin on macrophage inflammatory activity by measuring cytokine production and the effector molecules iNOS and arginase.

\textbf{Materials and methods}

\textit{In vitro cell culture}

The immortalized macrophage cell line J774A.1 (J774, ATCC TIB-67) was utilized to determine influence of azithromycin over macrophage phenotype in an \textit{in vitro} culture system. J774 cells are a mouse macrophage line derived from BALB/cN adult mice.\textsuperscript{24} Cells were grown in complete RPMI 1640 media (Invitrogen, Carlsbad, CA, USA) containing penicillin (100 U/mL), streptomycin (100 mg/L) and 1% fetal calf serum at 37°C and 5% CO\textsubscript{2}. Cells undergoing log-phase growth were incubated overnight with either IFNγ (100 ng/mL) or IL-4/IL-13 (30 μM), along with azithromycin (15–30 μM) at a density of 2 × 10\textsuperscript{5} cells/mL in 24-well tissue culture-treated plates. The next morning the cells were stimulated with lipopolysaccharide (LPS) (50 ng/mL) for 4, 8 or 24 h. Control conditions included cells treated without cytokines, without LPS and without azithromycin. Cells were harvested and centrifuged, and supernatants were collected and frozen at –80°C. Cells were washed with 2 mL of PBS, and an aliquot for arginase activity and intracellular protein quantification was also frozen at –80°C. Fresh single-cell suspensions were immediately utilized for phenotyping.

\textbf{Surface marker expression}

J774 cells were incubated with multiple fluorescently labelled monoclonal antibodies specific for mouse macrophage cell surface markers to delineate macrophages of the M1 and M2 phenotypes as described previously.\textsuperscript{25} The following antibodies were utilized: rat anti-mouse mannose receptor (MR) antibody conjugated to phycoerythrin (PE) (Seropec, Raleigh, NC, USA), rat anti-mouse CD23 antibody conjugated to PE-Cy5 (BD Biosciences, Mountain View, CA, USA) and rat anti-mouse CCR7 antibody conjugated to allophycocyanin (APC) (BioLegend, San Diego, CA, USA). These specific receptors were chosen based on their roles in alternatively and classically activated macrophages as cited in the literature.\textsuperscript{21,26–28} Single-cell suspensions were washed before and after staining with 2 mL of PBS containing 0.1% BSA and 0.02% sodium azide, and labelling was performed in 100 μL of this buffer.

All cells were analysed for phenotype by flow cytometric multiparameter analysis using a FACSCaliber Flow Cytometer (BD Biosciences). Greater than 50 000 events per sample were routinely examined. The percentage of cells staining positive for each surface protein was determined by comparing test samples with unstained and isotype control-stained samples.

\textbf{Inflammatory cytokine measurement}

Inflammatory cytokine production was measured in the supernatants of J774 cells cultured in the conditions defined above. Cytometric Bead Array (CBA) kits (BD Pharmingen, San Jose, CA, USA) were utilized to quantify the following cytokine concentrations simultaneously as previously described: IL-12, TNFα, IFNγ, IL-10 and IL-6.\textsuperscript{29,30} These cytokines are indicative of the inflammatory function of macrophages, which are produced at high levels (with the exception of IL-10) by the cells of the M1 phenotype.\textsuperscript{16} Bead populations with distinct fluorescence intensities coated with capture antibodies specific for each cytokine were incubated with fluorochrome-conjugated detection antibodies along with 50 μL of each sample for 3 h at room temperature. Fluorescence intensities were assayed by flow cytometry and compared with a standard curve generated for each cytokine to determine the concentration in each sample.

\textbf{Arginase activity}

The activity of intracellular arginase was determined for the \textit{in vitro} experiments above using the QuantiChrom Arginase Assay Kit (BioAssay Systems, Hayward, CA, USA), which measures the conversion of arginine into urea by arginase. Cells were harvested, pelleted and lysed with 1 mL of buffer containing 10 mM Tris–HCl, 0.4% (w/v) Triton X-100 and Roche Mini-tablet protease cocktail. Protein was quantified using the bicinechonic acid protein assay (BCA) (Pierce Biotechnology, Rockford, IL, USA), and 20 μL of each sample was incubated with 5 μL of arginine buffer at 37°C for 6 h. Urea detection reagent containing anti-isonitrosopropiophenone was then added and incubated at room temperature for 15 min, and the optical density (OD) was read using a 490 nm filter. Readings were standardized to both total protein and cell number against the OD of each sample’s control well (reaction without incubation step).

\textbf{iNOS protein quantification}

iNOS was quantified in the cell lysates of each \textit{in vitro} culture condition by western blotting. Protein concentrations were measured using the BCA kit, and 20 μg of total protein per sample was separated by SDS–PAGE using a 12% gel and transferred onto Immobilon-P membranes (Millipore) by electroph blotting. After blocking, iNOS was detected by incubation of blots with anti-mouse monoclonal antibody (BD Pharmingen) specific for the protein and then followed by a goat anti-mouse antibody conjugated to horseradish peroxidase (HRP) (Sigma-Aldrich, St Louis, MO, USA). Chemiluminescence was generated using the SuperSignal West Pico...
Chemiluminescent Substrate (Pierce Biotechnology) and measured using a Kodak Image Station 2000MM (Carestream Health, Rochester, NY, USA). Membranes were then stripped and re-probed with anti-mouse β-actin antibody (Sigma-Aldrich) to ensure even protein loading.

Statistics

Results are expressed as mean ± SD and compared using the SigmaStat statistics software package (Systat, San Jose, CA, USA). Data were compared by one-way analysis of variance (ANOVA) among groups followed by the Student–Neuman–Keul test for post hoc comparisons. Data that failed normality testing were compared using the Kruskal–Wallis one-way ANOVA on ranks. Differences were determined to be statistically significant when a P value of <0.05 was attained.

Results

Surface marker expression

In order to evaluate azithromycin’s effect on macrophage phenotype, we first examined the in vitro effects of the drug on surface marker expression of J774 cells incubated with either IFNγ or IL-4/IL-13. Cells were stimulated and surface expression of proteins that are up-regulated in M1 cells (CCR7) and M2 cells (MR and CD23) was analysed by flow cytometry (Figure 1). When cells were pre-treated with IL-4/IL-13 (Figure 1a) before stimulation with LPS, the proportions of cells that were MR positive and CD23 positive were significantly higher compared with those pre-treated with IFNγ (Figure 1b) (P = 0.006 and 0.001, respectively). To mimic the impact of azithromycin on the inflammatory process in the pulmonary environment during infection, we then exposed IFNγ pre-treated cells to LPS in the presence of azithromycin (Figure 1c). As shown, azithromycin caused an increased proportion of cells to become MR positive and CD23 positive compared with IFNγ-treated cells without the drug (P = 0.002 and <0.001, respectively). The proportion of cells that was CCR7 positive was lower in both the IL-4/IL-13 pre-treated cells (Figure 1a) and the cells incubated with azithromycin (Figure 1c) compared with cells pre-treated with IFNγ, although statistical significance was not reached (ANOVA P = 0.183). Thus, despite pre-incubation with the classical activation cytokine IFNγ, azithromycin shifted surface protein expression in macrophages to resemble that of IL-4/IL-13-driven alternatively activated macrophages.

Figure 1. Influence of azithromycin exposure on M2 cellular marker expression. J774 mouse macrophages were cultured with cytokines overnight and then stimulated with LPS for 4 h. Cells were harvested and stained with fluorescently labelled monoclonal antibodies specific for MR, CD23 and CCR7 and analysed by flow cytometry. Histograms show (a) cells pre-incubated with IL-4/IL-13, (b) cells pre-incubated with IFNγ and (c) cells incubated with IFNγ in the presence of azithromycin (30 μM). The mean ± SD for the percentage of cells that fluoresced positive for each surface protein (based upon negative and isotype control staining—region denoted by horizontal bars) are indicated on each graph (n = 3). An asterisk denotes a P value <0.05 compared with IFNγ-treated cells (b) using ANOVA and the Student–Neuman–Keul test for post hoc comparisons. Data are representative of three replicate experiments.

Cytokine production

Azithromycin has been shown to cause decreased production of pro-inflammatory cytokines both in vivo and in vitro.4,9 The concentrations of the pro-inflammatory cytokines IL-12 and IL-6 were significantly decreased in the presence of azithromycin (P < 0.001 for each), while the concentration of the anti-inflammatory cytokine IL-10 was increased by the drug (P = 0.003) (Figure 2). We did not detect differences in the pro-inflammatory molecule TNFα. The ratio of pro-inflammatory IL-12 to anti-inflammatory IL-10 was significantly decreased when azithromycin was introduced into the system (P = 0.002). These data are consistent with an altered macrophage phenotype and show that azithromycin alters cytokine production of macrophages stimulated with LPS to a decreased inflammatory status.

Arginase and iNOS

Study of macrophage responses to Th1- and Th2-infection types has led to the observation that expression of arginase is induced by Th2-type cytokines and that the balance of iNOS to arginase correlates with Th1- and Th2-types of responses.31,32 To define the effect that azithromycin confers in this context, we examined arginase activity in total protein isolated from lysates of cells exposed to azithromycin. Cells stimulated in culture with IL-4, IL-13 and LPS produced similar amounts of arginase activity as the unstimulated cells (data not shown). Figure 3 depicts, as expected, that cells exposed to IFNγ plus LPS down-regulated expression of arginase (no activity detected with control wells). However, when azithromycin was also added, arginase activity in the cells stimulated with IFNγ plus LPS increased to levels that were ~20- to 40-fold higher than that of the other treatment groups. When compared on a per milligram of total protein basis, statistical significance was not reached (P = 0.150), primarily because of high variability associated with the arginase assay. On a per cell basis, a statistically significant difference was observed between azithromycin-treated and -untreated cells (P = 0.002). We measured arginase in these different methods to account for the possibility that azithromycin increased other measurable but unidentified proteins that could affect the results, comparing the amounts of arginase relative to total protein.
iNOS is up-regulated in response to inflammatory stimuli as macrophages shift towards the M1 phenotype and become involved in phagocytosis, bacterial killing and initiation of the immune response. M1 macrophages but not M2 macrophages up-regulate iNOS to generate free radicals for phagocytosis.

iNOS protein expression was measured by western blotting from LPS-stimulated cells in the presence or absence of azithromycin. iNOS was detectable from cells that were pre-treated with IFN-γ and then stimulated with LPS (Figure 4, lane 3). However, this response was reduced in the presence of azithromycin (Figure 4, lane 4). Predictably, no iNOS was detected in cells cultured overnight with IL-4/IL-13 and then stimulated with LPS in the presence or absence of azithromycin (Figure 4, lanes 1 and 2). Negative control lanes (Figure 4, lanes 5–7) produced no detectable iNOS.

**Discussion**

We report here that azithromycin polarizes J774 cells towards an M2 phenotype. This polarization is defined by surface protein expression, cytokine production and arginase/iNOS activities. Consistent with previous reports,1–7 our data demonstrate that azithromycin has potent anti-inflammatory properties. In J774 cells, azithromycin decreased pro-inflammatory IL-12 and IL-6 and increased IL-10 production. Furthermore, the ratio of pro-inflammatory to anti-inflammatory IL-12/IL-10 was 2.5 times lower when azithromycin was added to IFN-γ/LPS stimulated macrophages. Additionally, the cytokine profile of azithromycin-treated cells resembled those that were alternatively activated by IL-4/IL-13. This alternative phenotype was also demonstrated by the fact that azithromycin increased arginase production and decreased iNOS. Finally, azithromycin’s ability to polarize macrophages to the alternative phenotype is also supported by the increased proportion of cells displaying up-regulated receptors that are increased on M2 cells (MR and CD23) and down-regulated numbers of the M1 receptor CCR7. In addition to the data reported here, we have observed similar
results both in a human macrophage cell line and in a limited number of human clinical specimens (data not shown).

The concentration of azithromycin used throughout this work (15–30 μM) was similar to that utilized in the aforementioned studies describing the drug’s anti-inflammatory properties. In patients with CF, azithromycin achieves a concentration in bronchial secretions of 2–4 mg/L.33 However, the drug is sequestered inside macrophages at an intracellular to extracellular ratio of up to 25:34 This gives the data generated here and in other studies a clinical relevance, indicating that the immunomodulatory properties of azithromycin are potentially useful.

Macrophages of alternative activation display differences in surface protein expression compared with classically activated cells, as reviewed by Mantovani et al.25 Signalling through the IL-4 receptor and the IL-13 receptor leads to expression of MR26 and CD23 (FcεRII)27 on macrophages.35,36 Ligation of these receptors stimulates macrophage anti-inflammatory cytokine production including IL-1037 and an increase in arginase production.32 Together, these function as part of a comprehensive host defence process that includes regulation, clearance of extracellular debris, tissue homeostasis and repair.38 The net outcome is a down-regulation of inflammatory markers and inflammatory effector molecules from the macrophage (M2).

Additionally, study of macrophage responses has led to the conclusion that expression of arginase is induced by Th2-type cytokines such as IL-4, and the balance of iNOS to arginase correlates with Th1- and Th2-types of responses.31,32 iNOS is up-regulated in response to inflammatory stimuli as macrophages shift towards the M1 phenotype and become involved in phagocytosis, bacterial killing and initiation of the adaptive immune response. The main role of M2 cells, conversely, is to scavenge debris, phagocytose apoptotic cells after inflammatory injury and orchestrate tissue remodelling and repair through the production of extracellular matrix proteins. This up-regulation of the arginase pathway functions in the healing phases of acute and chronic inflammatory diseases as it modulates airway remodelling with IL-13 and TGFβ.22,39 These same fibrotic changes occur over time in the CF lung as a result of chronic and acute inflammation. Our data demonstrating azithromycin’s ability to preference an alternative metabolic pathway of L-arginine metabolism are consistent with the differential activation of macrophages and provide a potential explanation for the association with Th2-type inflammation with enhanced fibrosis in CF patients.16

Furthermore, IFNγ has been shown to reciprocally inhibit arginase.39 We demonstrate that in J774 cells, this inhibition is ablated in the presence of azithromycin. These data provide further evidence of the ability of azithromycin to alter the phenotype of these cells, despite a classical activation signal. Macrophages from P. aeruginosa-infected CF patients have been shown to have increased arginase and decreased levels of iNOS during acute exacerbations versus those with stable disease.40 Therefore, this property of azithromycin could have a clinically significant impact.

How M2 cells impact these changes in CF and what long-term effect azithromycin has upon fibrosis development are yet to be determined. The role of M2 cells on pulmonary remodelling has, however, been demonstrated in other pulmonary fibrotic diseases, including idiopathic pulmonary fibrosis, hypersensitivity pneumonitis and sarcoidosis.41 Previous studies show that Th2 cytokines up-regulate gene expression of alternative macrophage activation chemokines which have been shown to correlate with increased collagen production and pulmonary fibrosis.42 Additionally, Hodge et al.43 demonstrated that azithromycin increases the phagocytosis of apoptotic bronchial epithelial cells in patients with chronic obstructive pulmonary disease, which is also a function of cells of the M2 phenotype.

There is increasing evidence that a Th1-dominated immune response might improve the prognosis of CF patients with chronic P. aeruginosa lung infection,44,45 and an overzealous Th2 inflammatory response to chronic P. aeruginosa infection leads to lung destruction in the context of CF.15,46–50 It has been shown that CF patients infected with P. aeruginosa have higher levels of IL-4 and IL-13 and lower levels of IFNγ than P. aeruginosa-uninfected CF patients.35 This type of cytokine profile and the cytokine profile we have found in J774 cells treated with azithromycin is more consistent with a Th2-type of inflammatory response. Since this type of inflammatory response is inconsistent with the short-term beneficial effects of azithromycin, a better understanding of the plasticity of macrophage phenotypes (M1 versus M2) in their response to an ever changing lung microenvironment is critical in the evaluation of their role in the pathology of acute and chronic CF exacerbations. A better understanding of macrophage function and phenotype from early infection to late colonization in the CF patient will delineate the activation, potential destruction and the azithromycin-induced alteration of host defence function. Longitudinal studies of chronically infected patients with CF are necessary to analyse the contribution of macrophage phenotype to the pulmonary outcome of P. aeruginosa-infected patients. Such studies will also help us to better understand the pathogenesis of P. aeruginosa infection in CF patients, and by identifying targets of immunoregulation, the principles learned could benefit CF patients who are not yet colonized.

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Transparency declarations

None to declare.

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