



Pulmonary, Gastrointestinal and Urogenital Pharmacology

Clarithromycin suppresses airway hyperresponsiveness and inflammation in mouse models of asthma

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ABSTRACT

Macrolide antibiotics, a class of potent antimicrobials, also possess immunomodulatory/anti-inflammatory properties. These properties are considered fundamental for the efficacy of macrolide antibiotics in the treatment of diffuse panbronchiolitis and cystic fibrosis. In patients with asthma, macrolide antibiotics have been reported to reduce airway hyperresponsiveness and improve pulmonary function. However, their beneficial actions in asthmatics possibly could be attributed to antimicrobial activity against atypical pathogens (e.g. *Chlamydia pneumoniae*), corticosteroid-sparing effect (inhibition of exogenous corticosteroid metabolism), and/or their anti-inflammatory/immunomodulatory effects. In order to investigate whether efficacy of macrolide antibiotics in asthma results from their immunomodulatory/anti-inflammatory activity, the influence of clarithromycin pretreatment (2 h before challenge) was examined on ovalbumin-induced airway hyperresponsiveness and airway inflammation in the mouse. Clarithromycin treatment (200 mg/kg intraperitoneally) decreased IL-4, IL-5, IL-13, CXCL2 and CCL2 concentrations in bronchoalveolar lavage fluid and markedly reduced inflammatory cell accumulation in bronchoalveolar lavage fluid and into the lungs, as revealed by histopathological examination. Furthermore, clarithromycin-induced reduction in inflammation was accompanied by normalization of airway hyperresponsiveness. In summary, in ovalbumin-induced mouse models, clarithromycin efficiently inhibited two important pathological characteristics of asthma, airway hyperresponsiveness and inflammation. These data suggest that the efficacy of clarithromycin, as well as of other macrolide antibiotics, in asthmatic patients could be attributed to their anti-inflammatory/immunomodulatory properties, and not only to their antimicrobial activity or exogenous corticosteroid-sparing effects.

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1. Introduction

Macrolide antibiotics with a 14- and 15-member lactone ring (erythromycin, clarithromycin and azithromycin) are a class of potent and well established antimicrobials that also possess anti-inflammatory and/or immunomodulatory properties (Čulić et al., 2001; López-Boado and Rubin, 2008; Shinkai et al., 2008). Anti-inflammatory/immunomodulatory activities of macrolide antibiotics include inhibition of inflammatory cytokine and chemokine production (Khan et al., 1999; Abe et al., 2000; Cigana et al., 2007), adhesion molecule expression (Miyanohara et al., 2000; Enomoto et al., 2002; Sanz et al., 2005), inflammatory cell accumulation and mucus hypersecretion (Kadota et al., 1993; Sanz et al., 2005; Ivetić Tkalčević et al., 2006; Leiva et al., 2008; Ou et al., 2008).

The most convincing demonstration of the anti-inflammatory/immunomodulatory effects of macrolide antibiotics has been in the treatment of diffuse panbronchiolitis (Kudoh et al., 1998) and cystic

fibrosis (Jaffe et al., 1998; Clement et al., 2006). In addition, macrolide antibiotics are also being evaluated for the therapy of other chronic inflammatory disorders of the lungs, including chronic obstructive pulmonary disease (Basyigit et al., 2004; Parnham et al., 2005), chronic sinusitis (Hatipoglu and Rubinstein, 2005) and asthma (Kostadima et al., 2004; Piacentini et al., 2007; Simpson et al., 2008).

Asthma is an inflammatory disease of the airways whose common characteristics include reversible airway obstruction, airway hyperresponsiveness, airway and lung inflammation and increased mucus production (Bousquet et al., 2000). It is one of the most common chronic inflammatory diseases, affecting approximately 4% to 10% of the population (Arif et al., 2003; Pearce et al., 2000). Although asthma is generally well controlled with existing therapies (such as inhaled corticosteroids and β_2 -agonists), there are several reasons why a research for novel treatment modalities for asthma is still ongoing: (a) the disease is still poorly controlled in patients with severe, corticosteroid-insensitive asthma; (b) there is a constant need for improvement of existing therapies in terms of more favorable side effect profile or oral formulation; and (c) the current asthma therapies are not cures and symptoms return soon after the treatment is stopped, even after long-term therapy (Adcock et al., 2008).

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Beneficial effects of macrolide antibiotics in asthma have been known for almost 50 years: in the 1950s, concomitant therapy with troleandomycin, a 14-member lactone ring macrolide antibiotic, improved clinical symptoms of the disease and reduced corticosteroid dosage in patients with corticosteroid-dependent asthma (Kaplan and Goldin, 1959). Since then, troleandomycin, as well as newer macrolide antibiotics such as clarithromycin, erythromycin or roxithromycin have been shown to reduce corticosteroid use by >50% in corticosteroid-dependent asthmatic patients (Zeiger et al., 1980; Ball et al., 1990; Garey et al., 2000), decrease airway hyperresponsiveness (Miyatake et al., 1991; Shimizu et al., 1994; Amayasu et al., 2000; Kostadima et al., 2004), as well as eosinophil and neutrophil number in sputum (Shoji et al., 1999; Amayasu et al., 2000; Simpson et al., 2008).

In the past, the reported therapeutic benefit of macrolide antibiotic treatment in patients with asthma was not attributed to their anti-inflammatory/immunomodulatory properties. Pharmacokinetic studies suggested that the beneficial effects of troleandomycin therapy, at least in part, may be due to the inhibition of metabolism of exogenous steroid, through the inactivation of hepatic cytochrome P450 by forming inactive P450-metabolite complexes (Szeffler et al., 1980; Szeffler et al., 1982; Periti et al., 1992; Fost et al., 1999). In addition to the effect on metabolism of exogenous steroids, macrolide antibiotics possess antimicrobial activity against atypical intracellular pathogens such as *Chlamydia pneumoniae* and *Mycoplasma pneumoniae* (Critchley et al., 2002) and according to some publications, macrolide antibiotic therapy improves lung function only in those patients whose asthma is associated with the presence of these pathogens (Black et al., 2001; Kraft et al., 2002). On the other hand, a low-dose of troleandomycin was shown to directly attenuate bronchial hyperresponsiveness in children with severe asthma and this effect was independent of its action on metabolism of exogenous glucocorticoids (Ball et al. 1990; Kamada et al., 1993). Additionally, the doses of macrolide antibiotics used for the treatment of respiratory diseases are lower than those used in standard antibiotic therapy and thus below the minimum inhibitory concentration for the most common respiratory pathogens (Nagai et al., 1991).

Therefore, we hypothesized that the efficacy of macrolide antibiotics in asthma results not only from possible exogenous corticosteroid-sparing or antimicrobial effects, but also from their immunomodulatory/anti-inflammatory activities. In order to test this hypothesis, we have investigated the influence of a 14-member ring macrolide antibiotic clarithromycin on ovalbumin-induced airway hyperresponsiveness and airway inflammation in a mouse model of asthma. The beneficial effects of the macrolide antibiotic in this model could be attributed exclusively to its anti-inflammatory/immunomodulatory properties, as all experiments were conducted on pathogen-free laboratory mice (thereby excluding antimicrobial effects) and no corticosteroid treatment was employed (thereby excluding exogenous corticosteroid-sparing effects).

2. Materials and methods

2.1. Mice

Studies were performed on 6–12 weeks old specific pathogen-free (SPF) male BALB/c (Charles River, Germany) or BALB/cJ (Charles River, France), maintained under standard laboratory conditions. Pelleted food (VRF-1, Altromin, Charels River, 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 (a) guidelines outlined by the NIH Guide for the Care and Use of Laboratory Animals; and (b) regional legislative equivalent, Animal protection law, published December 13th, 2006 in Official gazette No. 135.

2.2. Materials: chemicals, antibodies and drugs

Ovalbumin (grade VI) was from Sigma Chemical Co. (St Louis, MO, US). Luminex kits and antibodies for enzyme-linked immunosorbent assay (ELISA) were purchased from R&D Systems (MN, US). Dexamethasone was from Sigma Chemical Co. (St Louis, MO, US) and clarithromycin was from Spectrum Chemical Mfg. Corp. (Gardena, CA, US). All other reagents, if not indicated otherwise, were from Sigma Chemical Co. (St Louis, MO, US).

2.3. Sensitization and airway challenge

The experimental protocol for airway hyperresponsiveness and airway inflammation assessment was done according to literature data (Tomkinson et al., 2001) with slight modifications established in preliminary experiments. Briefly, for airway hyperresponsiveness assessment on experimental days 0 and 14 BALB/cJ mice were sensitized to ovalbumin by intraperitoneal administration of 20 µg of ovalbumin dissolved in 1.96 mg of aluminum hydroxide (Alu-Gel-S, SERVA Electrophoresis GmbH, Heidelberg, Germany) in a total volume of 0.1 ml. On experimental day 20, mice, under light anesthesia (combination of ketamine hydrochloride [2 mg/mouse; Narketan® Vetoquinol, Bern, Switzerland) and xylazinehydrochloride (0.07 mg/mouse; Rompun®, Bayer, Leverkusen, Germany)], were intranasally challenged with 50 µl of saline (controls) or 100 µg of ovalbumin in 50 µl of saline. Twenty-four hours after challenge airway hyperresponsiveness was assessed. From the same mice bronchoalveolar lavage samples were collected at 48 h after challenge as described later.

In order to examine allergic airway inflammation in mice in more detail, a second experimental scheme was employed as described earlier (Hrvačić et al., 2006). Briefly, BALB/c, mice were sensitized with ovalbumin on experimental days 0 and 14. Sensitization was achieved by intraperitoneal application of 10 µg of ovalbumin dissolved in 2% Alu-Gel-S (SERVA Electrophoresis GmbH, Heidelberg, Germany) in a total volume of 0.2 ml/mouse. On experimental day 20, mice, under light anesthesia, were instilled intranasally with 25 µg of ovalbumin dissolved in PBS in a total volume of 50 µl. Bronchoalveolar lavage samples and lungs were collected at time points indicated as described later.

2.4. Treatment of mice

Standard steroid, dexamethasone, was administered intraperitoneally on experimental days 18–21, once daily at the dose of 1 mg/kg (b.w.). Clarithromycin was administered once on experimental day 20, 2 h before intranasal challenge, at the dose of 200 mg/kg intraperitoneally. The treatment regimen was chosen according to the previously described clarithromycin efficacy in other inflammatory *in vivo* models (Ivetić Tkalečević et al., 2006); in rodents intraperitoneal administration of macrolide antibiotics is usually more effective than oral administration.

For administration, dexamethasone was first dissolved in dimethyl sulfoxide (DMSO) and then diluted with saline, while clarithromycin was first dissolved in DMSO and then diluted with 0.5% (w/v) methyl-cellulose (final concentration of DMSO was up to 5% (v/v)). Ovalbumin-challenged control mice received vehicle alone [5% (v/v) DMSO in 0.5% (w/v) methyl-cellulose] 2 h prior to challenge. The suspensions obtained were given intraperitoneally in a volume of 10 ml/kg (b.w.; for dexamethasone) or 20 ml/kg (b.w.; for vehicle and clarithromycin).

2.5. Determination of airway hyperresponsiveness

Airway hyperresponsiveness to methacholine in conscious, spontaneously breathing animals was measured by barometric plethysmography (Buxco Electronics Inc., Troy, NY, USA) as described earlier (Hamelmann et al., 1997). Briefly, plethysmography measures pressure differences between the main chamber of the system containing the mouse and a reference chamber. Mice were exposed to aerosolized

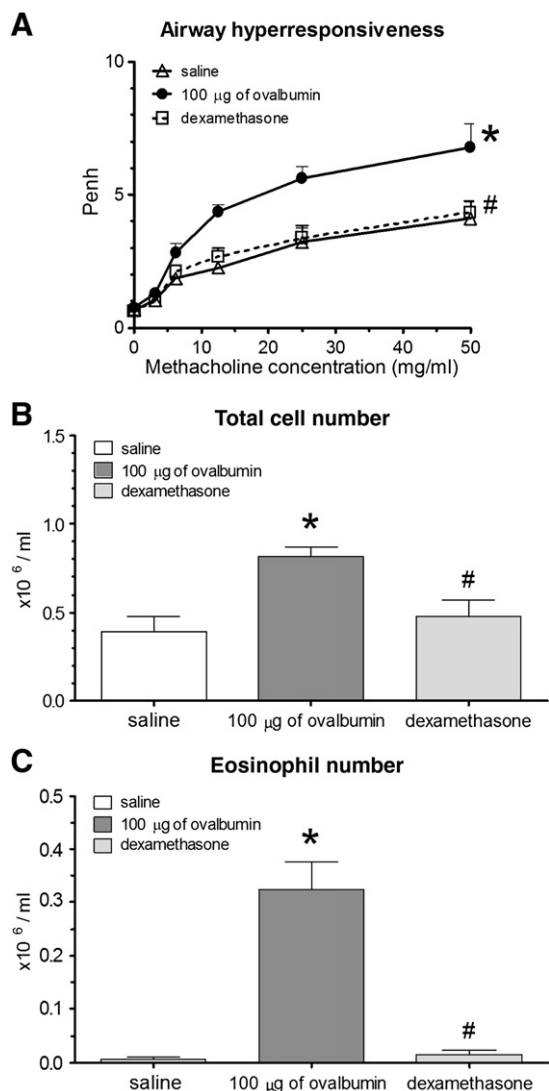


Fig. 1. (A) Airway hyperresponsiveness assessed as Penh using unrestricted barometric plethysmography in vehicle- and dexamethasone-treated mice 24 h after challenge with 100 µg of ovalbumin; control mice were challenged with saline. At 48 h after challenge of the same mice, total cell (B) and eosinophil number (C) was assessed. Dexamethasone was administered intraperitoneally at the dose of 1 mg/kg, 2 h before ovalbumin challenge. All data represent means $\pm$ S.E.M. ($n = 6-8$ mice per group). The asterisk (*) indicates significant difference ($P < 0.05$) between saline and ovalbumin-challenged groups, and the ladder (#) indicates significant difference ($P < 0.05$) between the ovalbumin-challenged group and clarithromycin-treated group.

PBS (for the baseline measurement) or methacholine (3.125 to 50 mg/ml) for 3 min and readings were taken and averaged for 3 min after each nebulization. Data obtained were presented as the enhanced pause (Penh, dimensionless parameter) vs. the methacholine concentration (mg/ml) used to generate the aerosol.

2.6. Bronchoalveolar lavage and accumulation of inflammatory cells

At the time points indicated, mice were killed by an intraperitoneal overdose of Thiopental® (Nycomed, Germany). Tracheostomy was performed and a plastic catheter was clamped into the trachea. The lungs were washed 3 times with PBS in a total volume of 1 ml (0.4, 0.3 and 0.3 ml). The bronchoalveolar lavage samples were centrifuged for 5 min at 10,000 g and 4 °C to collect whole cells in the pellet. The supernatant was carefully removed and stored at -80°C for subsequent analyses. The pellet of cells that remains after centrifugation of the bronchoalveolar lavage samples was resuspended in an

equal volume of fresh PBS and used for total and differential cell counts. The total number of cells in bronchoalveolar lavage fluid was counted in a haematology counter (Sysmex SF 3000, Sysmex Co., Kobe, Japan). The percentage of inflammatory cells was determined by morphological examination of at least 200 cells in cytocentrifuged preparations (Cytospin-3, Shandon Instruments, UK), stained with the Kwik-Diff staining set (Thermo Fisher Scientific Inc., Pittsburgh, PA, USA), according to standard morphologic criteria.

2.7. Measurement of bronchoalveolar lavage fluid cytokines

Samples were analyzed using xMAP technology, which enables simultaneous measurement of multiple biomarkers. Concentrations of GM-CSF, IL-1 β , IL-2, IL-4, IL-5, IL-13, IL-17, CCL2 (JE), CXCL1 (KC), CXCL2 (MIP-2) and TNF- α were determined using Fluorokine MAP multiplex kit (R&D Systems, MN, US) according to the manufacturer's protocol. Briefly, 50 µl of samples were incubated with antibody-coated microparticles for 3 h at

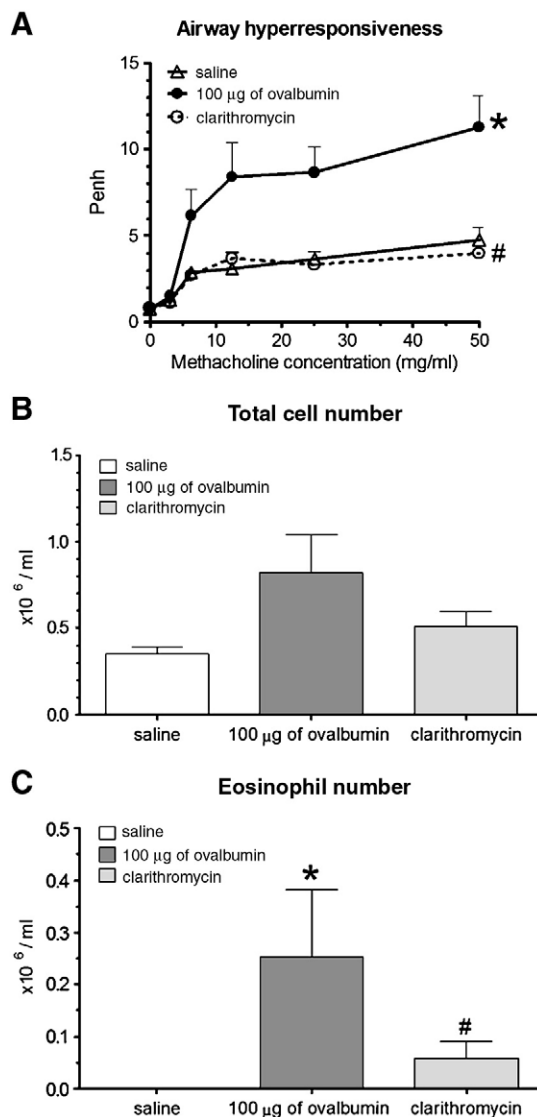


Fig. 2. (A) Airway hyperresponsiveness assessed as Penh using unrestricted barometric plethysmography in vehicle- and clarithromycin-treated mice 24 h after challenge with 100 µg of ovalbumin; control mice were challenged with saline. At 48 h after challenge of the same mice, total cell (B) and eosinophil number (C) were assessed. Clarithromycin was administered intraperitoneally at the dose of 200 mg/kg, 2 h before ovalbumin challenge. All data represent means $\pm$ S.E.M. ($n = 5-6$ mice per group). The asterisk (*) indicates significant difference ($P < 0.05$) between saline and ovalbumin-challenged groups, and the ladder (#) indicates significant difference ($P < 0.05$) between ovalbumin-challenged group and clarithromycin-treated group.

room temperature. Afterwards, washed beads were incubated with biotinylated detection antibody cocktail for 1 h at room temperature, washed and incubated for 30 min with streptavidin–phycoerythrin conjugate. After the final wash the microparticles were resuspended in buffer and analyzed with the Luminex 200™ (Luminex, Austin, TX, US) and StarStation software v2.3 (Applied Cytometry Systems, Sacramento, CA, US) using a five-parameter-logistic-curve fitting.

2.8. Histopathological analysis of the lungs

After bronchoalveolar lavage, lungs were fixed by immersion in 10% formalin, trimmed and paraffin embedded. For scoring of eosinophil and mononuclear cell infiltration, four 3 µm sections containing main stem bronchi from each lung specimen were cut and stained routinely with hematoxylin and eosin. Eosinophil and mononuclear cell infiltration into peribronchial/peribronchiolar areas were evaluated observer-blind and graded according to the following criteria:

- 0 no inflammatory cells (granulocytes or mononuclear cells);
- 1 few scattered inflammatory cells;
- 2 larger aggregates of inflammatory cells;
- 3 marked accumulation of inflammatory cells.

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

2.9. Statistical analysis

Values are presented as means ± S.E.M. For statistical comparison of airway hyperresponsiveness on the graph of Penh (y axis) vs. the methacholine concentration (mg/ml; x axis), area under curve was calculated for each experimental animal using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA). To define statistically significant differences among groups, the data were

subjected to ANOVA using GraphPad Prism. *P* values <0.05 were considered to be significant.

3. Results

3.1. Effect of clarithromycin on airway hyperresponsiveness and airway inflammation

The ability of clarithromycin to suppress two asthma hallmarks, airway hyperresponsiveness and eosinophil accumulation in bronchoalveolar lavage fluid, was firstly evaluated in comparison to standard steroid, dexamethasone. For this purpose, an experimental model of acute asthma in which ovalbumin-sensitized mice are intranasally challenged with 100 µg of ovalbumin was employed. In this model airway hyperresponsiveness can be detected using unrestrained plethysmography 24 h after challenge, whereas marked accumulation of eosinophils is present in bronchoalveolar lavage fluid at 48 h after challenge (Tomkinson et al., 2001).

As expected, single intranasal challenge with 100 µg of ovalbumin induced airway hyperresponsiveness to methacholine, as well as eosinophil accumulation in vehicle-pretreated mice (Figs. 1 and 2). Intraperitoneal administration of dexamethasone at the dose of 1 mg/kg 2 h before ovalbumin challenge completely abolished airway hyperresponsiveness and eosinophil accumulation in bronchoalveolar lavage fluid induced by ovalbumin challenge (Fig. 1). Intraperitoneal administration of macrolide antibiotic clarithromycin also significantly decreased airway hyperresponsiveness as well as a number of eosinophils, while total cells in bronchoalveolar lavage fluid also tended to be reduced, but failed to achieve statistical significance (Fig. 2).

3.2. Effect of clarithromycin on cell numbers in bronchoalveolar lavage fluid

The results presented so far indicated that clarithromycin efficiently suppresses the monitored asthma characteristics in the described mouse

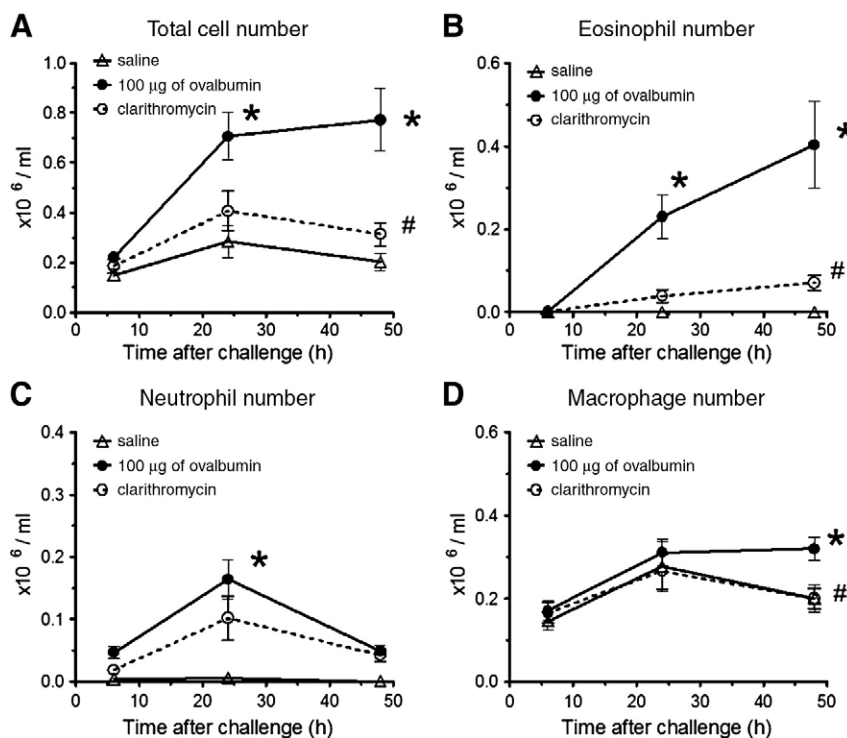


Fig. 3. Total number of cells (A), number of neutrophils (B), eosinophils (C) and macrophages (D) in bronchoalveolar lavage fluid in vehicle- and clarithromycin-treated mice challenged with 25 µg of ovalbumin. Clarithromycin was administered intraperitoneally at the dose of 200 mg/kg, 2 h before ovalbumin challenge. Control mice were challenged with saline. Data are shown as means ± S.E.M. (*n* = 8–15 mice per group). The asterisk (*) indicates significant difference (*P* < 0.05) between saline and ovalbumin-challenged groups, and the ladder (#) indicates significant difference (*P* < 0.05) between ovalbumin-challenged group and clarithromycin-treated group.

asthma model. In order to further dissect anti-inflammatory activity of clarithromycin, a second mouse model of asthma was employed, using lower sensitizing and challenging doses of ovalbumin. In saline-challenged mice, total cell number in bronchoalveolar lavage fluid ($0.23 \pm 0.03 \times 10^6$ cells/ml; mean $\pm$ S.E.M.), as well as its composition ($96 \pm 2\%$ macrophages and $4 \pm 1\%$ neutrophils; mean $\pm$ S.E.M.) remained unchanged (Fig. 3). In contrast, intranasal challenge with $25 \mu\text{g}$ of ovalbumin induced a significant increase in total cell number in bronchoalveolar lavage fluid at 24 h and 48 h (Fig. 3A). The number of neutrophils in bronchoalveolar lavage fluid started to increase already at 6 h, and maximal numbers were detected at 24 h after challenge (Fig. 3B). The number of eosinophils progressively increased from 6 h until 48 h (Fig. 3C), whereas macrophages were significantly increased at 48 h after ovalbumin challenge (Fig. 3D). Single intraperitoneal administration of clarithromycin at the dose of 200 mg/kg, 2 h before ovalbumin challenge, markedly inhibited total cell and neutrophil number in bronchoalveolar lavage fluid at 24 h after challenge. The decrease induced by clarithromycin was even more pronounced at 48 h after challenge, when total cell, eosinophil and macrophage number were significantly decreased in comparison to ovalbumin-challenged mice (Fig. 3).

3.3. Effect of clarithromycin on inflammatory cell accumulation into the lungs

Inflammatory cells were not detected in peribronchial/peribronchiolar lung areas of control mice (Figs. 4 and 5A). In contrast, ovalbumin challenge induced marked accumulation of eosinophils and mononuclear cells 24 and 48 h after challenge (Figs. 4 and 5B). Inflammatory cell accumulation in peribronchial/peribronchiolar lung

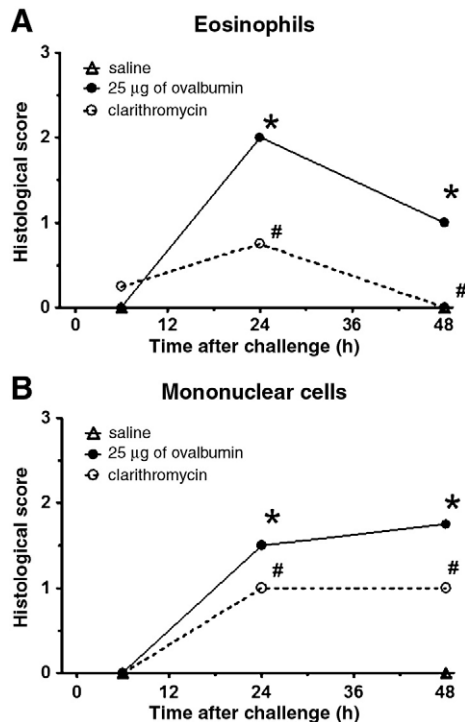


Fig. 4. Median histological scores of eosinophil (A) and mononuclear cell (B) infiltration in peribronchial/peribronchiolar lung areas of vehicle- and clarithromycin-treated mice challenged with $25 \mu\text{g}$ of ovalbumin ($n = 10\text{--}12$). Clarithromycin was administered intraperitoneally at the dose of 200 mg/kg, 2 h before ovalbumin challenge. Control mice ($n = 4$) were challenged with saline. The asterisk (*) indicates significant difference ($P < 0.05$) between saline and ovalbumin-challenged groups, and the ladder (#) indicates significant difference ($P < 0.05$) between the ovalbumin-challenged group and clarithromycin-treated group.

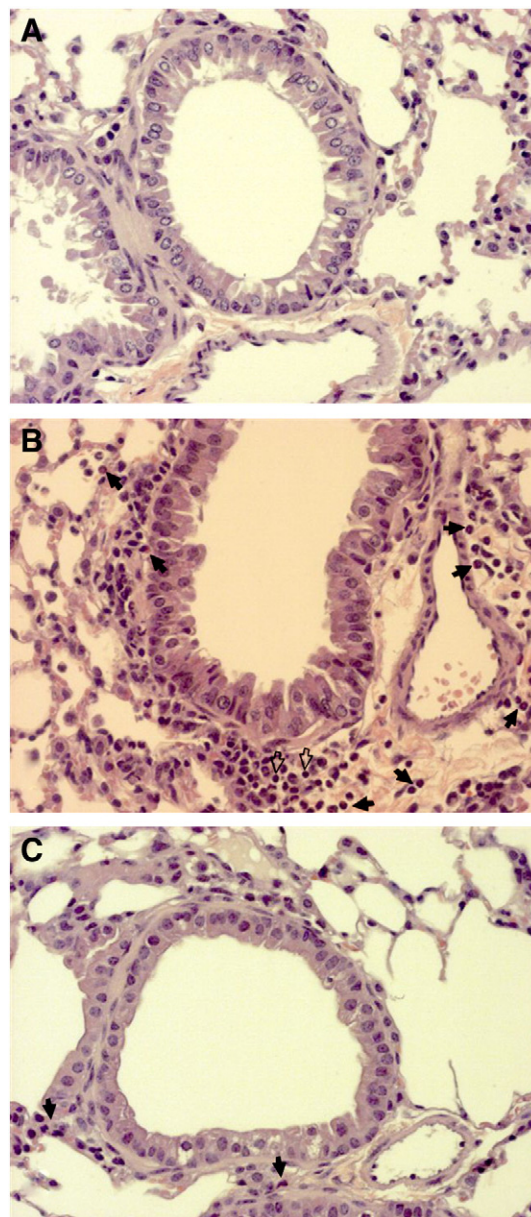


Fig. 5. Representative photomicrographs of lung tissue (hematoxylin and eosin, $\times 200$) at 24 h after challenge with saline (A) or ovalbumin (B and C). The saline-challenged group showed normal lung physiology (A). Ovalbumin challenge induced marked accumulation of eosinophils (filled arrows) and mononuclear cells (empty arrows) in vehicle-treated mice (B). Only rare inflammatory cells were detected in group intraperitoneally treated with clarithromycin (at the dose of 200 mg/kg) 2 h before ovalbumin challenge (C).

areas induced by ovalbumin challenge was significantly decreased in mice that received clarithromycin treatment (Figs. 4 and 5C).

3.4. Effect of clarithromycin on inflammatory mediators in bronchoalveolar lavage fluid

In addition to its effect on accumulation of inflammatory cells, the effect of clarithromycin on inflammatory mediators was also evaluated. Using Luminex xMAP technology, the presence of CCL2 (MCP-1), CXCL1 (KC), CXCL2 (MIP-2), IL-4, IL-5 and IL-13 in bronchoalveolar lavage fluid was observed after ovalbumin challenge, while concentrations of TNF- α , GM-CSF, IL-1 β and IL-2 were below the limit of detection (data not shown). At 24 h after challenge with ovalbumin in vehicle-treated mice, significant increase in IL-4, IL-5, IL-13, CCL2 and CXCL2 concentrations

was detected in comparison to saline-challenged mice. At the same time point, in clarithromycin-treated mice concentrations of all these inflammatory mediators were significantly decreased in comparison to vehicle-treated mice (Fig. 6). At 48 h after challenge only concentrations of IL-4 and IL-13 remained significantly increased in vehicle-treated mice, and the significant difference between vehicle- and clarithromycin-treated mice was no longer detectable (Fig. 6).

4. Discussion

There is extensive *in vitro* and growing *in vivo* evidence that macrolide antibiotics possess anti-inflammatory/immunomodulatory properties which are separate and distinct from their antibiotic actions (Čulić et al., 2001; López-Boado and Rubin, 2008; Shinkai et al., 2008). These anti-inflammatory/immunomodulatory properties of macrolide antibiotics are becoming widely acknowledged and used or tested in the treatment of various inflammatory disorders of the respiratory tract, including asthma. In order to test whether

beneficial effects of macrolide antibiotics in asthma could be attributed to their anti-inflammatory/immunomodulatory properties, we have investigated the ability of clarithromycin to suppress inflammation and airway hyperresponsiveness in models of acute experimental asthma.

In the first model employed in our study, the standard steroid dexamethasone at a dose of 1 mg/kg significantly decreased airway hyperresponsiveness as well as eosinophil number in bronchoalveolar lavage fluid. Interestingly, single intraperitoneal administration of clarithromycin at a dose of 200 mg/kg, 2 h before ovalbumin challenge, also almost completely inhibited accumulation of inflammatory cells in airway lumen (number in bronchoalveolar lavage fluid) and into the lungs. In addition to the effect on inflammatory cells, clarithromycin treatment completely abolished another important pathological characteristic of asthma, airway hyperresponsiveness to methacholine.

The effects of clarithromycin on inflammatory cell accumulation and airway hyperresponsiveness could be attributed to the decreased concentration of inflammatory mediators in bronchoalveolar lavage

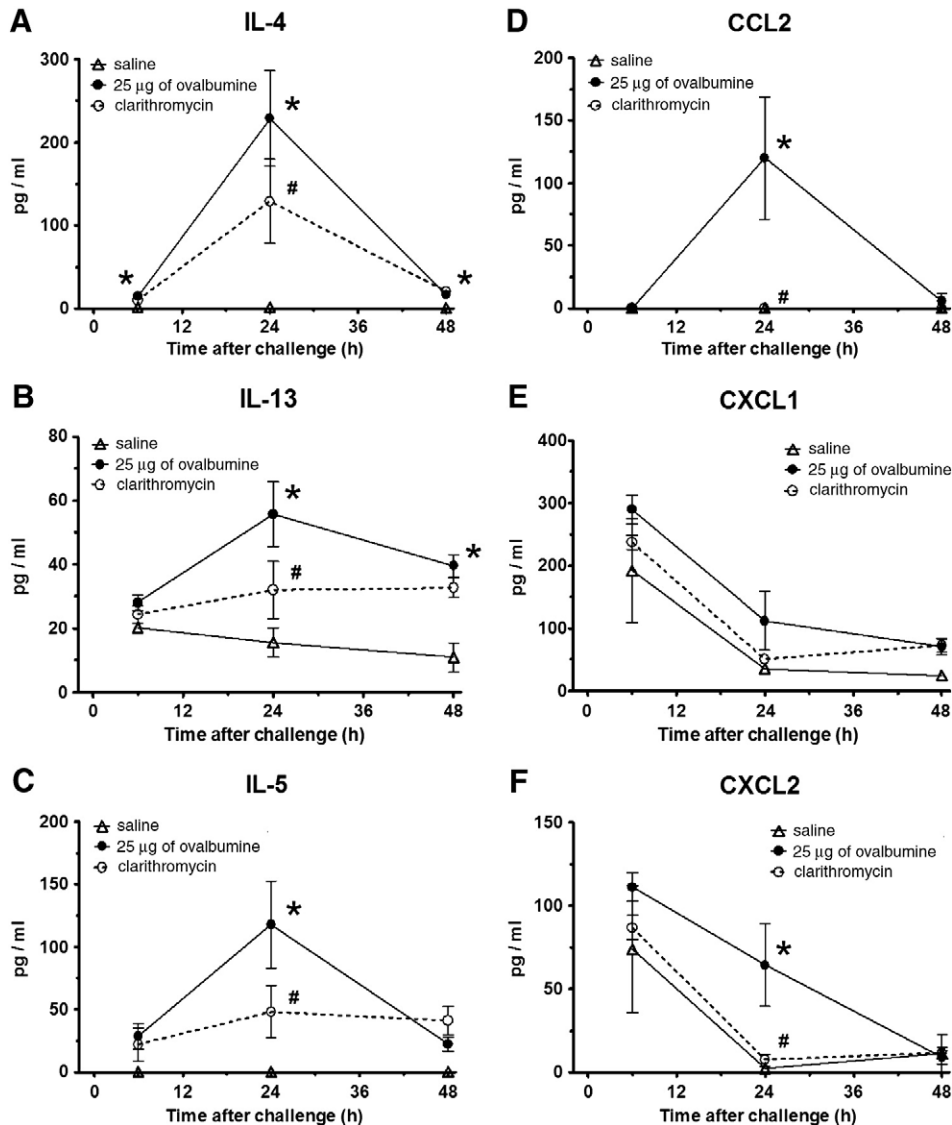


Fig. 6. Concentrations of IL-4 (A), IL-13 (B), IL-5 (C), CCL2 (D), CXCL1 (E) and CXCL2 (F) in bronchoalveolar lavage fluid in vehicle- and clarithromycin-treated mice challenged with 25 µg of ovalbumin ($n = 7-8$ mice per group). Clarithromycin was administered intraperitoneally at the dose of 200 mg/kg, 2 h before ovalbumin challenge. Control mice were challenged with saline ($n = 3$ per group). Data are presented as means $\pm$ S.E.M. The asterisk (*) indicates significant difference ($P < 0.05$) between saline and ovalbumin-challenged groups, and the ladder (#) indicates significant difference ($P < 0.05$) between the ovalbumin-challenged group and clarithromycin-treated group.

fluid. Clarithromycin significantly decreased concentrations of CXCL2, IL-5 and CCL2, mediators which are crucial for accumulation and/or survival of neutrophils (Lee et al., 1995), eosinophils (Sanderson et al., 1988) and macrophages (Boring et al., 1997), respectively. Moreover, clarithromycin treatment also significantly decreased concentrations of IL-4 and IL-13, two cytokines which have been shown to be crucial for the development of airway hyperresponsiveness (Wills-Karp et al., 1998; Venkayya et al., 2002).

Expression of all these inflammatory mediators is, at least in part, under the regulation of the transcription factors nuclear factor kappa B (NF- κ B) and activator protein 1 (AP-1), which are known to be involved in allergic inflammation (Lorentz et al., 2003; Blease et al., 2003). Macrolide antibiotics, on the other hand, have been reported to exert anti-inflammatory/immunomodulatory actions through inhibition of these two transcription factors (Kikuchi et al., 2002). The inhibition of NF- κ B and AP-1 seems, therefore, to be a likely mechanism by which clarithromycin could decrease the concentration of inflammatory mediators and exert its beneficial actions in the described model. This hypothesis should be investigated further in additional experiments.

In clinical investigations, macrolide antibiotics have been reported to decrease eosinophil and neutrophil number in sputum (Shoji et al., 1999; Amayasu et al., 2000; Simpson et al., 2008), as well as reducing airway hyperresponsiveness (Miyatake et al., 1991; Shimizu et al., 1994; Amayasu et al., 2000; Kostadima et al., 2004) in asthmatic patients. The results of our study suggest that the beneficial effects of macrolide antibiotics in asthmatic patients could be related, at least in part, to their distinct anti-inflammatory/immunomodulatory properties.

In contrast to this study, in which anti-inflammatory/immunomodulatory actions of clarithromycin were recorded after single intraperitoneal administration at a relatively high dose (200 mg/kg), macrolide antibiotics in clinical studies on asthmatic patients seem to require long oral treatment periods of at least 8 weeks (Shoji et al., 1999; Amayasu et al., 2000; Simpson et al., 2008; Miyatake et al., 1991; Shimizu et al., 1994; Kostadima et al., 2004). Similarly, in patients with diffuse panbronchiolitis, clinical effectiveness of macrolide antibiotic treatment was also shown after at least 2 to 3 months of use (reviewed in Yanagihara et al., 2001). Nevertheless we have previously shown that single intraperitoneal administration of azithromycin at a dose of 150 mg/kg, 2 h before lipopolysaccharide (LPS) challenge significantly suppresses pulmonary neutrophilia (Ivetić Tkalečević et al., 2006). Thus, single intraperitoneal administration of macrolide antibiotics at relatively high doses is able to suppress inflammation in animal models of lung inflammation induced by a single inflammatory stimulus. Interestingly, it has been shown previously that in patients with moderate to severe COPD only three doses of azithromycin were able to induce a number of anti-inflammatory actions, including decreases in blood leukocyte and platelet counts, serum acute phase protein (including C reactive protein) and soluble E-selectin concentrations and a transient decrease in serum IL-8 (Parnham et al., 2005). According to clinical findings that macrolide antibiotics require a low-dose, long-term treatment regimen in chronic inflammatory lung disorders, it would be interesting to test this treatment regimen in animal models of chronic diseases, such as chronic asthma (Jungsuwadee et al., 2004).

It was recently reported that 14-day oral erythromycin treatment was unable to prevent bronchial hyperreactivity and inflammatory response in ovalbumin-sensitized rats (Lin et al., 2008). These results could be explained by different treatment regimens (14-days of low-dose erythromycin vs. single high dose of clarithromycin), different pharmacokinetic characteristics, as well as differences in animal asthma models. Anti-inflammatory/immunomodulatory properties of macrolide antibiotics have been shown to depend on the experimental system used (Tsai and Standiford 2004), and it would be interesting to further investigate the causes of the differences between the results of these two studies.

In conclusion, in the mouse models of acute asthma used, clarithromycin effectively inhibited two important pathological characteristics of asthma, airway hyperresponsiveness and airway inflam-

mation, as well as decreasing the concentration of a number of inflammatory mediators, including IL-4, IL-5 and IL-13 in bronchoalveolar lavage fluid. These results indicate that the efficacy of clarithromycin, as well as of other macrolide antibiotics, in asthmatic patients could be attributed to their anti-inflammatory/immunomodulatory properties and not only to their antimicrobial activity or exogenous corticosteroid-sparing effects. Altogether, these findings support the hypothesis that macrolide antibiotic therapy represents a promising addition to the anti-asthma drug armamentarium (Hatipoglu and Rubinstein, 2004).

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References

- Abe, S., Nakamura, H., Inoue, S., Takeda, H., Saito, H., Kato, S., Mukaida, N., Matsushima, K., Tomoi, H., 2000. Interleukin-8 gene repression by clarithromycin is mediated by the activator protein-1 binding site in human bronchial epithelial cells. *Am. J. Respir. Cell Mol. Biol.* 22, 51–60.
- Adcock, I.M., Caramori, G., Chung, K.F., 2008. New targets for drug development in asthma. *Lancet* 372, 1073–1087.
- Amayasu, H., Yoshida, S., Ebana, S., Yamamoto, Y., Nishikawa, T., Shoji, T., Nakagawa, H., Hasegawa, H., Nakabayashi, M., Ishizaki, Y., 2000. Clarithromycin suppresses bronchial hyperresponsiveness associated with eosinophilic inflammation in patients with asthma. *Ann. Allergy Asthma Immunol.* 84, 594–598.
- Arif, A.A., Delclos, G.L., Lee, E.S., Tortolero, S.R., Whitehead, L.W., 2003. Prevalence and risk factors of asthma and wheezing among US adults: an analysis of the NHANES III data. *Eur. Respir. J.* 21, 827–833.
- Ball, B.D., Hill, M.R., Brenner, M., Sanks, R., Szefer, S.J., 1990. Effect of low-dose troleandomycin on glucocorticoid pharmacokinetics and airway hyperresponsiveness in severely asthmatic children. *Ann. Allergy* 65, 37–45.
- Basyigit, I., Yildiz, F., Ozkara, S.K., Yildirim, E., Boyaci, H., Ilgazli, A., 2004. The effect of clarithromycin on inflammatory markers in chronic obstructive pulmonary disease: preliminary data. *Ann. Pharmacother.* 38, 1400–1405.
- Black, P.N., Blasi, F., Jenkins, C.R., Scicchitano, R., Mills, G.D., Rubinfeld, A.R., Ruffin, R.E., Mullins, P.R., Dangain, J., Cooper, B.C., David, D.B., Allegra, L., 2001. Trial of roxithromycin in subjects with asthma and serological evidence of infection with *Chlamydia pneumoniae*. *Am. J. Respir. Crit. Care Med.* 164, 536–541.
- Blease, K., Lewis, A., Raymon, H.K., 2003. Emerging treatments for asthma. *Expert Opin. Emerg. Drugs* 8, 71–81.
- Boring, L., Gosling, J., Chensue, S.W., Kunkel, S.L., Farese Jr., R.V., Broxmeyer, H.E., Charo, I.F., 1997. Impaired monocyte migration and reduced type 1 (Th1) cytokine responses in C-C chemokine receptor 2 knockout mice. *J. Clin. Invest.* 100, 2552–2561.
- Bousquet, J., Jeffery, P.K., Busse, W.W., Johnson, M., Vingola, A.M., 2000. Asthma. From bronchoconstriction to airways inflammation and remodeling. *Am. J. Respir. Crit. Care Med.* 161, 1720–1745.
- Cigana, C., Assael, B.M., Melotti, P., 2007. Azithromycin selectively reduces tumor necrosis factor alpha levels in cystic fibrosis airway epithelial cells. *Antimicrob. Agents Chemother.* 51, 975–981.
- Clement, A., Tamalet, A., Leroux, E., Ravilly, S., Fauroux, B., Jais, J.P., 2006. Long term effects of azithromycin in patients with cystic fibrosis: a double blind, placebo controlled trial. *Thorax* 61, 895–902.
- Critchley, I.A., Jones, M.E., Heinze, P.D., Hubbard, D., Engler, H.D., Evangelista, A.T., Thornsberry, C., Karlowsky, J.A., Sahm, D.F., 2002. In vitro activity of levofloxacin against contemporary clinical isolates of *Legionella pneumophila*, *Mycoplasma pneumoniae* and *Chlamydia pneumoniae* from North America and Europe. *Clin. Microbiol. Infect.* 8, 214–221.
- Čulić, O., Eraković, V., Parnham, M.J., 2001. Anti-inflammatory effects of macrolide antibiotics. *Eur. J. Pharmacol.* 429, 209–229.
- Enomoto, F., Andou, I., Nagaoka, I., Ichikawa, G., 2002. Effect of new macrolides on the expression of adhesion molecules on neutrophils in chronic sinusitis. *Auris Nasus Larynx* 29, 267–269.
- Fost, D.A., Leung, D.Y., Martin, R.J., Brown, E.E., Szefer, S.J., Spahn, J.D., 1999. Inhibition of methylprednisolone elimination in the presence of clarithromycin therapy. *J. Allergy Clin. Immunol.* 103, 1031–1035.
- Garey, K.W., Rubinstein, I., Gotfried, M.H., Khan, I.J., Varma, S., Danziger, L.H., 2000. Long-term clarithromycin decreases prednisone requirements in elderly patients with prednisone-dependent asthma. *Chest* 118, 1826–1827.
- Hamelmann, E., Schwarze, J., Takeda, K., Oshiba, A., Larsen, G.L., Irvin, C.G., Gelfand, E.W., 1997. Noninvasive measurement of airway responsiveness in allergic mice using barometric plethysmography. *Am. J. Respir. Crit. Care Med.* 156, 766–775.
- Hatipoglu, U., Rubinstein, I., 2004. Low-dose, long-term macrolide therapy in asthma: an overview. *Clin. Mol. Allergy* 2, 4.

- Hatipoglu, U., Rubinstein, I., 2005. Treatment of chronic rhinosinusitis with low-dose, long-term macrolide antibiotics: an evolving paradigm. *Curr. Allergy Asthma Rep.* 5, 491–494.
- Hrvačić, B., Bošnjak, B., Tudja, M., Mesić, M., Merčep, M., 2006. Applicability of an ultrasonic nebulization system for the airways delivery of beclomethasone dipropionate in a murine model of asthma. *Pharm. Res.* 23, 1765–1775.
- Ivetić Tkalević, V., Bošnjak, B., Hrvačić, B., Bosnar, M., Marjanović, N., Ferenčić, Ž., Šitum, K., Čulić, O., Parnham, M.J., Eraković, V., 2006. Anti-inflammatory activity of azithromycin attenuates the effects of lipopolysaccharide administration in mice. *Eur. J. Pharmacol.* 539, 131–138.
- Jaffe, A., Francis, J., Rosenthal, M., Bush, A., 1998. Long-term azithromycin may improve lung function in children with cystic fibrosis. *Lancet* 351, 420.
- Jungsuwadee, P., Dekan, G., Stingl, G., Epstein, M.M., 2004. Inhaled dexamethasone differentially attenuates disease relapse and established allergic asthma in mice. *Clin. Immunol.* 110, 13–21.
- Kadota, J., Sakito, O., Kohno, S., Sawa, H., Mukae, H., Oda, H., Kawakami, K., Fukushima, K., Hiratani, K., Hara, K., 1993. A mechanism of erythromycin treatment in patients with diffuse panbronchiolitis. *Am. Rev. Respir. Dis.* 147, 153–159.
- Kamada, A.K., Hill, M.R., Iklé, D.N., Brenner, A.M., Szefer, S.J., 1993. Efficacy and safety of low-dose troleandomycin therapy in children with severe, steroid-requiring asthma. *J. Allergy Clin. Immunol.* 91, 873–882.
- Kaplan, M.A., Goldin, M., 1959. The use of triacetyltroleandomycin in chronic infectious asthma. In: Welsh, H., Marti-Ibanez, F. (Eds.), *Antibiotic Annual 1958–1959*. Interscience Publishers, New York, NY, pp. 273–276.
- Khan, A.A., Slifer, T.R., Araujo, F.G., Remington, J.S., 1999. Effect of clarithromycin and azithromycin on production of cytokines by human monocytes. *Int. J. Antimicrob. Agents* 11, 121–132.
- Kikuchi, T., Hagiwara, K., Honda, Y., Gomi, K., Kobayashi, T., Takahashi, H., Tokue, Y., Watanabe, A., Nukiwa, T., 2002. Clarithromycin suppresses lipopolysaccharide-induced interleukin-8 production by human monocytes through AP-1 and NF- κ B transcription factors. *J. Antimicrob. Chemother.* 49, 745–755.
- Kostadima, E., Tsiodras, S., Alexopoulos, E.I., Kaditis, A.G., Mavrou, I., Georgatou, N., Papamichalopoulos, A., 2004. Clarithromycin reduces the severity of bronchial hyperresponsiveness in patients with asthma. *Eur. Respir. J.* 23, 714–717.
- Kraft, M., Cassell, G.H., Pak, J., Martin, R.J., 2002. *Mycoplasma pneumoniae* and *Chlamydia pneumoniae* in asthma: effect of clarithromycin. *Chest* 121, 1782–1788.
- Kudoh, S., Azuma, A., Yamamoto, M., Izumi, T., Ando, M., 1998. Improvement of survival in patients with diffuse panbronchiolitis treated with low-dose erythromycin. *Am. J. Respir. Crit. Care Med.* 158, 1829–1832.
- Lee, J., Cacalano, G., Camerato, T., Toy, K., Moore, M.W., Wood, W.I., 1995. Chemokine binding and activities mediated by the mouse IL-8 receptor. *J. Immunol.* 155, 2158–2164.
- Leiva, M., Ruiz-Bravo, A., Jimenez-Valera, M., 2008. Effects of telithromycin in vitro and in vivo models of lipopolysaccharide-induced airway inflammation. *Chest* 134, 20–29.
- Lin, C.-C., Liaw, S.-F., Wu, K.-M., Lin, C.-H., 2008. Effect of erythromycin on bronchial hyperactivity and inflammation in ovalbumin-sensitized brown Norway rats. *Respir. Physiol. Neurobiol.* 161, 267–272.
- López-Boado, Y.S., Rubin, B.K., 2008. Macrolides as immunomodulatory medications for the therapy of chronic lung diseases. *Curr. Opin. Pharmacol.* 8, 286–291.
- Lorentz, A., Klopp, I., Gebhardt, T., Manns, M.P., Bischoff, S.C., 2003. Role of activator protein 1, nuclear factor- κ B, and nuclear factor of activated T cells in IgE receptor-mediated cytokine expression in mature human mast cells. *J. Allergy Clin. Immunol.* 111, 1062–1068.
- Miyanoara, T., Ushikai, M., Matsune, S., Ueno, K., Katahira, S., Kurono, Y., 2000. Effects of clarithromycin on cultured human nasal epithelial cells and fibroblasts. *Laryngoscope* 110, 126–131.
- Miyatake, H., Taki, F., Taniguchi, H., Suzuki, R., Takagi, K., Satake, T., 1991. Erythromycin reduces the severity of bronchial hyperresponsiveness in asthma. *Chest* 99, 670–673.
- Nagai, H., Shishido, H., Yoneda, R., Yamaguchi, E., Tamura, A., Kurashima, A., 1991. Long-term low-dose administration of erythromycin to patients with diffuse panbronchiolitis. *Respiration* 58, 145–149.
- Ou, X.M., Feng, Y.L., Wen, F.Q., Wang, K., Yang, J., Deng, Z.P., Liu, D.S., Li, Y.P., 2008. Macrolides attenuate mucus hypersecretion in rat airways through inactivation of NF- κ B. *Respirology* 13, 63–72.
- Parnham, M.J., Čulić, O., Eraković, V., Munić, V., Popović-Grle, S., Barišić, K., Bosnar, M., Brajša, K., Čepelak, I., Čuzić, S., Glojnaric, I., Manojlović, Z., Novak-Mircetić, R., Orešković, K., Pavičić-Beljak, V., Radošević, S., Sučić, M., 2005. Modulation of neutrophil and inflammation markers in chronic obstructive pulmonary disease by short-term azithromycin treatment. *Eur. J. Pharmacol.* 517, 132–143.
- Pearce, N., Sunyer, J., Cheng, S., Chinn, S., Björkstén, B., Burr, M., Keil, U., Anderson, H.R., Burney, P., 2000. Comparison of asthma prevalence in the ISAAC and the ECRHS. ISAAC Steering Committee and the European Community Respiratory Health Survey. International Study of Asthma and Allergies in Childhood. *Eur. Respir. J.* 16, 420–426.
- Periti, P., Mazzei, T., Mini, E., Novelli, A., 1992. Pharmacokinetic drug interactions of macrolides. *Clin. Pharmacokinet.* 23, 106–131.
- Piacentini, G.L., Peroni, D.G., Bodini, A., Pigozzi, R., Costella, S., Loiacono, A., Boner, A.L., 2007. Azithromycin reduces bronchial hyperresponsiveness and neutrophilic airway inflammation in asthmatic children: a preliminary report. *Allergy Asthma Proc.* 28, 194–198.
- Sanderson, C.J., Campbell, H.D., Young, I.G., 1988. Molecular and cellular biology of eosinophil differentiation factor (interleukin-5) and its effects on human and mouse B cells. *Immunol. Rev.* 102, 29–50.
- Sanz, M.J., Nabah, Y.N., Cerda-Nicolas, M., O'Connor, J.E., Issekutz, A.C., Cortijo, J., Morcillo, E.J., 2005. Erythromycin exerts in vivo anti-inflammatory activity downregulating cell adhesion molecule expression. *Br. J. Pharmacol.* 144, 190–201.
- Shimizu, T., Kato, M., Mochizuki, H., Tokuyama, K., Morikawa, A., Kurume, T., 1994. Roxithromycin reduces the degree of bronchial hyperresponsiveness in children with asthma. *Chest* 106, 458–461.
- Shinkai, M., Henke, M.O., Rubin, B.K., 2008. Macrolide antibiotics as immunomodulatory medications: proposed mechanisms of action. *Pharmacol. Ther.* 117, 393–405.
- Shoji, T., Yoshida, S., Sakamoto, H., Hasegawa, H., Nakagawa, H., Amayasu, H., 1999. Anti-inflammatory effect of roxithromycin in patients with aspirin-intolerant asthma. *Clin. Exp. Allergy* 29, 950–956.
- Simpson, J.L., Powell, H., Boyle, M.J., Scott, R.J., Gibson, P.G., 2008. Clarithromycin targets neutrophilic airway inflammation in refractory asthma. *Am. J. Respir. Crit. Care Med.* 177, 148–155.
- Szefer, S.J., Rose, J.Q., Ellis, E.F., Spector, S.L., Green, A.W., Jusko, W.J., 1980. The effect of troleandomycin on methylprednisolone elimination. *J. Allergy Clin. Immunol.* 66, 447–451.
- Szefer, S.J., Brenner, M., Jusko, W.J., Spector, S.L., Flesher, K.A., Ellis, E.F., 1982. Dose- and time-related effect of troleandomycin on methylprednisolone elimination. *Clin. Pharmacol. Ther.* 32, 166–171.
- Tomkinson, A., Cieslewicz, G., Duez, C., Larson, K.A., Lee, J.J., Gelfand, E.W., 2001. Temporal association between airway hyperresponsiveness and airway eosinophilia in ovalbumin-sensitized mice. *Am. J. Respir. Crit. Care Med.* 163, 721–730.
- Tsai, W.C., Standiford, T.J., 2004. Immunomodulatory effects of macrolides in the lung: lesions from *in-vitro* and *in-vivo* models. *Curr. Pharm. Des.* 10, 3081–3093.
- Venkayya, R., Lam, M., Willkom, M., Grunig, G., Corry, D.B., Erle, D.J., 2002. The Th2 lymphocyte products IL-4 and IL-13 rapidly induce airway hyperresponsiveness through direct effects on resident airway cells. *Am. J. Respir. Cell Mol. Biol.* 26, 202–208.
- Wills-Karp, M., Luyimbazi, J., Xu, X., Schofield, B., Neben, T.Y., Karp, C.L., Donaldson, D.D., 1998. Interleukin-13: central mediator of allergic asthma. *Science* 282, 2258–2261.
- Yanagihara, K., Kadoto, J., Kohno, N., 2001. Diffuse panbronchiolitis – pathophysiology and treatment mechanisms. *Int. J. Antimicrob. Agents* 18, S83–S87.
- Zeiger, R.S., Schatz, M., Sperling, W., Simon, R.A., Stevenson, D.D., 1980. Efficacy of troleandomycin in outpatients with severe, corticosteroid-dependent asthma. *J. Allergy Clin. Immunol.* 66, 438–446.