Azithromycin and Clarithromycin Inhibit Lipopolysaccharide-Induced Murine Pulmonary Neutrophilia Mainly through Effects on Macrophage-Derived Granulocyte-Macrophage Colony-Stimulating Factor and Interleukin-1β

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ABSTRACT

Macrolide antibiotics possess immunomodulatory/anti-inflammatory properties. These properties are considered fundamental for the efficacy of macrolide antibiotics in the treatment of chronic inflammatory diseases like diffuse panbronchiolitis and cystic fibrosis. However, the molecular mechanisms and cellular targets of anti-inflammatory/immunomodulatory macrolide activity are still not fully understood. To describe anti-inflammatory effects of macrolides in more detail and to identify potential biomarkers of their activity, we have investigated the influence of azithromycin and clarithromycin on the inflammatory cascade leading to neutrophil infiltration into lungs after intranasal lipopolysaccharide challenge in mice. Azithromycin and clarithromycin pretreatment reduced total cell and neutrophil numbers in bronchoalveolar lavage fluid and myeloperoxidase concentration in lung tissue. In addition, concentrations of several inflammatory mediators, including CCL2, granulocyte-macrophage colony stimulating factor (GM-CSF), interleukin-1β (IL-1β), tumor necrosis factor α, and sE-selectin in lung homogenates were decreased after macrolide treatment. Inhibition of cytokine production observed in vivo was also corroborated in vitro in lipopolysaccharide-stimulated monocytes/macrophages, but not in an epithelial cell line. In summary, results presented in this article confirm that macrolides can suppress neutrophil-dominated pulmonary inflammation and suggest that the effect is mediated through inhibition of GM-CSF and IL-1β production by alveolar macrophages. Besides GM-CSF and IL-1β, CCL2 and sE-selectin are also identified as potential biomarkers of macrolide anti-inflammatory activity in the lungs.

Macrolide antibiotics (macrolides) are a well established class of antimicrobial agents characterized by the presence of a highly substituted macrocyclic lactone ring. Erythromycin, a natural product isolated from Saccharopolyspora erythraea, was the first macrolide to be introduced to clinical use over 50 years ago. Afterward, several semisynthetic derivatives of erythromycin, like clarithromycin (6-O-methylerythromycin A) and azithromycin (9-deoxy-9a-aza-9a-methyl-9a-homoerythromycin A), were designed to broaden the antimicrobial spectrum, reduce gastrointestinal side effects, and increase acid stability and bioavailability in this class of antibiotics (Whitman and Tunkel, 1992). Nowadays, macrolides are widely used in the treatment of respiratory tract and soft tissue infections.

In addition to their efficacy in treatment of bacterial infections, many studies over the past 20 years have demonstrated that macrolides are effective in the treatment of various chronic inflammatory disorders of the respiratory tract. Introduction of erythromycin to the treatment of diffuse panbronchiolitis (DPB) in the 1980s drastically increased the 10-year survival rate, decreased frequency of exacerbations, and restored lung function (Kudoh et al., 1998). Afterward, macrolides were successfully used in the treatment of cystic fibrosis (CF), which shares a number of

ABBREVIATIONS: DPB, diffuse panbronchiolitis; BALF, bronchoalveolar lavage fluid; BEBM, bronchial epithelial basal medium; CF, cystic fibrosis; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; GM-CSF, granulocyte-macrophage colony stimulating factor; siCAM-1, soluble intracellular adhesion molecule 1; IL, interleukin; LPS, lipopolysaccharide; macrocles, macrolide antibiotics; MPO, myeloperoxidase; PBS, phosphate-buffered saline; TNF-α, tumor necrosis factor α; sVCAM-1, soluble vascular adhesion molecule 1; ANOVA, analysis of variance.
similarities in clinical and pathological characteristics with DPB. In CF patients, macrolide treatment was shown to significantly improve lung function and reduce frequency of exacerbations. Consequently, macrolides are now first-line therapy for DPB and recommended for patients with CF, and they are also being evaluated in the therapy of chronic obstructive lung disease, chronic sinusitis, asthma, bronchiectasis, and bronchiolitis obliterans (reviewed in Crosbie and Woodhead, 2009).

Beneficial effects of macrolide treatment in chronic inflammatory lung diseases can not be attributed solely to their antimicrobial activity, because the doses used are lower than those used in standard antibiotic therapy and thus below the minimum inhibitory concentration for the most common respiratory pathogens. In addition, numerous in vitro and in vivo studies have shown that macrolides also possess a number of anti-inflammatory and immunomodulatory properties. They were shown to inhibit expression of proinflammatory cytokines including TNF-α, IL-1β and IL-6, chemokines, and adhesion molecules. Moreover, macrolides inhibit recruitment and migration of inflammatory cells and mucus hypersecretion (reviewed in Culić et al., 2001; Shinkai et al., 2008). Each or a combination of these anti-inflammatory or immunomodulatory properties could, therefore, account for the efficacy of macrolides in chronic inflammatory lung diseases.

Development of novel macrolide compounds that have anti-inflammatory properties, but are devoid of antimicrobial activity, has been hindered by the lack of knowledge of molecular mechanisms and cellular targets of their anti-inflammatory/immunomodulatory activity. Because several groups, including ours, have reported that various macrolides inhibit the lipopolysaccharide-induced pulmonary inflammatory response (Kadota et al., 1993; Tamaoki et al., 1995; Sanz et al., 2005; Ivetić Tkalević et al., 2006; Leiva et al., 2008; Ou et al., 2008), we have used this model to perform an in-depth investigation of the effect of azithromycin and clarithromycin on the inflammatory cascade leading to neutrophil infiltration into lungs after intranasal lipopolysaccharide (LPS) challenge in mice. The effect of macrolides on various cytokines, chemokines, and adhesion molecules induced by LPS challenge was carefully assessed to identify potential biomarkers of their anti-inflammatory activity. The data gathered from the model allowed us to establish a simple in vitro test by which the anti-inflammatory activity of novel macrolide compounds could be measured. Finally, based on in vitro and in vivo experiments, a hypothesis for the possible mechanism of inhibition of lung neutrophil infiltration was proposed.

**Materials and Methods**

**Mice.** Studies were performed on 10-week-old male BALB/cJ mice (Charles River Laboratories, Lyon, France). Mice were kept on wire mesh floors with irradiated maize granulate bedding (Scobis Due, Mucedola, Settimo Milanese, Italy) and maintained under standard laboratory conditions (temperature, 23–24°C; relative humidity, 60 ± 5%; 15 air changes per hour; artificial lighting with circadian cycle of 12 h). Pelleted food (VRF-1, Altromin; Charles River, Isaszág, Hungary) and tap water were provided ad libitum.

All procedures on animals were approved by the ethics committee of GlaxoSmithKline Research Centre Zagreb Limited, and performed in accordance with the European Economic Community Council Directive 86/609.

**Materials: Chemicals, Antibodies, and Drugs.** LPS from *Escherichia coli* serotype 0111:B4 was obtained from Sigma-Aldrich (St Louis, MO). Lumixen kits and antibodies for enzyme-linked immunosorbent assay (ELISA) were purchased from R&D Systems (Minneapolis, MN). Azithromycin was from PLIVA Inc. (Zagreb, Croatia) and clarithromycin was from Spectrum Chemical Mfg. Corp. (Gardena, CA). All other reagents, if not indicated otherwise, were from Sigma-Aldrich.

**LPS-Induced Pulmonary Neutrophilia.** Experimental pulmonary neutrophilia was induced as described earlier (Ivetić Tkalević et al., 2006). In brief, mice, under light anesthesia, were instilled intranasally with 2 μg of LPS from *E. coli* (60 μl of phosphate-buffered saline (PBS)). Vehicle, clarithromycin, and azithromycin were administered orally by gavage 4 h before intranasal challenge with LPS. For administration, macrolides were first dissolved in dimethyl sulfoxide (DMSO) and then diluted with 0.5% (w/v) methylcellulose (final concentration of DMSO was 5% (v/v)). Azithromycin was further solubilized by addition of an equimolar quantity of citric acid. Solutions obtained were applied orally in a volume of 20 ml/kg (body weight). First, macrolides were tested at doses of 150, 300, and 600 mg/kg to determine the lowest effective dose at which compounds statistically significantly decreased total cell and neutrophil numbers in bronchoalveolar lavage fluid 24 h after challenge with LPS. Based on the results obtained, a dose of 600 mg/kg was used in subsequent time course experiments.

**Bronchoalveolar Lavage and Determination of Total and Relative Cell Number in Bronchoalveolar Lavage Fluid.** Immediately before (0 h) or at various time points after LPS application, the animals were euthanized by an intraperitoneal overdose of thiopental (Inresa Arzneimittel GmbH, Freiburg, Germany). After preparation and cannulation of tracheas, bronchoalveolar lavage was performed with PBS in a total volume of 1 ml (0.4, 0.3 and 0.3 ml). After bronchoalveolar lavage, lungs were excised and fixed in 10% buffered neutral formalin fixative.

The bronchoalveolar lavage samples were centrifuged (4°C, 100g, 5 min), the pellet of cells resuspended in an equal volume of fresh PBS and used for total and differential cell counts. Total number of cells in bronchoalveolar lavage fluid (BALF) was counted with a hematological analyzer (Sysmex SF 3000; Sysmex Corp., Kobe, Japan). Percentages of neutrophils and macrophages were determined by morphological examination of at least 200 cells on smears prepared by cytocentrifugation (Cytospin-3, Thermo Fisher Scientific, Waltham, MA) and stained with Kwik-Diff staining set (Thermo Fisher Scientific). The number of neutrophils (and macrophages) in BALF was calculated for each sample according to the formula: Number of neutrophils = total number of cells × (neutrophil percentage/100%).

**Histopathological Examination of Lungs.** After collection of BALF, lungs from all animals were formalin-fixed, paraffin-embedded in toto, cut at 3 μm, and stained routinely with hematoxylin and eosin. For each lung specimen, neutrophilic granulocyte infiltration into peribronchial, periarterial, and perivenular areas, in the interstitium and alveolar space, was examined by an observer blinded to the experimental design and was graded according to the following criteria:

0. No granulocytes
1. Few scattered granulocytes
2. Larger aggregates of granulocytes
3. Marked accumulation of granulocytes.

In borderline cases, an intermediate grade was used (0–1, 1–2, 2–3), extending the scoring to a total of seven grades.

**Preparation of Lung Homogenates for Determination of Inflammatory Mediators and Adhesion Molecules.** In separate groups of mice, nonlavaged lungs were collected for determination of the concentrations of inflammatory mediators. Lungs were homogenized on ice in PBS with protease inhibitors (1 μg/ml leupeptin, 2
µg/ml aprotinin, 1 µg/ml pepstatin, and 17 µg/ml phenylmethylsulfonyl fluoride); 4 ml of PBS with protease inhibitors was added per gram of lung tissue. Homogenates were centrifuged (4°C, 2500g, 15 min) and stored at −80°C until analysis.

**Determination of Protein Concentration.** Protein concentration in lung homogenates was determined by BCA Protein Assay (Thermo Fisher Scientific) according to the manufacturer's recommendation.

**Measurement of Inflammatory Mediators in Lungs.** Samples were analyzed by use of xMAP technology (Luminex, Austin, TX), which enables simultaneous measurement of multiple biomarkers. Concentrations of GM-CSF, interferon γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-13, IL-17, CCL2 (JE), CXCL1 (KC), CXCL2 (MIP-2), and TNF-α were determined by use of fluorokine MAP multiplex kit (RD Systems) according to the manufacturer's protocol. In brief, 50 µl of samples was incubated with antibody-coated microparticles for 3 h at room temperature. Afterward, washed beads were incubated with biotinylated detection antibody cocktail for 1 h at room temperature, washed, and incubated for 30 min with streptavidin-phycocerythrin conjugate. After the final wash, the microparticles were resuspended in buffer and analyzed with the Luminex 200 (Luminex) and STarStation software v2.3 (Applied Cytometry Systems, Sheffield, UK) with use of a five-parameter, logistic-curve fitting.

Myeloperoxidase (MPO) concentration in lung homogenates was determined by Mouse MPO ELISA kit (Hycult Biotechnology, Uden, The Netherlands) according to the manufacturer's recommendations. Optical density was measured at 450 nm by use of a microplate reader (SpectraMax 190; Molecular Devices, Sunnyvale, CA). MPO concentration was determined by interpolation from standard curves with SoftMax Pro v 4.3.1 software (Molecular Devices).

Concentration of cytokines in lung homogenates was further normalized to protein concentration in the samples and expressed as picograms of analyte per milligram of protein.

**Measurement of Adhesion Molecules in Lungs.** Concentrations of sE-selectin, soluble intracellular adhesion molecule 1 (sICAM-1) and soluble vascular adhesion molecule 1 (sVCAM-1) were determined by use of a mouse cardiovascular disease panel LINCplex kit (Lincor Research, St. Charles, MO) according to the manufacturer's protocol. In brief, 25 µl of sample was incubated with antibody-coated microparticles overnight at +4°C. Afterward, washed beads were incubated with biotinylated detection antibody cocktail for 1 h at room temperature followed by a 30-min incubation with streptavidin-phycocerythrin conjugate. After the final wash the microparticles were resuspended in sheath fluid and analyzed with the Luminex 200 (Luminex) and STarStation software v2.3 (Applied Cytometry Systems, Sheffield, UK) with use of a five-parameter, logistic-curve fitting. Concentration of adhesive molecules in lung homogenates was further normalized to protein concentration in the samples and expressed as picograms or nanograms of analyte per milligram of protein.

**Cells.** The murine monocytic/macrophage cell line, J774.2 was obtained from the European Collection of Cell Cultures (Porton Down, Wiltshire, UK), whereas murine lung epithelial cells, MLE 12, were from American Type Culture Collection (Manassas, VA). J774.2 cells were grown in Dulbecco’s modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (BFS) (BioWest, Nuaillé, France). MLE 12 cells were maintained in bronchial epithelial basal medium (BEBM; Lonza Walkersville, Walkersville, MD) supplemented with growth factors (bovine pituitary extract, hydrocortisone, human epidermal growth factor, insulin, and transferrin), gentamicin, and amphotericin-B (all provided as BEGM SingleQuots; Lonza Walkersville) and 2% BFS.

**Cell Stimulation and Inhibition.** For all in vitro experiments macrodilines were dissolved in DMSO at a concentration of 50 mM and a series of 2-fold dilutions in DMSO were prepared. Macrolide DMSO stock solutions were diluted 1000-fold in cell culture medium to the desired concentrations. Therefore, DMSO concentration was 0.1% in all samples. J774.2 cells were seeded in a 24-well plates in Dulbecco’s modified Eagle’s medium with 10% BFS at a concentration of 3 × 10^5 cells per well. The next day, cells were preincubated with macrolides for 2 h and stimulated overnight with 1 µg/ml LPS from E. coli serotype 0111:B4. At the end of the incubation period, supernatants were collected and stored at −20°C until assayed.

MLE 12 cells were seeded in 24-well plates in supplemented BEBM at a concentration of 2.5 × 10^5 cells per well and grown to confluence. Afterward, cells were preincubated with macrolides in BEBM supplemented with 2% BFS for 2 h and stimulated overnight with 10 ng/ml LPS from E. coli serotype 0111:B4. At the end of the incubation period, supernatants were collected and stored at −20°C until assayed. Optimal concentrations of LPS for stimulation of J774.2 and MLE were established in preliminary experiments.

**ELISA.** Cytokine concentrations were determined in cell culture supernatants by sandwich ELISA using capture and detection antibodies according to the manufacturer’s instructions. Sensitivity of the assay was 0.1 pg/ml for CCL2, 12.2 pg/ml for CXCL1, 154.1 pg/ml for CXCL2, 4.6 pg/ml for GM-CSF, 20.2 pg/ml for IL-1β, 5.5 pg/ml for IL-6, and 25.5 pg/ml for TNF-α. Optical density was measured at 450 nm by use of a microplate reader (SpectraMax 190, Molecular Devices). Concentration of cytokines was determined by interpolation from standard curves with SoftMax Pro v4.3.1 software (Molecular Devices).

**Statistical Analysis.** All values are presented as means ± S.E.M. To define statistically significant differences among vehicle- and macrolide-treated mice after LPS challenge, the data were subjected to two-way ANOVA followed by a Bonferroni post test using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA). To define statistically significant differences between vehicle and macrolide-exposed cells in vitro, the data were subjected to one-way ANOVA followed by Dunnett’s post-test using GraphPad Prism (GraphPad Software). The level of significance was set at p < 0.05 in all cases.

**Results**

**Effect of Macrolides on Inflammatory Cell Accumulation in Airways and Lungs.** The lowest effective oral dose at which azithromycin and clarithromycin statistically significantly decreased total cell and neutrophil numbers in bronchoalveolar lavage fluid, 24 h after challenge with LPS, is 600 mg/kg (body weight) (Fig. 1, A and B). Therefore, this dose was used in subsequent time course experiments, in which effects of macrolides on the inflammatory cascade leading to neutrophil infiltration into the lungs was assessed after intranasal LPS challenge.

An increase in the number of inflammatory cells in airways (BALF) was observed at 6 h and progressed until 24 h after LPS instillation. As expected, infiltrated cells were neutrophils, whereas macrophage numbers did not change significantly. Macrolide treatment almost completely blocked initial inflammatory cell accumulation in BALF at 6 h, and significantly decreased total cell and neutrophil numbers in BALF (~50%) at 24 h after LPS challenge (Fig. 2, A and B). To quantitatively measure neutrophil levels in the lungs, the concentration of a neutrophil-specific enzyme, MPO, in lung homogenates was determined. MPO concentration in lungs reached maximal levels by 4 h after LPS challenge and remained increased for at least 24 h (Fig. 3). The effect of both tested macrolides on LPS-induced increase of MPO was even more striking than their effects on inflammatory cell accumulation in BALF (Fig. 3). We were surprised to find that MPO concentration was significantly increased 4 h after...
azithromycin administration to naive animals, but not after clarithromycin administration.

Histopathological examination of the lungs confirmed accumulation of neutrophils around arteries, veins, and bronchi and in alveoli of LPS-treated animals (Fig. 4B). Treatment with azithromycin decreased the number of neutrophils around arteries and bronchi (Fig. 4C). In addition, azithromycin postponed and reduced the perivascular and intra-alveolar accumulation of neutrophils. Clarithromycin had a similar effect on neutrophil accumulation as azithromycin (Fig. 4D).

**Effect of Macrolides on Inflammatory Mediators in Lungs.** After the demonstration that macrolides efficiently inhibit LPS-induced pulmonary infiltration of inflammatory cells, their effects on inflammatory mediators were evaluated. In a preliminary experiment, LPS-mediated increases in GM-CSF, IL-1β, IL-6, CCL2 (JE), CXCL1 (KC), CXCL2 (MIP-2), and TNF-α in lung homogenates were observed, whereas interferon γ, IL-2, IL-4, IL-5, IL-10, IL-12p70, IL-13, and IL-17 were below the limit of detection (data not shown) and therefore not measured in the subsequent experiments.

Azithromycin and clarithromycin did not inhibit the production of two major neutrophil chemoattractants in mice (CXCL1 and CXCL2) which were strongly induced immediately after LPS challenge, peaked 4 h after challenge, and gradually decreased afterward (Fig. 5, A and B). Similar to the effects observed on neutrophil infiltration into lungs, azithromycin pretreatment increased CXCL1 concentration in naive mice (at 0 h). On the other hand, azithromycin and clarithromycin decreased LPS-induced TNF-α, IL-6, GM-CSF, CCL2, and IL-1β levels. Whereas IL-6 concentration was only slightly decreased between 4 and 8 h after LPS challenge (Fig. 5D), TNF-α concentration was significantly decreased (for ∼50%) at all time points after LPS challenge, with the exception of 24 h when TNF-α levels had almost returned to baseline (Fig. 5C). The most striking effect of macrolides was seen on GM-CSF and CCL2: both azithromycin and clarithromycin almost completely inhibited LPS-induced release of these two cytokines, which peaked at 4 and 8 h after LPS challenge, respectively (Fig. 5, E and F). In contrast to other measured inflammatory mediators, IL-1β concentration increased at 4 h but remained elevated even 24 h after LPS challenge, and it was significantly inhibited by both compounds (Fig. 5G).

**Effect of Macrolides on Adhesion Molecules in Lungs.** In addition to the modulation of production of cytokines, macrolides could also affect neutrophil infiltration into the lungs by decreasing the expression of adhesion molecules. Therefore, concentrations of several adhesion molecules were determined in lung homogenates. LPS-induced increase in sE-selectin concentration between 4 and 8 h after challenge was strongly decreased by both tested macrolides (Fig. 6A). In contrast to sE-selectin, a continuous increase in sICAM-1 concentration was observed up to 24 h after LPS challenge, and the macrolide effect on sICAM-1 concentration was observed only at the latest time point investigated (Fig. 6B). On the other hand, sVCAM-1 concentration in lung homogenates was not changed after LPS administration (Fig. 6C).

**Effect of Macrolides on Cytokine Production by LPS-Stimulated J774.2 and MLE 12 Cells.** The results presented so far indicate that macrolides efficiently suppress LPS-induced neutrophil accumulation, which could be explained by the decreased production and/or release of inflammatory mediators and adhesion molecules. The main mouse lung cell types that express Toll-like receptor 4 and coreceptors required for LPS recognition, and seem to be responsible for initiation of inflammation in lungs after LPS challenge, are macrophages and epithelial cells (Martin, 2000; Saito et al., 2005). Therefore, to validate the biomarkers detected in vivo, we examined the ability of macrolides to suppress LPS-induced cytokine production from lung epithelial cells, MLE 12, and the monocyte-macrophage cell line, J774.2.

In the epithelial cell line, MLE 12, macrolides did not influence production of any of the LPS-stimulated cytokines (IL-6, GM-CSF, CXCL1, and CCL2; Fig. 7). On the other hand, in the macrophage cell line, J774.2, macrolide pretreatment dose-dependently suppressed the LPS-induced production of IL-1β, IL-6, and GM-CSF (Fig. 8), while having no inhibitory effect on LPS-stimulated production of TNF-α, CCL2, CXCL1, and CXCL2 (data not shown). The viability of cells was not affected by treatment with macrolides because
no significant increase in adenylate kinase was observed in supernatants at the end of incubation (data not shown).

Discussion

Neutrophil infiltration is prominent in many lung diseases but its resistance to corticosteroid therapy prompted a search for drugs that inhibit neutrophil inflammation (Barnes, 2007). Investigations in humans and animals, including our experimental findings presented here with the use of cell counts, histology, and MPO determinations, show that macrolide treatment efficiently suppresses neutrophil-dominated pulmonary inflammation. However, the mechanism is not entirely clear, so we studied the inflammatory cascade leading to lung neutrophil accumulation after LPS challenge. We aimed to identify potential biomarkers of macrolide anti-inflammatory activity, subsequently applicable to a relatively simple in vitro test to profile non-antibacterial, anti-inflammatory macrolide compounds.

The dose showing anti-inflammatory effects in this animal model (600 mg/kg) is higher than those exerting therapeutic effects in humans (ranging from 250 mg every other day to 500 mg every day). However, to achieve therapeutic effects in chronic inflammatory diseases, macrolides have to be administered for long periods of several months or even years (Crosbie and Woodhead, 2009). This may explain why, in our acute animal model of inflammation, a single high dose is needed. Moreover, Criqui et al. (2000) showed that, even in clinical settings, short-term treatment with azithromycin (500 mg on day 1 followed by 250 mg daily for 3 days) is not sufficient to reduce an acute inflammatory response induced by ozone.
Within 2 h of intranasal LPS challenge, lung inflammation is characterized by production of three mediators: CXCL1, CXCL2, and TNF-α. We were surprised to find that macrolides did not inhibit production of the major murine neutrophilic chemoattractants, CXCL1 and CXCL2, either at this early or at later time points. Moreover, macrolides failed to inhibit LPS-induced production of these chemokines in epithelial and macrophage cell lines in vitro. In contrast, production of TNF-α was weakly, but statistically significantly decreased by azithromycin and clarithromycin (33 and 54%, respectively, at peak levels, 4 h after LPS challenge). This weak inhibitory activity of the macrolides was not corroborated by in vitro findings. Because various cell types contribute to lung inflammation, the inhibition of TNF-α production observed in vivo could be the consequence of effects on cell

Fig. 5. Concentration of CXCL1 (A), CXCL2 (B), TNF-α (C), IL-6 (D), GM-CSF (E), CCL2 (F), and IL-1β (G) in lung homogenates at different time points after LPS challenge. Azithromycin (△) or clarithromycin (○) were administered orally 4 h before LPS challenge at a dose of 600 mg/kg. Data are presented as means ± S.E.M. *p < 0.05, two-way ANOVA; Bonferroni post test.
macrolide-mediated inhibition of TNF-α is unlikely to be a prerequisite for inhibition of lung neutrophil accumulation.

That inflammatory mediators produced early after LPS challenge (CXCL1, CXCL2, and TNF-α) are unininvolved in macroside-induced decreases in neutrophil accumulation agrees with the findings of Sanz et al. (2005). They reported that erythromycin treatment reduced BALF neutrophil counts and lung tissue MPO activity in LPS-challenged rats without effects on CXCL2 (MIP-2) and TNF-α levels in BALF.

In contrast to effects on early inflammatory mediators, macrolide treatment significantly attenuated increases in IL-6, GM-CSF, CCL2, and IL-1β concentrations from 4 h after LPS challenge. Azithromycin and clarithromycin weakly inhibited—like TNF-α—IL-6 production (~30%) between 4 h and 8 h after LPS challenge in vivo, and IL-6 production by macrophages in vitro. IL-6 plays a dual role in LPS-induced neutrophil accumulation, possessing both anti- and pro-inflammatory properties. LPS administration to IL-6−/− mice induced significantly higher neutrophilic responses and increases in TNF-α and CXCL2 (MIP-2) compared with responses in wild-type mice (Xing et al., 1998). However, IL-6 was required for leukocyte migration into the lungs, and for the neutrophil to monocyte recruitment transition during inflammation (Kaplanski et al., 2003). Therefore, mild IL-6 inhibition induced by macrolides probably does not directly affect neutrophil accumulation, but could ameliorate later stages of the inflammatory response to LPS, when transition to monocyte accumulation occurs.

Although macrolides inhibited TNF-α and IL-6 only moderately, they almost completely blocked LPS-induced GM-CSF generation in vivo. GM-CSF production by macrophages in vitro was also inhibited. GM-CSF is a survival factor for neutrophils, and its neutralization by anti-GM-CSF antibodies suppresses LPS-induced neutrophilic inflammation in lungs (Bozinovski et al., 2004). Moreover, anti-GM-CSF antibody treatment did not influence expression of CXCL2 (MIP-2), and reduced peak TNF-α levels only by 37%, in keeping with effects of macrolides in our study. Thus, macrolides by inhibiting GM-CSF could reduce neutrophil life-span, increase apoptosis, and subsequently reduce their number in LPS-challenged lungs. This suggestion is strengthened by findings that the number of apoptotic granulocytes in blood of healthy volunteers was significantly increased after 3-day dosing with azithromycin (500 mg/day p.o.) (Culić et al., 2002). Moreover, macrolides decreased neutrophil survival in vitro through inhibition of GM-CSF release from activated human airway epithelial cells (Yamasawa et al., 2004). In our study, macrolides failed to inhibit GM-CSF production by mouse epithelial cells, implying a species-dependent, differential cell target for the anti-inflammatory effects of macrolides, currently under investigation in our laboratory. Nevertheless, inhibition of GM-CSF production by macrolides could well account for reduced LPS-induced cellular accumulation into mouse lungs.

Azithromycin and clarithromycin also dramatically (73% and 96%, respectively) suppressed lung CCL2. Although neutrophils do not express the CCL2 receptor, CCR2, CCL2 still seems to be important for lung neutrophil accumulation. Treatment of mice with anti-CCL2 antibodies reduced both monocyte and neutrophil recruitment during pulmonary...
Cryptococcus neoformans infection (Huffnagle et al., 2000). Moreover, intratracheal challenge with CCL2 and LPS strongly augmented neutrophil influx into alveolar spaces, compared with challenge with LPS alone (Maus et al., 2003). Therefore, inhibition of CCL2 production by macrolides could contribute to reduced neutrophil influx into LPS-challenged mouse lungs. Inhibition of CCL2 production was not observed in LPS-stimulated macrophages and epithelial cells in vitro. This contradictory result can be explained by the finding that CCL2 production is increased both by LPS and the induced cytokines, TNF-α and IL-1β (Standiford et al., 1991). Consequently, it is likely that the inhibition of CCL2 production after macrolide treatment in vivo was secondary to reduction of TNF-α and IL-1β levels, whereas in epithelial and macrophage monocultures in vitro, TNF-α and thereby CCL2 production remained unchanged.

In contrast to other inflammatory mediators, the pulmonary IL-1β increased between 2 and 4 h, remaining elevated until 24 h after LPS challenge. IL-1β alone is sufficient to induce substantial lung neutrophil accumulation (Ulich et al., 1991; Lappalainen et al., 2005). Therefore, its inhibition by macrolides in vivo and in alveolar macrophages in vitro seems to be a major mechanism for reduction of cellular influx into lungs. IL-1β was, in fact, the only significantly decreased proinflammatory cytokine in BALF after 12 weeks of azithromycin treatment of patients with chronic obstructive lung disease (Hodge et al., 2008). These data further support the validity of IL-1β as a biomarker of macrolide anti-inflammatory activity in the lungs.

Because the spectrum of cytokine inhibition in vivo was mirrored in monocyte/macrophages, but not in epithelial cells in vitro, it is likely that alveolar macrophages are the target cells for macrolide activity in our in vivo model. Nevertheless, macrolides may also act on other pulmonary cell types, either directly inhibiting LPS signaling or that of proinflammatory cytokines.

Macrolide treatment also reduced levels of two up-regulated adhesion molecules, sE-selectin and sICAM-1. Inhibition of adhesion molecule expression by macrolides was reported in a similar animal model (Sanz et al., 2005) and in clinical studies (Parchure et al., 2002; Hillis et al., 2004). However, because these adhesion molecules are induced by both LPS and proinflammatory cytokines, such as TNF-α and IL-1β, the observed inhibition may be indirect, through effects of macrolides on cytokines.

Azithromycin, but not clarithromycin, increased lung CXCL1 and MPO concentrations at the time of challenge. This proinflammatory activity of azithromycin corresponds to a biphasic effect reported previously (Culić et al., 2002; Shinkai et al., 2006). Azithromycin (500 mg/day for 3 days) initially stimulated blood neutrophil degranulation and oxidative burst to particulate stimuli in healthy volunteers, a response followed by a continual fall in IL-8, growth-regulated oncogene alpha, and IL-6 concentrations, a delayed down-regulation of the oxidative burst, and an increase in apoptosis of neutrophils (Culić et al., 2002). Likewise, azithromycin and clarithromycin increased IL-8 production by normal human bronchial epithelial cells at 24 h after LPS...
stimulation, but after prolonged incubation (for 5 days), decreased IL-8 levels compared with LPS alone (Shinkai et al., 2006). The early proinflammatory activity of azithromycin in our model in vivo, however, is unrelated to its anti-inflammatory effects on the monitored inflammatory parameters, because, in general, these were comparable with those of clarithromycin.

In conclusion, the effective suppression by macrolide antibiotics of pulmonary neutrophil-dominated inflammation seems to involve inhibition of GM-CSF and IL-1β production by alveolar macrophages. Measurement of these mediators in LPS-stimulated macrophages in vitro could represent a simple test for novel anti-inflammatory macrolides. In addition, CCL2 and sE-selectin show potential as biomarkers for macrolide anti-inflammatory activity in the lungs. If GM-CSF and IL-1β are indeed major targets for macrolides in reducing murine lung neutrophilia, inflammation induced by one or both of these mediators should be resistant to macrolides. This will be addressed in future studies. In addition, in vitro mechanistic studies will be extended to human bronchial epithelial cells and monocyte-derived macrophages.

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References


Fig. 8. Effect of macrolides on IL-6 (A), GM-CSF (B), and IL-1β (C) production by LPS-stimulated J774.2 cells. Cells were preincubated with compounds for 2 h followed by an overnight LPS stimulation. Data are presented as means ± S.E.M. from four different experiments. *, p < 0.05, one-way ANOVA; Dunnett’s post test.


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