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A novel macrolide solithromycin exerts superior anti-inflammatory effect via NF-κB inhibition

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Abbreviation

CAP; community-acquired pneumonia

COPD; chronic obstructive pulmonary disease

DPB; diffuse panbronchiolitis

LPS; lipopolysaccharide

MMP9; matrix metalloproteinase 9

NF-κB; nuclear factor-κB

PBMCs; peripheral blood mononuclear cells

PMA; phorbol 12-myristate 13-acetate

TNFα; tumor necrosis factor α

Abstract

Macrolides are reported to reduce exacerbation of chronic inflammatory respiratory disease such as chronic obstructive pulmonary disease (COPD), and also show anti-inflammatory effects *in vitro* and *in vivo*. However the anti-inflammatory efficacies of current macrolides are relatively weak. Here we found that a novel macrolide/fluoroketolide solithromycin (CEM-101) showed superior anti-inflammatory effects to macrolides in current clinical use. The effects of solithromycin (SOL) on LPS-induced TNFα and/or CXCL8 release, PMA-induced MMP9 activity and NF-κB activity under conditions of oxidative stress have been evaluated and compared with the effects of erythromycin, clarithromycin, azithromycin and telithromycin in human monocytic U937 cells and peripheral blood mononuclear cells (PBMC) obtained from COPD patients. We also examined effect of SOL on cigarette smoke-induced airway inflammation in mice. SOL exerted superior inhibitory effects on TNFα/CXCL8 production and MMP9 activity in U937 cells. In addition, SOL suppressed TNFα release and MMP9 activity in PBMC from COPD patients at 10μM, which is 100 times more potent than the other macrolides tested. Activated NF-κB due to oxidative stress was completely reversed by SOL. SOL also inhibited cigarette smoke-induced neutrophilia and proMMP9 production *in vivo* although erythromycin did not inhibit them. Thus, SOL showed better anti-inflammatory profiles compared with macrolides currently used in the clinic, and may be a promising anti-inflammatory and anti-microbial macrolide for the treatment of COPD in the future.

Introduction

It has long been recognized that macrolides are exert not only antibacterial activity, but also anti-inflammatory and immunoregulatory effects (Kanoh and Rubin, 2010). Erythromycin was found to induce a dramatic and unexpected improvement in diffuse panbronchiolitis (DPB) (Kudoh et al., 1998), leading to an investigation of its anti-inflammatory actions. Several studies indicate that macrolides inhibit pro-inflammatory cytokine production, such as CXCL8 and TNFα, as the mechanism of the effects of macrolides on DPB (Sakito et al., 1996). Macrolides also inhibit release of leukotriene B_4 (LTB_4), a neutrophil chemotactic mediator (Oda et al., 1995), and soluble adhesion molecules (Mukae et al., 1997), resulting in reduction of neutrophil chemotaxis and infiltration into the airway epithelium. It has also been reported that macrolides increase antioxidant activity in alveolar macrophages (Morikawa et al., 2000), enhance apoptosis of neutrophils (Inamura et al., 2000), decrease superoxide anion release by neutrophils (Tamaoki et al., 2004) and reduce mucus secretion in the airways (Kaneko et al., 2003).

Macrolides were found to be effective for DPB at doses below the concentrations required for their anti-bacterial activity, by inhibiting not only biofilm-formation and virulence factors produced from *Pseudomonas aeruginosa*, but also quorum-sensing systems in which bacteria coordinately regulate specific virulence genes (Schultz, 2004; Tateda et al., 2004). Thus, macrolides suppress airway inflammation observed in DPB, resulting in an improvement of lung function and prolonged survival (Oda et al., 1995; Kudoh et al., 1998; Kadota et al., 2003).

In addition to DPB, it has been reported that macrolides provide beneficial effects, although to a lesser degree, in other inflammatory airway diseases, such as

bronchiectasis, cystic fibrosis, sarcoidosis, asthma and chronic obstructive pulmonary disease (COPD) (Trisolini et al., 2008; Kanoh and Rubin, 2010; Albert et al., 2011). Although DPB is geographically limited to East Asia, COPD is a serious worldwide health problem without any safe and effective anti-inflammatory therapy (Ito and Barnes, 2009). There are few reports investigating the clinical benefit of macrolides for COPD, with some studies showing reduced exacerbations after long-term therapy COPD (Simpson et al., 2008; He et al., 2010; Albert et al., 2011). However, the reduction in exacerbations could be due to the antibiotic action of macrolides rather than any anti-inflammatory effect and there is no evidence for any significant effects on inflammatory biomarkers in these studies.

The anti-inflammatory effects of macrolides have been previously investigated. For example, azithromycin inhibits pro-inflammatory cytokines from M1 macrophages in cystic fibrosis (Meyer et al., 2009). Clarithromycin and erythromycin inhibit airway inflammation in a cigarette smoke model (Nakanishi et al., 2009; Mikura et al., 2011). One of the molecular mechanisms of anti-inflammatory effects of macrolides is inhibition of pro-inflammatory transcription factors, such as nuclear factor-κB (NF-κB) (Li et al., 2012) and activated protein-1 (AP-1) (Bosnar et al., 2011).

Solithromycin (CEM-101)) has been developed as a novel oral and intravenous fluoroketolide antibacterial agent related to 14-member-ring macrolides that is currently in clinical development for severe community-acquired bacterial pneumonia (CABP). Solithromycin exhibits a greater potency and a wider spectrum of *in vitro* activity against respiratory tract pathogens associated with CABP, including macrolide-resistant strains, compared with other available macrolides (Farrell et al., 2010; McGhee et al., 2010). However, the anti-inflammatory efficacies of solithromycin

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have not yet been explored.

Here we compared the anti-inflammatory effects of solithromycin on release of pro-inflammatory mediators, such as TNFα, CXCL8 and MMP9, and activation of NF-κB compared to other macrolides currently used clinically, such as erythromycin, clarythromycin, azithromycin and telithromycin

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Methods

Reagents.

Solithromycin((3a*S*,4*R*,7*S*,9*R*,10*R*,11*R*,13*R*,15*R*,15a*R*)-1-[4-[4-(3-aminophenyl)-1*H*-1,2,3-triazol-1-yl]butyl]-4-ethyl-7-fluorooctahydro-11-methoxy-3a,7,9,11,13,15-hexamethyl-10-{[3,4,6-trideoxy-3-(dimethylamino)-β-D-*xylo*-hexopyranosyl]oxy}-2*H*-Oxacyclotetradecino[4,3-d]oxazole-2,6,8,14(1*H*,7*H*,9*H*)-tetrone) and telithromycin ((1*S*,2*R*,5*R*,7*R*,8*R*,9*S*,11*R*,13*R*,14*R*)-8-[(2*S*,3*R*,4*S*,6*R*)-4-dimethylamino-3-hydroxy-6-methyl-oxan-2-yl]oxy-2-ethyl-9-methoxy-1,5,7,9,11,13-hexamethyl-15-[4-(4-pyridin-3-yl imidazol-1-yl)butyl]-3,17-dioxa-15-azabicyclo[12.3.0]heptadecane-4,6,12,16-tetrone) were provided by Cempra Pharmaceuticals, Inc (Chapel Hill, NC). Erythromycin ((3*R*,4*S*,5*S*,6*R*,7*R*,9*R*,11*R*,12*R*,13*S*,14*R*)-6-{[(2*S*,3*R*,4*S*,6*R*)-4-(dimethylamino)-3-hydroxy-6-methyloxan-2-yl]oxy}-14-ethyl-7,12,13-trihydroxy-4-{[(2*R*,4*R*,5*S*,6*S*)-5-hydroxy-4-methoxy-4,6-dimethyloxan-2-yl]oxy}-3,5,7,9,11,13-hexamethyl-1-oxacyclotetradecane-2,10-dione), clarithromycin ((3*R*,4*S*,5*S*,6*R*,7*R*,9*R*,11*S*,12*R*,13*S*,14*S*)-6-{[(2*S*,3*R*,4*S*,6*R*)-4-(dimethylamino)-3-hydroxy-6-methyloxan-2-yl]oxy}-14-ethyl-12,13-dihydroxy-4-{[(2*R*,4*S*,5*S*,6*S*)-5-hydroxy-4-methoxy-4,6-dimethyloxan-2-yl]oxy}-7-methoxy-3,5,7,9,11,13-hexamethyl-1-oxacyclotetradecane-2,10-dione), azithromycin ((2*R*,3*S*,4*R*,5*R*,8*R*,10*R*,11*R*,12*S*,13*S*,14*R*)-2-ethyl-3,4,10-trihydroxy-3,5,6,8,10,12,14-heptamethyl-15-oxo-11-{[3,4,6-trideoxy-3-(dimethylamino)-β-D-*xylo*-]oxy}-1-oxa-6-azacyclopentadec-13-yl-2,6-dideoxy-3-*C*-methyl-3-*O*-methyl-α-L-*ribo*-hexopyranoside)), hydrogen peroxide, phorbol 12-myristate 13-acetate (PMA), and lipopolysaccharide (LPS) were purchased from Sigma Aldrich (Poole, UK).

Cells. The human monocytic cell line U937 was obtained from the American Type

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Culture Collection (ATCC, Rockville, MD). PBMCs from COPD patients were obtained from Brompton hospital and separated by AccuSPIN (Sigma–Aldrich). The characteristics of subjects are shown in Supplemental Table 1. Cells were cultured in complete growth medium (RPMI 1640) (Sigma–Aldrich) supplemented with 10% fetal bovine serum (FBS) and 1% L-glutamine at 37°C in a humidified atmosphere with 5% CO_2. U937 cells were differentiated into adherent macrophage-like morphology by exposure to PMA (50 ng/ml) for 48 hrs in complete growth medium. Cell viability was assessed microscopically by trypan blue staining. Cell toxicity was determined by MTT assay as needed. This study was approved by the ethics committee of the Royal Brompton and Harefield Hospitals, and all subjects gave written informed consent.

Cell Lysis. Whole cell extracts were prepared as previously described (Kobayashi et al., 2011). Briefly, cell protein extracts were prepared using modified RIPA buffer (50 mM Tris HCL pH 7.4, 0.5% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl with freshly added complete protease inhibitor cocktail (Roche, Mannheim, Germany)). Protein concentration was determined using the BCA Protein Assay (Thermo Fisher Scientific, Waltham, MA).

Cytokine ELISA. TNFα and IL-8 concentrations in the supernatant of cell cultures were determined by sandwich ELISA according to the manufacturer's instructions (R&D Systems Europe, Abingdon, UK).

Zymography. MMP9 enzyme activity was measured by gelatin zymography. Cell culture supernatants were diluted with equal amount of Laemli sample buffer (Bio-Rad,

Hertfordshire, UK) and loaded on a Novex® 10 % Zymogram (Gelatin) gel (Invitrogen Ltd, Paisley, UK). After electrophoresis, gels were incubated and rinsed with Novex® zymogram renaturing buffer (Invitrogen) for 30 min at room temperature. The gels were then rinsed in Novex® zymogram developing buffer (Invitrogen) for 30 min at room temperature prior to overnight incubation in the developing buffer at 37 °C. After incubation, the gels were stained using a Colloidal Blue Staining Kit (Invitrogen) to visualize the zymogen bands.

NF-κB activity. The activation of NF-κB (p65 binding activity to NF-κB binding sequence) was determined using a TransAM NF-κB p65 Assay kit (Active Motif, Inc., Carlsbad, CA) according to the manufacturer's instruction. As shown above, whole cell extracts were prepared from PMA-differentiated U937 cells, and 20 μl of each extract was used for this study. Results were determined by measuring the spectrophotometric absorbance at 450 nm with a reference wavelength of 655 nm.

Cigarette smoke exposure to mice. C57BL/6J mice (male, 4 weeks) were purchased from CLEA Japan, Inc, (Tokyo, Japan) and adapted for 1 week. Mice were exposed to cigarette smoke (4% cigarette smoke diluted with compressed air) for 30 min/day for 12 days or 4 days using the commercially marketed un-filtered Peace cigarettes (28 mg of tar and 2.3 mg of nicotine per cigarette; Japan Tobacco Inc., Tokyo, Japan) using a Tobacco Smoke Inhalation Experiment System for small animals (Model INH06-CIGR02A; MIPS, Osaka, Japan) as described previously (Nakamaru et al., 2009).

In cohort 1, solithromycin (100 mg/kg, orally) was suspended into 0.5%

carboxylmethylcysteine and administered orally for the last 8 days of 12 days tobacco smoke exposure (1hr before each tobacco smoke exposure). In cohort 2, solithromycin (100 mg/kg, orally) or erythromycin (100 mg/kg, orally) was suspended into 0.5% carboxylmethylcysteine and administered orally at 1hr before each tobacco smoke exposure (1hr before each tobacco smoke exposure) for 4 times. Bronchoalveolar lavage was performed at 24hrs after the last cigarette smoke exposure as previously described (Nakamaru et al., 2009), and the number of alveolar macrophages and neutrophils were determined from Diff-Quick stained specimens. The proMMP-9 protein concentration in BAL fluid supernatant was assessed using a commercially available mouse proMMP-9 ELISA kit (R&D, Abingdon, UK) according to the manufacturer's instructions. This animal study was approved by The Experimental Animal Ethics Committee in Kyorin University.

Statistical analysis. The results were expressed as the mean ± SEM. Comparisons of data in two groups were performed using the Student's *t* test or the Wilcoxon signed rank test. Multiple comparisons were made by one-way ANOVA with *post hoc* test (Dunnett's) as appropriate. The difference was considered significant at $p < 0.05$. IC_{50} values (50% inhibitory concentration) for macrolides for production of cytokines or MMP9 were calculated using Prism 4.0 (GraphPad Software Inc., San Diego, CA).

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Results

Anti-inflammatory effects of macrolides in U937 cells. LPS significantly increased TNFα and CXCL8 production in PMA-differentiated U937 cells (TNFα, 63.1 ± 2.6 fold in LPS vs. non-stimulated; and CXCL8, 2.0 ± 0.1 fold in LPS vs. non-stimulated cells, n=3). Solithromycin significantly inhibited both TNFα and CXCL8 at 100 μM (Fig. 1A and Fig. 2A). Although clarithromycin showed modest effects on both TNFα and CXCL8 production at a higher concentration (333 μM) (Fig. 1 C and Fig. 2C), erythromycin and azithromycin had no inhibitory effect (Fig. 1 B, D, Fig.2 B, D). Telithromycin (100μM) was similarly without effect on TNFα and CXCL8 (Fig. 1E, Fig. 2E). The IC_{50} values for solithromycin on TNFα and CXCL8 release were 41.6 ± 1.9 μM and 78.2 ± 9.5 μM, respectively, and were superior to those for clarithromycin ($IC_{50,}$ 426.3 ± 63.9 μM for TNFα and 506.5 ± 44.0 μM for CXCL8) (Table 1). None of compounds affected cell viability evaluated by MTT assay at maximal concentrations used in this test.

We also investigated the effects of macrolides on MMP9 activity, which was clearly elevated by PMA stimulation in U937 cells (9.9 ± 2.0 fold in PMA vs. non-stimulation, n=3). Solithromycin markedly reduced MMP9 activity, with an IC_{50} of 14.9 ± 3.1 μM (Fig. 3A and Table 1). In contrast, clarithromycin and azithromycin showed 10-fold lower inhibitory effects than solithromycin, whereas erythromycin showed no effect (Fig. 3B, C, D and Table 1). Telithromycin also inhibited MMP9 activity, although to lesser extent than solithromycin, with an IC_{50} of 83.4 μM (Fig. 3E).

Anti-inflammatory effects of macrolides in PBMC from COPD patients.

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Anti-inflammatory effects of solithromycin were evaluated in PBMC from COPD patients. Solithromycin (10 μM) slightly but significantly inhibited LPS-induced TNFα release from PBMC of COPD patients, while erythromycin, clarithromycin and azithromycin failed to suppress TNFα release, even at a higher concentration (100 μM) (Fig. 4 A-D). Furthermore, solithromycin clearly reduced MMP9 activity in PBMCs from COPD patients at 10 μM. Although clarithromycin slightly but significantly inhibited MMP9 activity at a higher concentration (100 μM), erythromycin and azithromycin showed no effect (Fig. 4 E-H). Telithromycin showed cytotoxicity at less than 100 μM, and could not get meaningful data.

Solithromycin inhibited NF-κB activity under conditions of oxidative stress. As the secretion of TNFα, CXCL8 and MMP9 are reported to be NF-κB dependent, we investigated the effects of solithromycin on NF-κB activity as defined by p65 binding activity to NF-κB DNA binding sequence under conditions of oxidative stress. NF-κB activity was already high in PMA-differentiated U937 cells and 4hrs exposure of H_2O_2 (200 μM) significantly enhanced NF-κB activity (114.1 ± 1.1 % of non-treatment control, n=4). Clarithromycin and azithromycin restored H_2O_2-induced enhancement of NF-κB activity at a high concentration (100 μM) (Fig. 5C,D), whereas solithromycin provided remarkable inhibitory effects even at a lower concentration (10 μM) (Fig. 5A). Erythromycin did not show any effects (Fig. 5B).

Inhibition of inflammatory cell accumulation in cigarette smoke-exposed mice *in vivo* by solithromycin. C57BL/6J mice were exposed to cigarette smoke (4%) for 12

days (30 min/day) and treated with solithromycin at 100 mg/kg orally once daily 1hr before each smoke exposure during the last 8 days of the 12 day smoking course. At 24hrs after the last cigarette smoke exposure, bronchoalveolar lavage fluid was collected. Cigarette smoke exposure significantly increased the number of alveolar macrophages (air: 48828 ± 2847 cells/ml, smoke + vehicle: 240712 ± 22917) (Fig. 6A), neutrophils (air: 30.9 ± 20.3 cells/ml, smoke + vehicle: 5702.0 ± 725.4) (Fig. 6B) and lymphocytes (Air: 32.9 ± 21.7 cells/ml, smoke + vehicle: 1024.0 ± 138.1) in bronchoalveolar lavage fluid.

Solithromycin did not inhibit alveolar macrophage accumulation (smoke + SOL: 280660 ± 40121) (Fig. 6A), but significantly inhibited neutrophil accumulation by 49% (smoke + SOL: 2635.0 ± 829.3, $p < 0.05$) (Fig. 6B) and lymphocyte accumulation by 75% (smoke + SOL: 276.5 ± 202.2, $p < 0.05$). In addition, although cigarette smoke exposure decreased body weight, solithromycin significantly restored the body weight (Fig. 6C).

The effects of erythromycin were also compared to that of solithromycin. Head-to-head comparison was conducted in 4 days smoking model. C57BL/6J mice were exposed to cigarette smoke (4%) for 4 days (30 min/day) and treated with vehicle, solithromycin at 100 mg/kg or erythromycin at 100 mg/kg orally once daily 1hr before each smoke exposure. At 24hrs after the last cigarette smoke exposure, bronchoalveolar lavage fluid was collected. As shown in Fig. 6D, although solithromycin significantly inhibited airway neutrophilia, erythromycin did not inhibit it (air: 46.3 ± 25.7 cells/ml, smoke + vehicle: 13500 ± 1360, smoke + SOL: 8320 ± 1160, $p < 0.05$, smoke + EM: 15100 ± 1140, *not significant*). In addition, proMMP9 (not active form) was also elevated in BAL by smoke exposure, and significantly inhibited by solithromycin, but not by

erythromycin (air: 1.6 ± 0.21 ng/ml, smoke + vehicle: 3.9 ± 0.29, smoke + SOL: 2.5 ± 0.17, $p < 0.05$, smoke + EM: 3.9 ± 0.70, *not significant*)

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Discussion

In this study, we demonstrated that a novel macrolide/fluoroketolide, solithromycin has beneficial anti-inflammatory effects in addition to the anti-microbial ability previously shown. The inhibitory activities on production of TNFα, CXCL8 and MMP9, as well as NF-κB activation, were superior to those of other macrolide antibiotics currently used in the clinic. Even more importantly, solithromycin also inhibited pro-inflammatory mediators released by PBMC obtained from COPD patients and inhibited neutrophilia and proMMP9 production in cigarette smoke-exposed mice *in vivo,* which are known to be corticosteroid resistant.

TNFα and CXCL8 were increased in bronchoalveolar lavage, sputum and serum in COPD (Stankiewicz et al., 2002; Vernooy et al., 2002) and are believed to play an important role in the pathogenesis of COPD (Barnes, 2009). There are many *in vitro* studies indicating that macrolides inhibit pro-inflammatory mediators, such as CXCL8 and TNFα released from monocytes (Iino et al., 1992; Kikuchi et al., 2002), neutrophils (Tsuchihashi et al., 2002), bronchial epithelial cells (Takizawa et al., 1997) and induced sputum cells (Marjanovic et al., 2011). Macrolides also exerted inhibitory effects on neutrophil-dominant inflammation in *in vivo* models (Leiva et al., 2008; Bosnar et al., 2009). Furthermore, macrolides inhibited release of inflammatory mediators including TNFα and CXCL8 from sputum cells obtained from patients with COPD (Marjanovic et al., 2011). In addition to these *in vitro* results, clinical benefits by long-term macrolide therapy have been reported in COPD patients. Although short-term treatment did not provide any advantages for patients with stable COPD (Banerjee et al., 2005), long-term (more than 6 months) macrolide therapy reduces exacerbation of COPD (Seemungal et al., 2008; Blasi et al., 2010; He et al., 2010).

Macrolides have also been reported to exert beneficial effects in asthma. Low-dose erythromycin therapy in children with asthma for 6 months improved their symptoms and serum CXCL8 levels (Korematsu et al., 2010). Telithromycin showed beneficial effects on acute exacerbations of adult asthma (Johnston et al., 2006). Even short-term (8 week) clarithromycin treatment reduced sputum CXCL8, neutrophils, neutrophil elastase and MMP9 and also improved QOL scores in patients with refractory neutrophilic asthma (Simpson et al., 2008). Some *in vitro* studies have supported these clinical findings. Erythromycin and clarithromycin reduced CXCL8 release from human eosinophils in patients with allergic diseases, including asthma (Kohyama et al., 1999). Moreover, azithromycin inhibited IL-5 release from Th0 and Th2 cells in atopic asthmatic children (Lin et al., 2011). Thus, macrolides have shown great promise in their ability to reduce airway inflammation, including improvements in quality of life and exacerbation rates in airways diseases such as COPD and asthma (Simpson et al., 2009), but the efficacies are relatively moderate.

Excessive or inappropriate expression of MMP is closely associated with pathogenesis of tissue destructive processes in a wide variety of diseases including lung diseases such as COPD (Ohbayashi, 2002). In peripheral lung from COPD patients, MMP9 expression and activity are up-regulated and related to disease severity (Nakamaru et al., 2009). Furthermore, overexpression of human MMP9 in macrophages induced adult onset emphysema in mice and was associated with the loss of alveolar elastin (Foronjy et al., 2008). MMP9 produced from macrophages might be important in the development of emphysema (Russell et al., 2002). Ribeiro et al. demonstrated that azithromycin prevented the up-regulation of MMP9 genes, which were triggered by inflammatory stimuli (Ribeiro et al., 2009). In addition, there were a few *in vivo* studies

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evaluating effect of macrolide on MMP9 activity (Simpson et al., 2008; Ogawa et al., 2009). Simpson et al. showed that MMP9 concentrations were reduced in sputum from refractory asthmatics receiving clarithromycin. As shown in Figure 3 and 4, the effect of solithromycin on MMP9 production was more potent than that of clarithromycin, promising higher efficacy of solithromycin. The inhibition of MMP9 by solithromycin was also confirmed in smoke exposed mice (Fig. 6E)

The inflammation in COPD is amplified by increased oxidative stress (Rahman and Adcock, 2006). Oxidative stress activates NF-κB, a transcription factor, that orchestrates the expression of multiple inflammatory genes, including TNFα, CXCL8 and MMP9 (Barnes, 2009). In fact, NF-κB is reported to be regulated by macrolides. For example, azithromycin inhibited pro-inflammatory mediators, such as CXCL8, via inhibition of NF-κB *in vitro* models (Aghai et al., 2007; Matsumura et al., 2011), although it inhibited LPS-induced pulmonary neutrophilia and IL-1β with concomitant suppression of AP-1, but not NF-κB *in vivo* (Bosnar et al., 2011). In addition, erythromycin and clarithromycin also inhibited CXCL8 with inactivation of NF-κB and/or AP-1 in human bronchial epithelial cells in vitro (Abe et al., 2000; Desaki et al., 2000) as the effects were moderate. In this study, we also confirmed that solithromycin inhibited activated NF-κB under oxidative stress and the efficacy was superior to those of other macrolides currently used clinically. As the mechanism of NF-κB inactivation by macrolides, it is reported that macrolides are able to suppress the degradation of IκBα, an inhibitor of NF-κB, and/or to affect downstream of the dissociation from IκBα in the NF-κB signalling pathway (Desaki et al., 2004; Aghai et al., 2007).

There have been some negative findings on the anti-inflammatory effects of macrolides. For example, neither erythromycin nor azithromycin directly inhibited inflammatory mediators such as CXCL8 and MMPs in human gingival cells (Kamemoto et al., 2009). Neither clarithromycin nor azithromycin had beneficial effects on CXCL8 release in human whole blood cells and alveolar macrophages (Kurdowska et al., 2001). These inconsistent results on anti-inflammatory effects could be due to time-dependent responses to macrolides via cross talk of signaling pathway (Shinkai et al., 2006) , due to weak/moderate efficacies, or variable cellular intake, the results became variable.

Telithromycin, a commercially available ketolide, has also been reported to exert anti-inflammatory effects through NF-κB inhibition (Leiva et al., 2008). We therefore compared the effect of telithromycin with that of solithromycin. We found that telithromycin inhibited MMP9 activity (IC_{50}; 83.4 μM), although to a lesser extent than solithromycin (IC_{50}; 14.9 μM). However, it did not inhibit TNFα or CXCL8 release in macrophage system. In PBMC, as telithromycin showed cytotoxicity (significant at 100μM), we could not evaluate the efficacies properly. In addition, the clinical use of telithromycin is limited due to its side effects that include liver failure, exacerbation of myasthenia gravis, and visual disturbances. The pyridine-imidazole-containing moiety, which is a part of telithromycin, could induce inhibition of the nicotinic acetyl-choline receptors at the vagus nerve innervating the liver, the neuromuscular junction receptors, and the ciliary ganglion, resulting in these side effects, whereas solithromycin does not contain this moiety (Bertrand et al., 2010).

In conclusion, our findings revealed that a novel macrolide/ketolide, solithromycin exerts potent anti-inflammatory effects compared with other macrolides

used clinically, possibly through NF-κB inhibition. This suggests that with its superior anti-microbial and anti-inflammatory activities, solithromycin might be a potential therapy for refractory chronic inflammatory respiratory diseases such as COPD.

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Authorship Contributions

Participated in research design: Kobayashi, Goto, Wada, Barnes, and Ito.

Conducted experiments: Kobayashi, Wada, Rossios, Takagi, Higaki, and Mikura.

Performed data analysis: Kobayashi, Wada and Ito.

Wrote or contributed to the writing of the manuscript: Kobayashi, Barnes and Ito.

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Footnotes

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Legend for figures

Fig. 1. Effects of macrolides on LPS-induced TNFα release in PMA-differentiated U937 cells. Cells were pretreated with solithromycin (A) (10 to 100 μM) or erythromycin (B), clarithromycin (C), azithromycin (D) (33 to 333 μM), telithromycin (E) (10 to 100 μM) for 1 hr, followed by LPS (100 ng/ml) stimulation for 4 hrs. LPS-induced TNFα release was evaluated by ELISA. Values represent means of three experiments ± SEM. $^{\#\#}$ $p < 0.01$ (vs. non-treatment control), * $p < 0.05$, ** $p < 0.01$ (vs. treatment with LPS only).

Fig. 2. Effects of macrolides on LPS-induced CXCL8 release in PMA-differentiated U937 cells. Cells were pretreated with solithromycin (A) (10 to 100 μM) or erythromycin (B), clarithromycin (C), azithromycin (D) (33 to 333 μM), telithromycin (E) (10 to 100 μM) for 1 hr, followed by LPS (100 ng/ml) stimulation for 4 hrs. LPS-induced CXCL8 release was evaluated by ELISA. Values represent means of three experiments ± SEM. $^{\#\#}$ $p < 0.01$ (vs. non-treatment control), * $p < 0.05$, ** $p < 0.01$ (vs. treatment with LPS only).

Fig. 3. Effects of macrolides on PMA-induced MMP9 activation in U937 cells. Cells were pretreated with solithromycin (A) (10 to 100 μM) or erythromycin (B), clarithromycin (C), or azithromycin (D) (33 to 333 μM), or telithromycin (E) (10 to 100 μM) for 1 hr, followed by PMA (50 ng/ml) treatment for 48 hrs. After 48 hrs supernatants were collected for zymography. MMP9 enzyme activity was measured by gelatin zymography. Data are expressed relative to standard. Values represent means of

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four (A) or three (B, C and D) experiments ± SEM. $^{\#\#}$ $p < 0.01$ (vs. non-treatment control), * $p < 0.05$, ** $p < 0.01$ (vs. treatment with PMA only).

Fig. 4. Effects of macrolides on TNFα release and MMP9 production by PBMC from COPD patients. PBMC were incubated with macrolide compounds (solithromycin (A, E), 10 μM; erythromycin (B, F), clarithromycin (C, G) and azithromycin (D, H), 100 μM) overnight. TNFα release was evaluated by ELISA. MMP9 enzyme activity in supernatants was measured by gelatin zymography. Seven or eight individual values are shown. $^{\#\#}$ $p < 0.01$, $^{\#}$ $p < 0.05$ (vs. non-treatment control).

Fig. 5. Effects of macrolides on NF-κB activity under conditions of oxidative stress. PMA-differentiated U937 cells were pretreated with solithromycin (A) (10 and 33 μM) or erythromycin (B), clarithromycin (C), or azithromycin (D) (33 and 100 μM) for 1 hr, followed by H_2O_2 (200 μM) stimulation for 4 hrs. Binding activity of p65 to the NF-κB binding sequence was measured by spectrophotometer. Data were expressed relative to standard (1 mg of recombinant p65). Values represent means of three experiments ± SEM. $^{\#\#}$ $p < 0.01$ (vs. non-treatment control), * $p < 0.05$, ** $p < 0.01$ (vs. treatment with H_2O_2 only).

Fig. 6. Anti-inflammatory effects of solithromycin in cigarette smoke-exposed mice. (A-C) C57BL/6J mice were exposed to cigarette smoke (4%) for 30min/day for 12 days. Solithromycin (100mg/kg orally: SOL) or vehicle was administered once daily 1hr before cigarette smoke exposure for the last 8 days of smoke exposure. The number of

alveolar macrophages (A) and neutrophils (B) in BAL fluid were calculated. (C) The effects of solithromycin on an increase in body weight over 12 days. . (D,E) C57BL/6J mice were exposed to cigarette smoke (4%) for 30min/day for 4 days. Solithromycin or erythromycin (100mg/kg orally: SOL/EM) or vehicle was administered once daily 1hr before cigarette smoke exposure. Neutrophils (D), proMMP9 in BAL (E). $p < 0.05$ vs. smoke control (SM).NS: not significant

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Table 1. Effect of macrolides on inhibition of LPS-induced CXCL8 and TNFα release, and PMA-induced MMP9 activation in U937 cells

	IC_{50} (μM)				
	Solithro-mycin	Erythro-mycin	Clarithro-mycin	Azithro-mycin	Telithro-mycin
LPS-induce CXCL8 release	78.2	NE at 333μM	506.5	NE at 333μM	NE at 100μM
LPS-induced TNFα release	41.6	NE at 333μM	426.3	NE at 333μM	NE at 100μM
PMA-induced MMP9 activation	14.9	NE at 333μM	118.0	212.1	83.4

NE: no effect

Figure 1

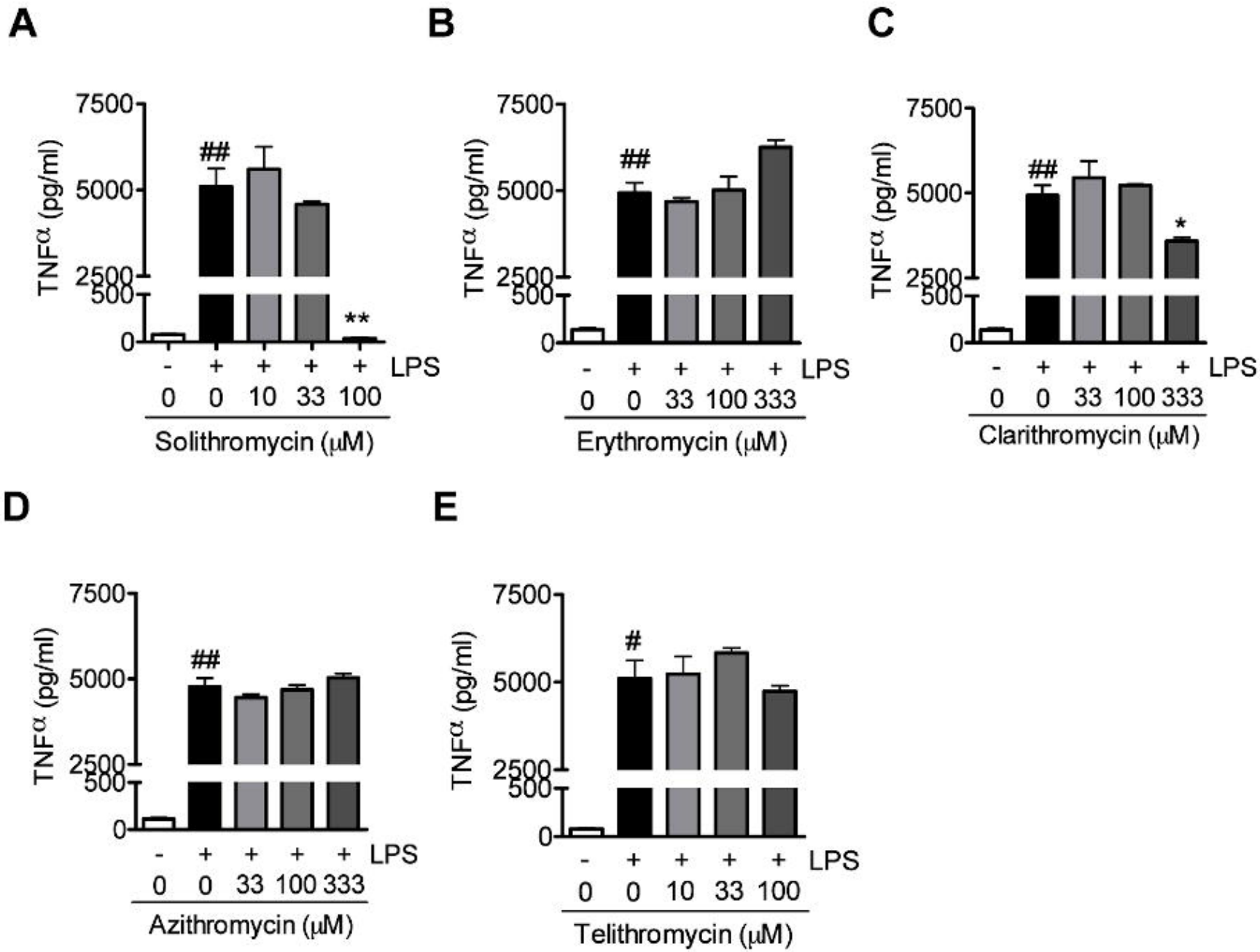

A
B
C
D
E
TNFα (pg/ml)
7500
5000
2500
500
0
##
#
**
*
- + + + + LPS
0 0 10 33 100
Solithromycin (μM)
0 0 33 100 333
Erythromycin (μM)
Clarithromycin (μM)
Azithromycin (μM)
0 0 10 33 100
Telithromycin (μM)

Figure 2

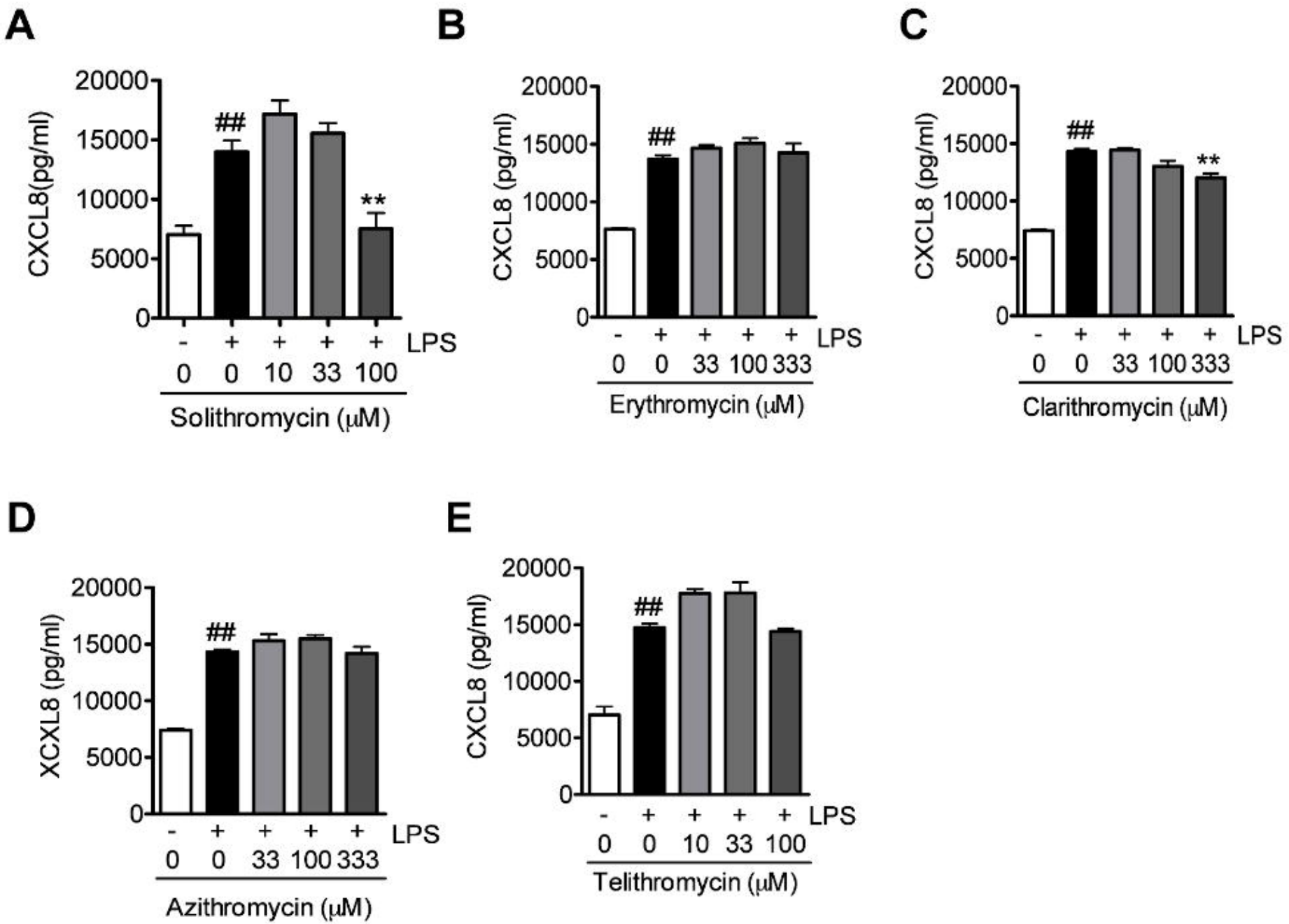

A
CXCL8(pg/ml)
20000
15000
10000
5000
0
##
**
- + + + + LPS
0 0 10 33 100
Solithromycin (μM)
B
CXCL8 (pg/ml)
20000
15000
10000
5000
0
##
- + + + + LPS
0 0 33 100 333
Erythromycin (μM)
C
CXCL8 (pg/ml)
20000
15000
10000
5000
0
##
**
- + + + + LPS
0 0 33 100 333
Clarithromycin (μM)
D
XCXL8 (pg/ml)
20000
15000
10000
5000
0
##
- + + + + LPS
0 0 33 100 333
Azithromycin (μM)
E
CXCL8 (pg/ml)
20000
15000
10000
5000
0
##
- + + + + LPS
0 0 10 33 100
Telithromycin (μM)

Figure 3

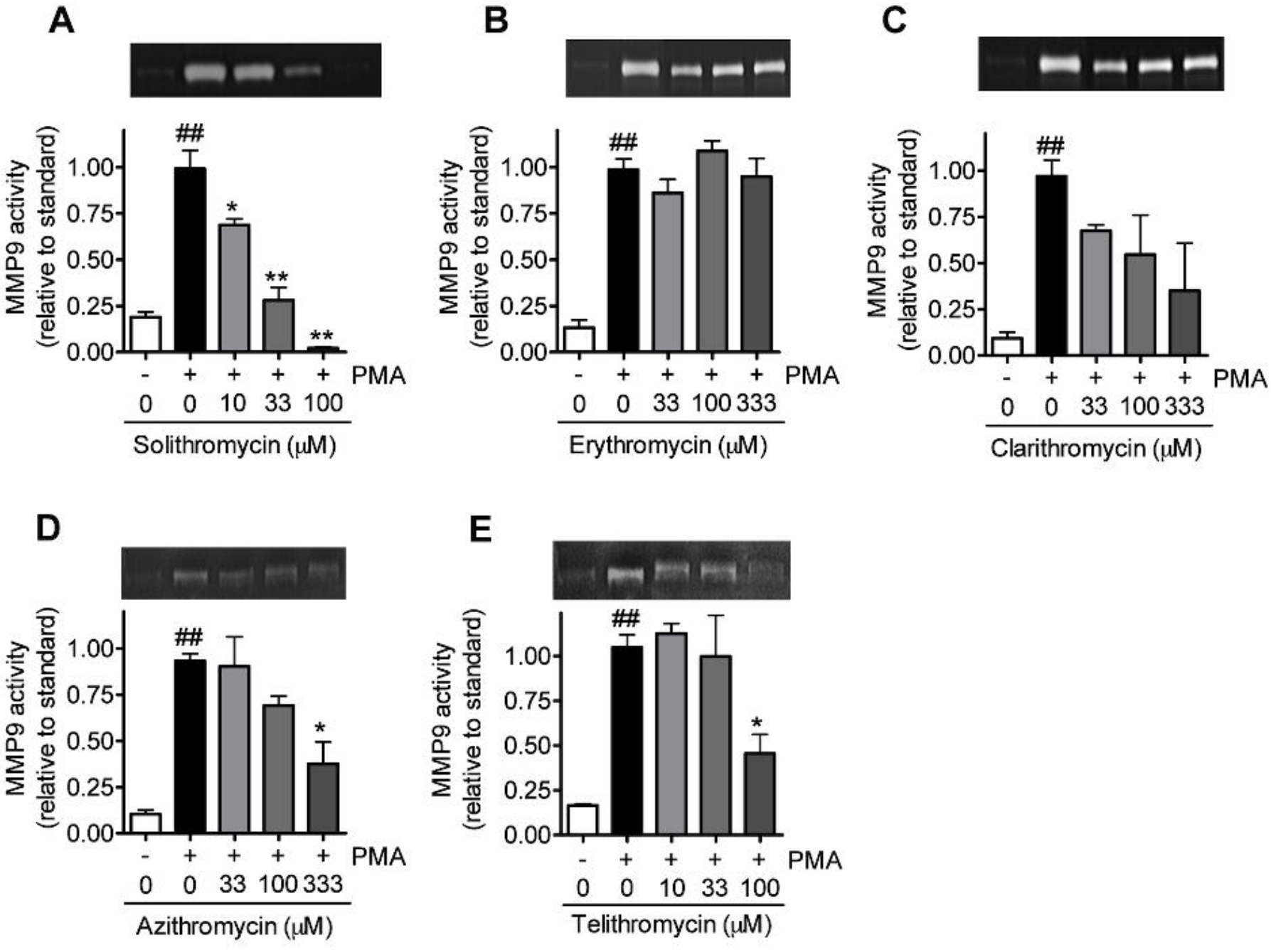

A
B
C
D
E
MMP9 activity (relative to standard)
1.00
0.75
0.50
0.25
0.00
##
*
**
- + + + + PMA
0 0 10 33 100
Solithromycin (μM)
0 0 33 100 333
Erythromycin (μM)
0 0 33 100 333
Clarithromycin (μM)
0 0 33 100 333
Azithromycin (μM)
0 0 10 33 100
Telithromycin (μM)

Figure 4

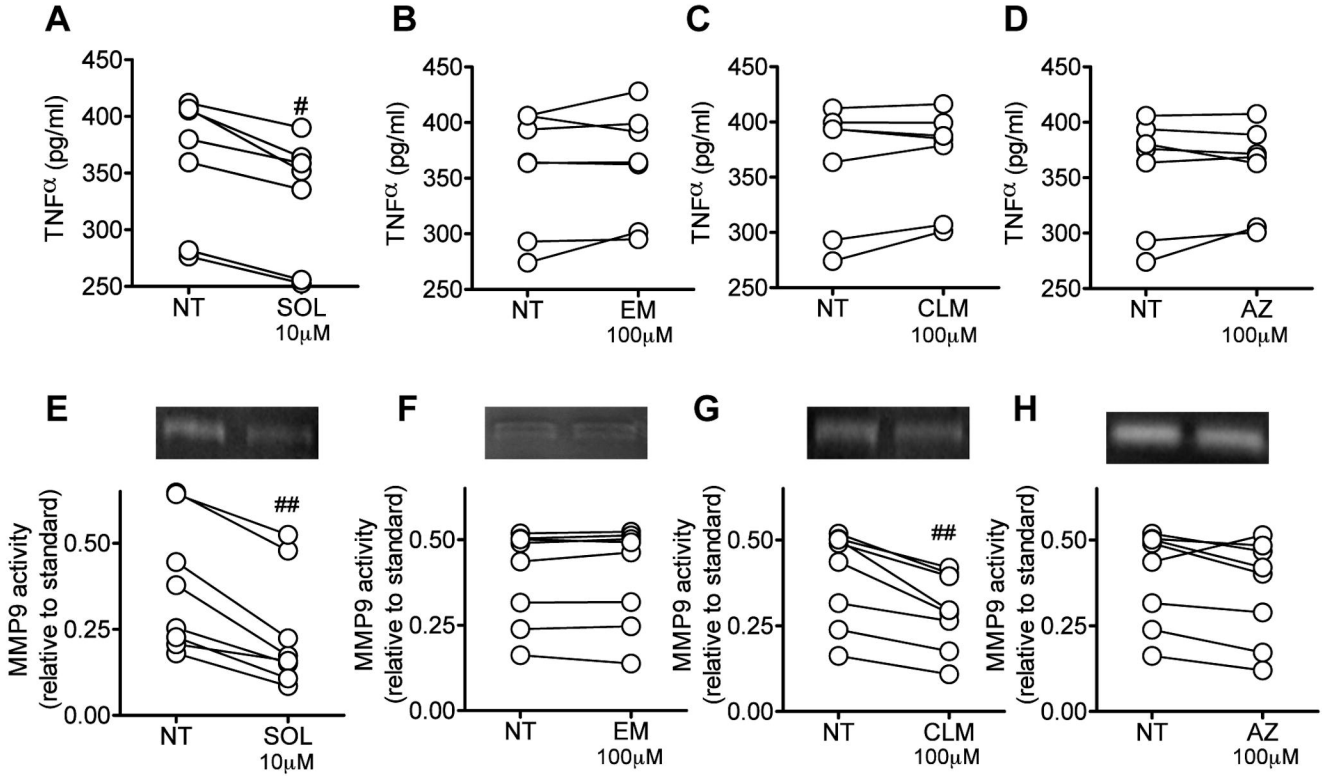

A
B
C
D
TNFα (pg/ml)
450
400
350
300
250
#
NT
SOL
10μM
EM
100μM
CLM
100μM
AZ
100μM
E
F
G
H
MMP9 activity
(relative to standard)
0.50
0.25
0.00
##

Figure 5

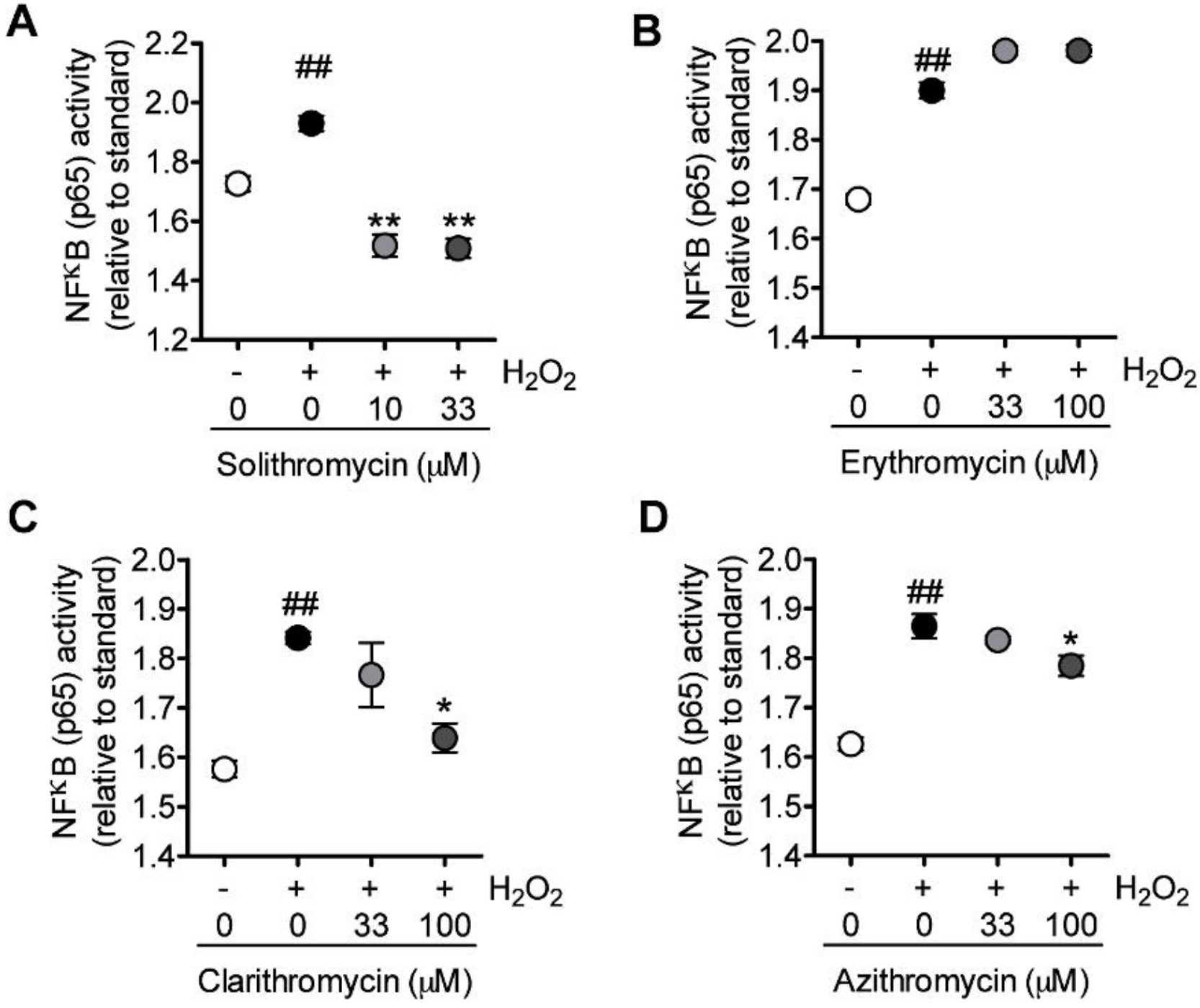
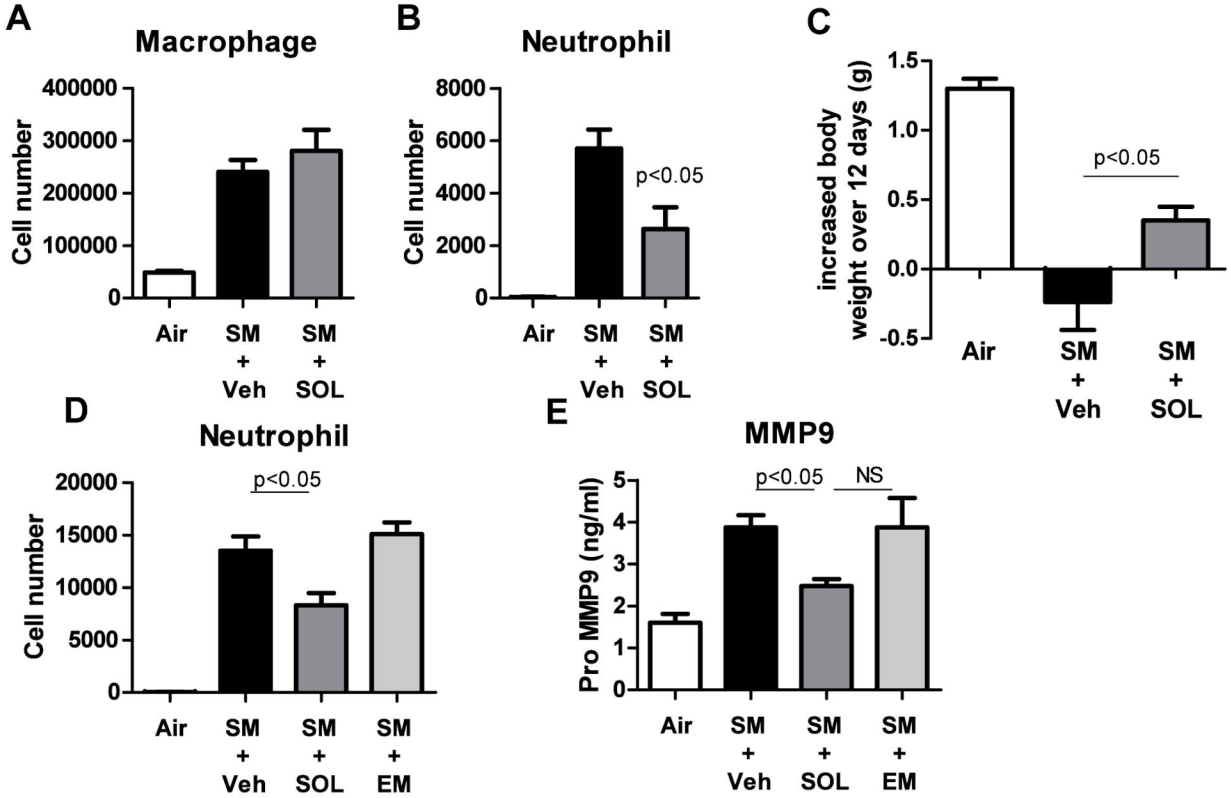

A
NFκB (p65) activity (relative to standard)
2.2
2.0
1.8
1.6
1.4
1.2
##
**
**
- + + + H2O2
0 0 10 33
Solithromycin (μM)
B
NFκB (p65) activity (relative to standard)
2.0
1.9
1.8
1.7
1.6
1.5
1.4
##
- + + + H2O2
0 0 33 100
Erythromycin (μM)
C
NFκB (p65) activity (relative to standard)
2.0
1.9
1.8
1.7
1.6
1.5
1.4
##
*
- + + + H2O2
0 0 33 100
Clarithromycin (μM)
D
NFκB (p65) activity (relative to standard)
2.0
1.9
1.8
1.7
1.6
1.5
1.4
##
*
- + + + H2O2
0 0 33 100
Azithromycin (μM)

Figure 6


A
Macrophage
Cell number
400000
300000
200000
100000
0
Air
SM + Veh
SM + SOL
B
Neutrophil
Cell number
8000
6000
4000
2000
0
p<0.05
Air
SM + Veh
SM + SOL
C
increased body weight over 12 days (g)
1.5
1.0
0.5
0.0
-0.5
p<0.05
Air
SM + Veh
SM + SOL
D
Neutrophil
Cell number
20000
15000
10000
5000
0
p<0.05
Air
SM + Veh
SM + SOL
SM + EM
E
MMP9
Pro MMP9 (ng/ml)
5
4
3
2
1
0
p<0.05
NS
Air
SM + Veh
SM + SOL
SM + EM