PRP Pharmacology Research & Perspectives
Open Access

Azithromycin inhibits nuclear factor-κB activation during lung inflammation: an in vivo imaging study

Fabio F. Stellari[1], Angelo Sala[2,3], Gaetano Donofrio[4], Francesca Ruscitti[5], Paola Caruso[1], Thomas M. Topini[1], Kevin P. Francis[6], Xiaojian Li[7], Chiara Carnini[1], Maurizio Civelli[1] & Gino Villetti[1]

[1]Chiesi Farmaceutici S.p.A, Parma, Italy
[2]Dipartimento di Scienze Farmacologiche e Biomolecolari, Università di Milano, Milano, Italy
[3]Consiglio Nazionale delle Ricerche, IBIM, Palermo, Italy
[4]Dipartimento di Scienze Medico-Veterinarie, Università di Parma, Parma, Italy
[5]Dipartimento di Scienze Biomediche, Biotecnologiche e Traslazionali, Università di Parma, Parma, Italy
[6]Perkin Elmer Discovery Services, Hanover, Maryland
[7]Perkin Elmer, Alameda, California

Keywords
Acute lung inflammation, azithromycin, bioluminescence, in vivo imaging, nuclear factor-κB

Correspondence
Angelo Sala, Dipartimento di Scienze Farmacologiche e Biomolecolari, Via Balzaretti 9, 20133 Milano. Tel: +39 0250318312; Fax: +39 0250318297; E-mail: angelo.sala@unimi.it

Funding Information
Supported by Institutional Funds from Chiesi Farmaceutici S.p.A.

Received: 24 March 2014; Revised: 8 May 2014; Accepted: 21 May 2014

Pharma Res Per, 2 (5), 2014, e00058, doi: 10.1002/prp2.58

doi: 10.1002/prp2.58

Abstract

We studied in vivo the potential involvement of nuclear factor-κB (NF-κB) pathway in the molecular mechanism of the anti-inflammatory and immunomodulatory activity of azithromycin in the lung. Mice transiently transfected with the luciferase gene under the control of a NF-κB responsive element were used to assess in vivo NF-κB activation by bioluminescence imaging. Bioluminescence as well as inflammatory cells and concentrations of proinflammatory cytokines in bronchoalveolar lavage fluids, were monitored in an acute model of pulmonary inflammation resulting from intratracheal instillation of lipopolysaccharide. Lipopolysaccharide (LPS) instillation induced a marked increase in lung bioluminescence in mice transiently transfected with the luciferase gene under the control of an NF-κB responsive element, with significant luciferase expression in resident cells such as endothelial and epithelial cells, as assessed by duoplex immunofluorescence staining. Activation of NF-κB and inflammatory cell lung infiltration linearly correlated when different doses of bortezomib were used to inhibit NF-κB activation. Pretreatment with azithromycin significantly decreased lung bioluminescence and airways cell infiltration induced by LPS, also reducing proinflammatory cytokines concentrations in bronchoalveolar lavages and inhibiting NF-κB nuclear translocation. The results obtained using a novel approach to monitor NF-κB activation, provided, for the first time, in vivo evidence that azithromycin treatment results in pulmonary anti-inflammatory activity associated with the inhibition of NF-κB activation in the lung.

Introduction

Macrolide antibiotics were shown to have immunomodulatory and anti-inflammatory, as well as antibacterial effects (Culic et al. 2001; Martinez et al. 2008; Kanoh and Rubin 2010). In vitro azithromycin inhibits the release of the neutrophil chemoattractant interleukin(IL)-8 and of granulocyte-macrophage colony-stimulating factor (GM-CSF) by lipopolysaccharide (LPS)-activated, human primary bronchial epithelial cells (Murphy et al. 2008), and in vivo azithromycin has been shown to inhibit LPS-induced mouse pulmonary neutrophilia (Bosnar et al. 2009). In potential agreement with these activities, azithromycin significantly decreased the rate of exacerbation in chronic obstructive pulmonary disease (COPD) patients (Albert et al. 2011) as well as in non-cystic fibrosis bronchiectasis patients (Wong et al. 2012), but the molecular mechanism of its pulmonary anti-inflammatory activity has not yet been elucidated.

Airway infiltration by polymorphonuclear leukocytes (PMNs) in response to bacterial and viral infections represents a pivotal feature of lung inflammatory reactions and is both directly and indirectly involved in most lung pathologies (Murugan and Peck 2009). Mobilization of PMNs to inflamed lungs is modulated by a complex interplay between cytokines, endothelial cells, and neutrophils.

During the acute-phase response to infectious agents in ruminants and humans, inflammatory cytokines such as IL-1β, tumor necrosis factor(TNF)-α and IL-8, secreted by a variety of immune and nonimmune cell types, induce a strong PMNs infiltration that sometimes fail to control the infection, and may even contribute to the development of lesions in the lung (Malazdrewich et al. 2001; Mukaida 2003; Murugan and Peck 2009).

Since its discovery in 1986 (Sen and Baltimore 1986), the role of ubiquitous nuclear factor-κB (NF-κB) transcription factor has been extensively studied in tumorigenesis, immunity, and inflammatory responses, and its dysregulated activation has been associated with malignancies as well as to inflammatory pathological conditions (Bours et al. 2000; Salminen et al. 2008; Tornatore et al. 2012).

In the lungs, several noxious/inflammatory stimuli activate NF-κB, including intact bacteria, Gram-negative bacterial LPS, ozone, and silica delivered directly to the airways, as well as systemic inflammatory insults such as sepsis, hemorrhage, and direct liver injury (Blackwell et al. 1994, 2000). In rodent models of lung inflammation induced by LPS, pretreatment with relatively nonspecific inhibitors of NF-κB activation was found to decrease lung inflammation (Lauzurica et al. 1999), and mice deficient in both RelA (the transactivating subunit of NF-κB) and type 1 TNF receptor (TNFR1) showed impaired neutrophil recruitment to the lungs in response to LPS (Alcamo et al. 2001).

To dynamically investigate in vivo, the involvement of NF-κB in the inflammatory response, a transgenic mouse model was developed expressing the minimal promoter of NF-κB and the luciferase gene as a reporter, allowing a direct monitoring of NF-κB activation by bioluminescence (Carlsen et al. 2002; Everhart et al. 2006; Kielland and Carlsen 2010). BLI is based on the detection of light emission from cells or tissues (Doyle et al. 2004), and requires an expression cassette consisting of the bioluminescence reporter gene (in this case was luciferase) under the control of a selected promoter (in this experimental setting the NF-κB gene promoter) driving the reporter. To induce light production, the substrate luciferin must be provided by intravascular or intraperitoneal injection immediately prior to bioluminescence imaging (BLI) evaluation. BLI has proven to be a very powerful technique, facilitating real-time, molecular level analysis of disease progression. Nevertheless the generation, characterization, and colony management of transgenic mice may be difficult, and typically very expensive. An alternative, relatively inexpensive approach is represented by the use of transient transgenic mice obtained through the infusion of a polyethylenimine (PEI)/DNA complex containing plasmids with specific responsive elements and luciferase as a reporter gene. This technique yields significant transfection in vivo, as was recently shown by imaging pulmonary NF-κB activation in LPS-treated mice (Ansaldi et al. 2011).

In this work, we studied the correlation between lung bioluminescence and lung inflammatory cell infiltration in mice transiently transfected with the luciferase (*luc*) gene under the control of an NF-κB responsive element, upon intratracheal challenge with LPS, and we took advantage of this unique model to provide novel, in vivo evidence that the molecular mechanism of action of the anti-inflammatory activity of the macrolide antibiotic azithromycin involves the modulation of NF-κB activation in lung resident cells.

Materials and Methods

Animals

Female FVB (7–8 week-old) mice were purchased from Harlan Laboratories Italy (S. Pietro al Natisone, Udine, Italy). Animals were maintained under conventional housing conditions. Prior to use, animals were acclimated for at least 5 days to the local vivarium conditions (room temperature: 20–24°C; relative humidity: 40–70%), having free access to standard rat chow and tap water. All animal experiments were carried out in agreement with the revised "Guide for the Care and Use of Laboratory Animals" (1996) and were approved by the Institutional Animal Care and Use Committee at Chiesi Farmaceutici. The study adhered to the ARRIVE guidelines (McGrath et al. 2010).

Reagents

LPS (from Escherichia coli 0111:B4, product n.L3012) was from Sigma (St. Louis, MO); azithromycin (Zitromax) was from Pfizer Inc (Latina, Italy); JetPEI was from Polyplus-transfection Inc (Euroclone, Milano, Italy); NF-κB vector (pGL4.32[luc2P/NF-κB-RE/Hygro]) was from Promega (Madison, WI); bortezomib (Velcade) was from Millennium Pharmaceuticals (Cambridge, MA).

Vector characteristic and in vivo gene delivery

The pGL4.32[luc2P/NF-κB-RE/Hygro] vector (GenBank/EMBL Accession Number EU581860) contains five copies of an NF-κB responsive element (NF-κB-RE) that drives transcription of the luciferase reporter gene luc2P (Photinus pyralis). Luc2P is a synthetically derived luciferase sequence with humanized codon optimization that is designed for high expression and reduced anomalous transcription.

The luc2P gene contains hPEST, a protein destabilization sequence. The protein encoded by luc2P responds more quickly than the protein encoded by the luc2 gene upon induction. The vector backbone contains a resistance gene to allow selection in *E. coli* and a mammalian selectable marker for hygromycin resistance.

JetPEI (Wu et al. 2004; Oh et al. 2013) was applied in vivo as a carrier for delivering DNA to lung tissues. The DNA and JetPEI were formulated according to the product manual. Briefly, 40 μg of NF-κB-luc reporter and 7 μL of JetPEI were each diluted into 100 μL 5% glucose. The two solutions were then mixed and incubated for 15 min at room temperature.

The entire mixture (app.ly 200 μL) was injected into the tail vein of mice.

In vivo bioluminescence imaging

Transfection per se causes a mild lung inflammatory response and NF-κB activation that is detectable by BLI up to 3–4 days after DNA injection and disappears completely after 1 week. Therefore 1 week after DNA delivery, the transient transgenic mice were injected with luciferin i.p. and BLI was recorded to check the baseline activation of the NF-κB pathway. Briefly, following intraperitoneal injection of luciferin (150 mg/kg) mice were lightly anesthetized with isoflurane (2.5%) and images were obtained using an IVIS imaging system (Caliper Life Sciences, Alameda, CA) at 10 and 15 min after luciferin: an average of photons emitted from the chest of the mice was quantified using Living Image® software (Caliper Life Sciences). The following day, mice were intratracheally challenged with LPS (12.5 μg/mouse) and BLI was recorded at 2, 4, 7, and 24 h after LPS instillation, 15 min after i.p. injection of luciferin (150 mg/kg).

Immunofluorescence staining of luciferase, CD31 and cytokeratin 18 (CK18)

To identify the type of cell expressing luciferase protein, duoplex immunofluorescence staining was performed with anti-luciferase antibody and either an anti-CD31 (an endothelial cell marker) or anti-CK18 (an epithelial cell marker). Briefly, during necropsy, 4% paraformaldehyde was first injected through the right ventricle/pulmonary artery to inflate the blood vessel within the lung tissue, and subsequently injected through the trachea to inflate the alveoli. Lung samples were then embedded in paraffin and cut into 5-μm sections, deparaffinized, microwaved in Citra Plus (pH 6; BioGenex, Fremont, CA) for antigen retrieval, and blocked with Antibody Diluent (Dako, Carpintiria, CA). Sections were then incubated with anti-luciferase antibody (Novus Biologicals, Littleton, CO) at 1:100 dilution and 4°C overnight, followed by anti-goat secondary antibody conjugated with Alexa Fluor 594 (Invitrogen, Grans Island, NY) for 1 h. Thereafter, sections were incubated with anti-CD31 antibody (Abcam, Cambridge, MA) at 1:100 dilution for 1 h, followed by anti-mouse secondary antibody conjugated with Alexa Fluor 488 for 1 h, and finally by 4′,6-diamidino-2-phenylindole (DAPI) for 5 min for nuclear staining. For Luciferase/CK18 duoplex staining, after deparaffinization, microwaving and blocking, sections were incubated with an anti-CK18 antibody (Abcam) at 1:500 dilution for 1 h, followed by SuperPicture horseradish peroxidase-conjugated anti-mouse secondary antibody (Invitrogen) for 5 min, and tyramide signal amplification conjugated fluorescein (PerkinElmer, Alameda, CA) for 10 min. Thereafter, sections were microwaved again, blocked and then incubated with anti-luciferase antibody at 1:100 overnight, followed by anti-goat secondary antibody conjugated with Alexa Fluor 594 for 1 h, and DAPI for 5 min for nuclear staining. Images were captured with a Vectra-2 Imaging System (PerkinElmer).

Acute pulmonary inflammation: effect of bortezomib and azithromycin

Intratracheal (i.tr.) challenge with LPS was carried out using 50 μL of LPS solution (250 μg/mL in phosphate buffered solution [PBS]) and a 22-gauge intubator, resulting in a final dose of LPS of 12.5 μg/mouse, with the control group receiving 50 μL of saline i.tr.. In a limited number of mice, 4 h after LPS challenge and luciferin administration, lungs were rapidly excised and BLI recorded at 10 and 15 min after luciferin, as described for intact animals.

Bortezomib was administered by injection into the tail vein at different doses (0.5–1 mg/kg, Psallidas et al. 2010) 1 h before LPS i.tr. instillation.

Azithromycin (Zitromax solution) was administered p.o. by gavage at different doses (100–600 mg/kg) (Bosnar et al. 2009) 4 h before LPS i.tr. instillation.

Bronchoalveolar lavage and cytokine determination

Twenty-four hours after LPS challenge, animals were weighted, anaesthetized with isoflurane and sacrificed by bleeding from the abdominal aorta for bronchoalveolar lavage fluid (BALF) collection, performed as previously described (Nassini et al. 2012). Bronchoalveolar lavage (BAL) fluid supernatants were frozen at −80°C for simultaneous quantitation of multiple cytokines/chemokines using a Bio-Plex™ Cytokine Assay Kit (Bio-Rad Laboratories, Segrate, Milano, Italy).

The cell pellet was resuspended in 0.2 mL of PBS and cell counts were obtained using a particle counter (Dasit XT 1800J, Cornaredo, Milano, Italy).

p65 nuclear translocation

Lungs were excised and homogenized using a trans Turrax homogenizer. Cytoplasmatic and nuclear extracts were obtained using Nuclear Extraction Kit (Active Motif, La Hulpe, Belgium), and determination of p65 was carried out using TransAM NFkB p65 transcription factor assay kit (Active Motif), according to the manufacturer instructions.

Data analysis

As tests for normality were positive, statistical analysis was performed on raw data using one-way analysis of variance (ANOVA) followed by Dunnett's *t* post-hoc test for comparison with control groups. Experimental values were expressed as the mean and standard error of the mean (SEM) of *n* observations. (*$P < 0.05$, **$P < 0.01$).

Results

As previously reported, LPS intratracheal instillation 1 week after DNA delivery of the luciferase reporter construct caused activation of NF-κB that could be easily monitored by BLI, showing a very marked increase when compared to control animals (Fig. 1A). Ex-vivo imaging of isolated lungs confirmed that the bioluminescence observed in vivo was associated to the activation of NF-κB in the lungs (Fig. 1B). *Luc* expression driven by NF-κB activation was detectable by BLI as early as 2 h after LPS challenge, peaked at 4 h after treatment, with a 10-fold induction over baseline. This signal was still significantly enhanced 7 h after LPS, but returned to baseline levels 24 h after LPS challenge (Fig. 1C). Double staining immunofluorescence analysis performed to identify which cell type(s) is(are) targeted by the injected plasmid DNA showed LPS-induced expression of luciferase in both epithelial (Fig. 2A–D) or endothelial (Fig. 2E–H) cells. While regular staining was observed for CK18 and CD31 (Fig. 2I and K, and Fig. S1A and C), no signal for luciferase was observed in LPS-challenged wild type animals (Fig. 2J) or in saline-challenged NF-κB-luc animals (Fig. S1B and D).

Bortezomib (Velcade), a known proteasome inhibitor that interferes with the degradation of IkB and therefore the activation of NF-κB, dose dependently inhibited luciferase expression 4 h after LPS administration (Fig. 3A and B), lending support to the quantitative correlation between NF-κB activation and bioluminescence determination in the transient transgenic mice. In agreement with the fact that the expression of adhesion molecules leading to white blood cells (WBC) extravasation and infiltration are results of NF-κB activation (Read et al. 1995; Kalogeris et al. 1999; Dagia and Goetz 2003), increased numbers of WBC and neutrophils were also recovered by BAL 24 h after LPS challenge (Fig. 4A and B) and this increase was also significantly inhibited by bortezomib treatment. A strong correlation was observed between bioluminescence observed 4 h after LPS administration and the number of WBC and neutrophils recovered by BAL 24 h after LPS challenge (Fig. 4C), further supporting the ability of this transient transgenic mouse model to provide an accurate, in vivo determination of NF-κB activation in the lung.

Pre-treatment of transient transgenic mice with azithromycin per os, dose-dependently inhibited bioluminescence induced by LPS tracheal instillation (9.5 ± 1.74 fold, 6.9 ± 1.82 and 4.16 ± 0.78 fold of induction over baseline at 4 h in LPS-control, azithromycin 100 mg/Kg p.o., and azithromycin 600 mg/Kg p.o., respectively) (Fig. 5A and

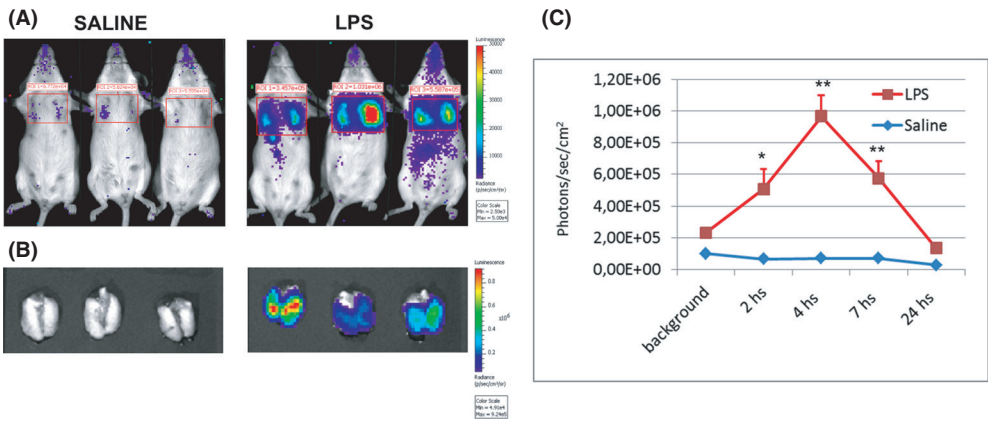

Figure 1. (A) In vivo imaging of NF-κB activation 4 h after LPS challenge. LPS (12.5 μg/mouse) was administered intratracheally. Representative mice are shown for saline and LPS groups. (B) Ex vivo imaging of NF-κB activation in lungs excised 4 h after LPS treatment. Representative lungs are shown for saline and LPS groups. (C) Time-course of luciferase induction in mice challenged with intratracheal LPS. Values are shown as mean ± SEM, $n = 12$ at each time point $P < 0.05$, **$P < 0.01$ versus saline group (Dunnett's *t* test).

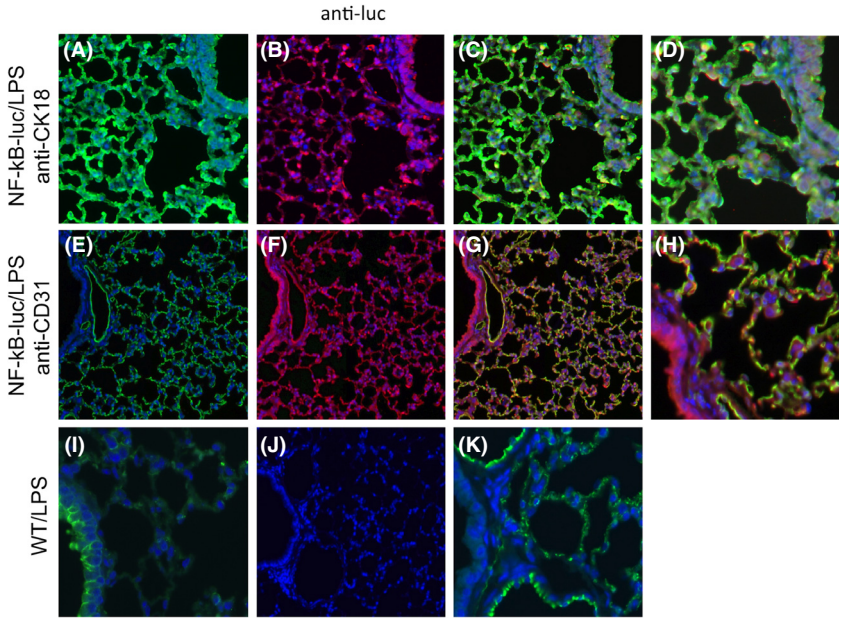

Figure 2. Double immunofluorescence staining of mice lungs. Duoplex immunofluorescence staining of luciferase/CK18 (epithelial cell marker) or luciferase/CD31(endothelial cell marker) were performed on paraformaldehyde-fixed lung sections, as described in Materials and Methods. Double staining showed that in LPS-challenged NF-kB-luc mice both epithelial cells (A) and endothelial cells (E) were expressing luciferase (B and F), and displayed yellow immunofluorescence when the two images were merged (C and G, respectively); larger magnifications of the merged images are reported in (D and H). Immunofluorescence staining of CK18 (I), luciferase (J) and CD31 (K) in LPS-challenged wild type mice, confirmed the absence of signal for luciferase. Original magnification: (A–C, E–G, and J) 210X; (D, H, I, and K) 600X.

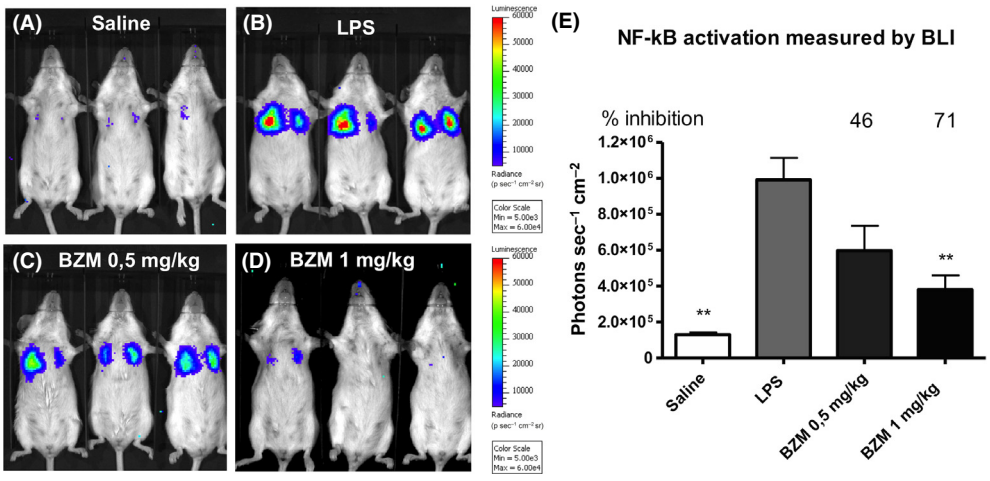

Figure 3. (A–D) In vivo imaging of NF-κB activation 4 h after LPS treatment: effects of pretreatment with bortezomib. Transient NF-κB-luc transgenic mice were pretreated with saline or bortezomib (BZM, 0.5–1 mg/kg) i.v. 1 h before LPS intratracheal instillation. Representative mice are shown for saline (A), LPS (B), LPS+BZM 0.5 mg/kg (C) and LPS+BZM 1 mg/kg (D and E). Quantification of NF-κB activation by BLI, 4 h after LPS intratracheal instillation. Values are shown as mean ± SEM, $n = 8$ for each group, and% inhibition versus LPS group is reported. $^{**}P < 0.01$ versus LPS group (Dunnett's *t* test)

B). As observed with bortezomib, the inhibition of BLI was followed by a significant inhibition of WBC and neutrophil infiltration at 24 h confirming the anti-inflammatory activity of this compound (Fig. 6A and B).

The inhibitory activity of azithromycin on NF-κB activation, also resulted in a statistically significant inhibition in the BAL concentrations of several pro-inflammatory cytokines, such as TNF-α, granulocyte colony-stimulating

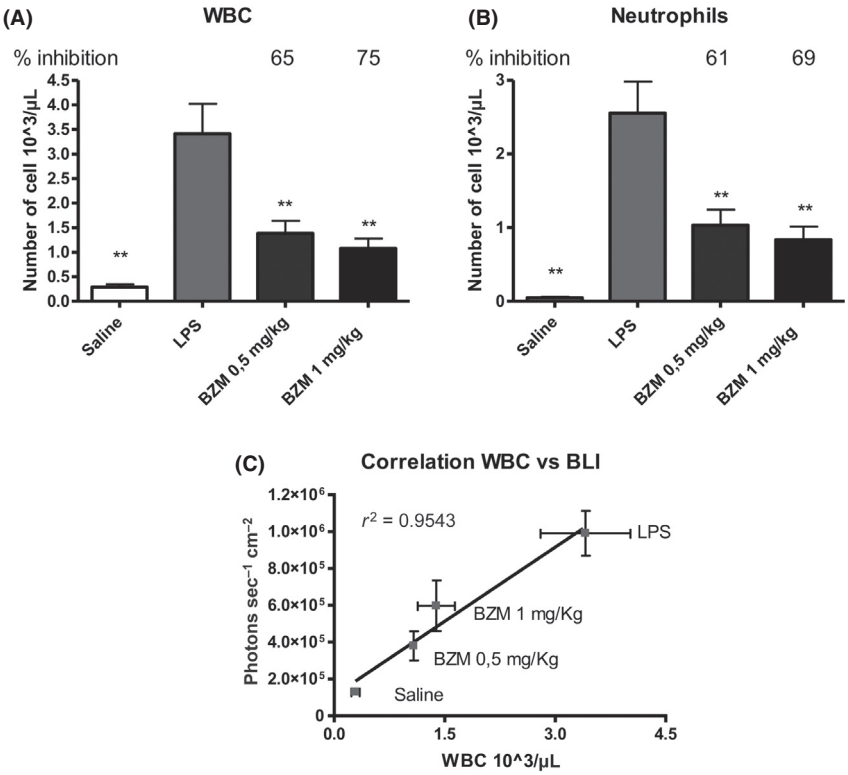

Figure 4. (A and B) LPS-induced neutrophil and white blood cells (WBC) recruitment in the airways: effects of bortezomib. Transient NF-κB-luc transgenic mice were pretreated with saline or bortezomib (BZM, 0.5–1 mg/kg, i.v.) 1 h before LPS, and sacrificed 24 h after LPS administration by tracheal instillation. Values are shown as mean ± SEM, $n = 8$ for each group, and% inhibition versus LPS group is reported. $^{**}P < 0.01$ versus LPS group (Dunnett's test, C). Correlation between NF-κB activation as measured by BLI, 4 h after LPS instillation, and the concentration of WBC in BAL lavage fluids, obtained 24 h after LPS instillation, in saline, LPS, LPS+BZM 0.5 mg/kg and LPS+BZM 1 mg/kg groups.

factor (G-CSF) and monocyte chemoattractant protein-1 (MCP-1) (Fig. 7A–F), while other cytokines upregulated by LPS were not affected (Fig. S2A–E). A complete list of cytokines analyzed is reported in Table 1S.

Time-course of p65 nuclear translocation paralleled *luc* signal induction, with maximal accumulation occurring at 4 h, while by 24 h p65 was again localized primarily in cytoplasm (data not shown); azithromycin treatment inhibited by 52% ($P < 0.05$) the nuclear translocation of activated NF-κB observed in lung homogenates 4 h after LPS tracheal instillation.

Discussion

The results obtained provided, for the first time, in vivo evidence that azithromycin treatment results in pulmonary anti-inflammatory activity associated with the inhibition of NF-κB activation in the lung, as followed by bioluminescence monitoring in mice transiently transfected with luciferase gene under the control of an NF-κB responsive element (Ansaldi et al. 2011).

While a limited number of evidence obtained in vitro using different cell types, including airways cells, pointed to the inhibition of NF-κB as part of the mechanism of the anti-inflammatory activity of azithromycin (Aghai et al. 2007; Cigana et al. 2007; Matsumura et al. 2011; Vrancic et al. 2012), the only evidence available in vivo was obtained in a model of ocular inflammation, where it was shown that azithromycin treatment decreased the amount of NF-κB protein detected by western blot in conjunctival homogenates, a measurement that does not actually evaluate changes in NF-κB activation. On the other side, studying the activity of azithromycin in a model of LPS-induced pulmonary neutrophilia Boznar and co. could not show any inhibitory effect on NF-κB activation in alveolar macrophages (Bosnar et al. 2011). The ability, offered by the animal model used in this study, to evaluate and monitor NF-κB activation in vivo at the whole organ level, clearly showed that a significant activation of NF-κB is indeed taking place in the lung upon intratracheal LPS challenge, and that azithromycin anti-inflammatory activity significantly inhibited

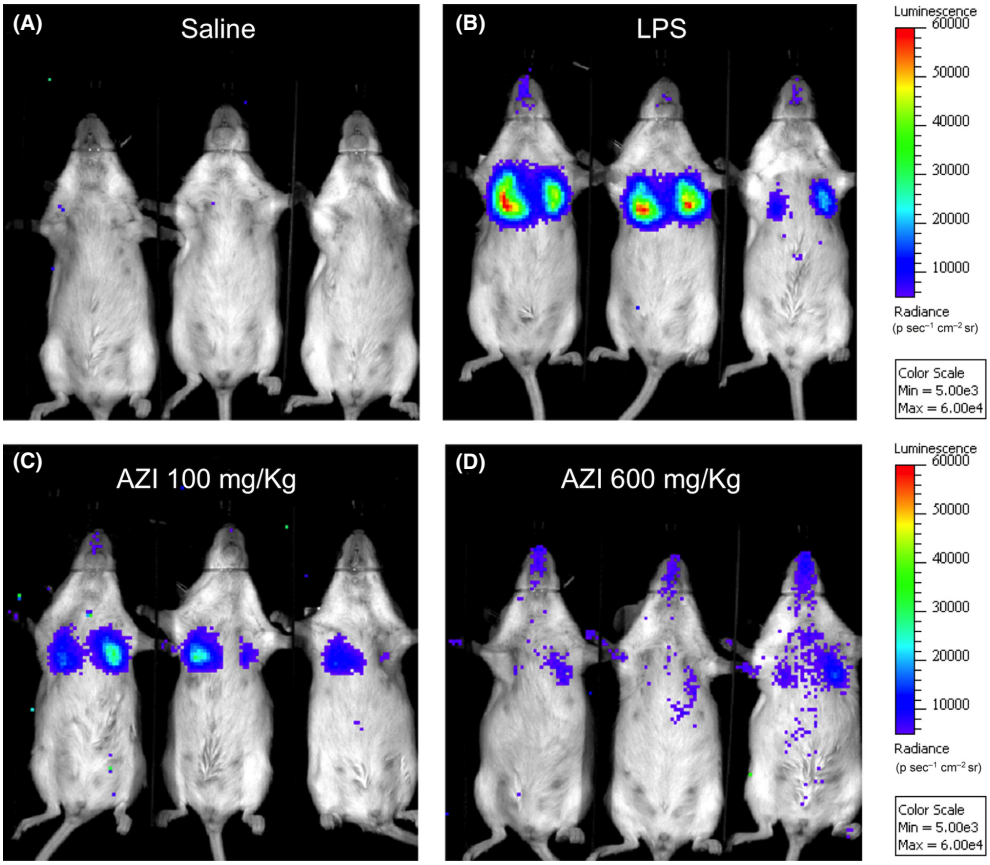

Figure 5. (A and B) In vivo imaging of NF-κB activation 4 h after LPS treatment: effect of azithromycin. Transient NF-κB-luc transgenic mice were pretreated with saline or azithromycin (100–600 mg/Kg, *per os)* 4 h before LPS intratracheal instillation. Representative mice are shown for saline (A), LPS (B), LPS+AZI 100 mg/kg (C) and LPS+AZI 600 mg/kg (D).

NF-κB activation-dependent bioluminescence. Luciferase co-expression with epithelial and endothelial cell-specific markers, as assessed by duoplex immunofluorescence, suggests that these cells may therefore represent potential targets of the activity of azithromycin. It must be noted that although cytokeratins are considered a typical marker of epithelial differentiation, they can also be expressed in certain vascular smooth muscle cells (Bar et al. 2001).

Although no infiltrating inflammatory cells showed up significantly expressing luciferase, this does not rule out the activation of NF-κB in neutrophils, as their rapid turnover likely prevents them from being detected at the time-points used for LPS challenge after transient transfection.

NF-κB activation upon LPS challenge resulted in a rather striking correlation between bioluminescence induced by NF-κB activation and inflammatory biomarkers such as WBC and neutrophils airways infiltration, as shown by the parallelism between changes in these parameters induced by LPS alone or in the presence of increasing doses of a potent proteasome inhibitor (bortezomib) known to inhibit NF-κB activation, by blocking the degradation of IκB, the inhibitory unit of the NF-κB complex, carried out by the 26S proteasome.

Dysregulation of NF-κB activation has been involved in lung diseases such as asthma, chronic bronchitis, and COPD (Donovan et al. 1999; Teramoto and Kume 2001), but its specific contribution to disease progression is currently unknown (Lawrence et al. 2001). BLI provides a noninvasive approach to monitor gene expression in vivo and represent an important tool to evaluate the potential contribution of NF-κB to the evolution of acute inflammatory reactions.

BLI is a powerful technique based on the detection of visible light produced during luciferase-mediated oxidation of a molecular substrate in the presence of the enzyme resulting from its expression in vivo as a molecular reporter. Bioluminescence arising from luciferase can be imaged as deep as several centimeters within tissues, allowing at least organ-level resolution. Being simple to execute and minimally invasive, BLI enables monitoring and serial quantification of biological processes without sacrificing the experimental animal. This powerful technique can

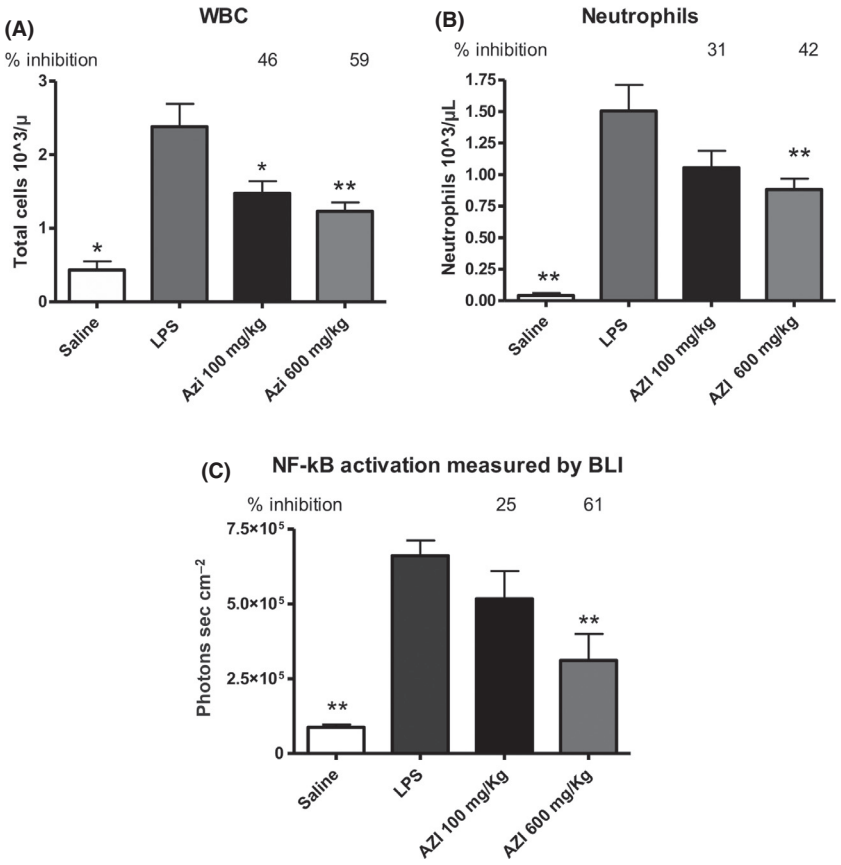

Figure 6. (A and B) LPS-induced WBC (A) and neutrophil (B) recruitment in the airways: effect of azithromycin. Transient NF-κB-luc transgenic mice were pretreated with saline or Azithromycin (AZI, 100–600 mg/kg, *per os)* 4 h prior to LPS, and sacrificed 24 h after LPS administration by tracheal instillation. (C) Quantitation of NF-κB activation by BLI, 4 h after LPS intratracheal instillation: effect of azithromycin. Values are shown as mean ± SEM, $n = 8$ for each group and% inhibition versus LPS group is reported. $P < 0.05$, $^{**}P < 0.01$ versus LPS group (Dunnett's t test).

therefore reduce the number of animals required for experimentation because multiple measurements can be made in the same animal over time, also minimizing the effects of biological variation.

In this study, we confirm the activity of azithromycin against pulmonary inflammation induced by LPS (Ianaro et al. 2000), showing a reduction in WBC and neutrophil airways infiltration and a significant decrease in the concentrations of proinflammatory cytokines in BAL. It must be noted that in this study azithromycin has been used prophylactically to prevent an acute inflammatory response mainly because we have been trying to establish a link between its anti-inflammatory activity and NF-κB activation in vivo. Indeed, taking advantage of BLI on transiently transfected animals, we provide unequivocal evidence that this activity is associated with a significant inhibition of NF-κB activation in vivo. Double immunofluorescence staining with antibodies targeting luciferase protein and specific cell type antigens, showed that in transient NF-κB-luc transgenic mice intratracheal challenge with LPS induces luciferase expression both in epithelial and endothelial cells, suggesting a key role for these cell types in the inflammatory response to bacterial infection. Although no infiltrating inflammatory cells resulted in expressing luciferase, this does not rule out the activation of NF-κB in these cells, but it rather reflects their rapid turnover.

This innovative analytical approach to the in vivo monitoring of pulmonary inflammation through the analysis of bioluminescence in transiently transfected mice, can certainly be used to test different macrolide antibiotics for their effects on NF-κB activation, or, taking advantage of different vectors, to monitor different models of pathology (fibrosis, asthma etc.).

As previously reported, azithromycin was also able to significantly reduce the concentrations of G-CSF within the airways, and this activity may result in a decrease in epithelial cells-dependent neutrophil survival within the airways (Yamasawa et al. 2004), and contribute to the reported effects, among others, on COPD exacerbations (Albert et al. 2011; Yamaya et al. 2012).

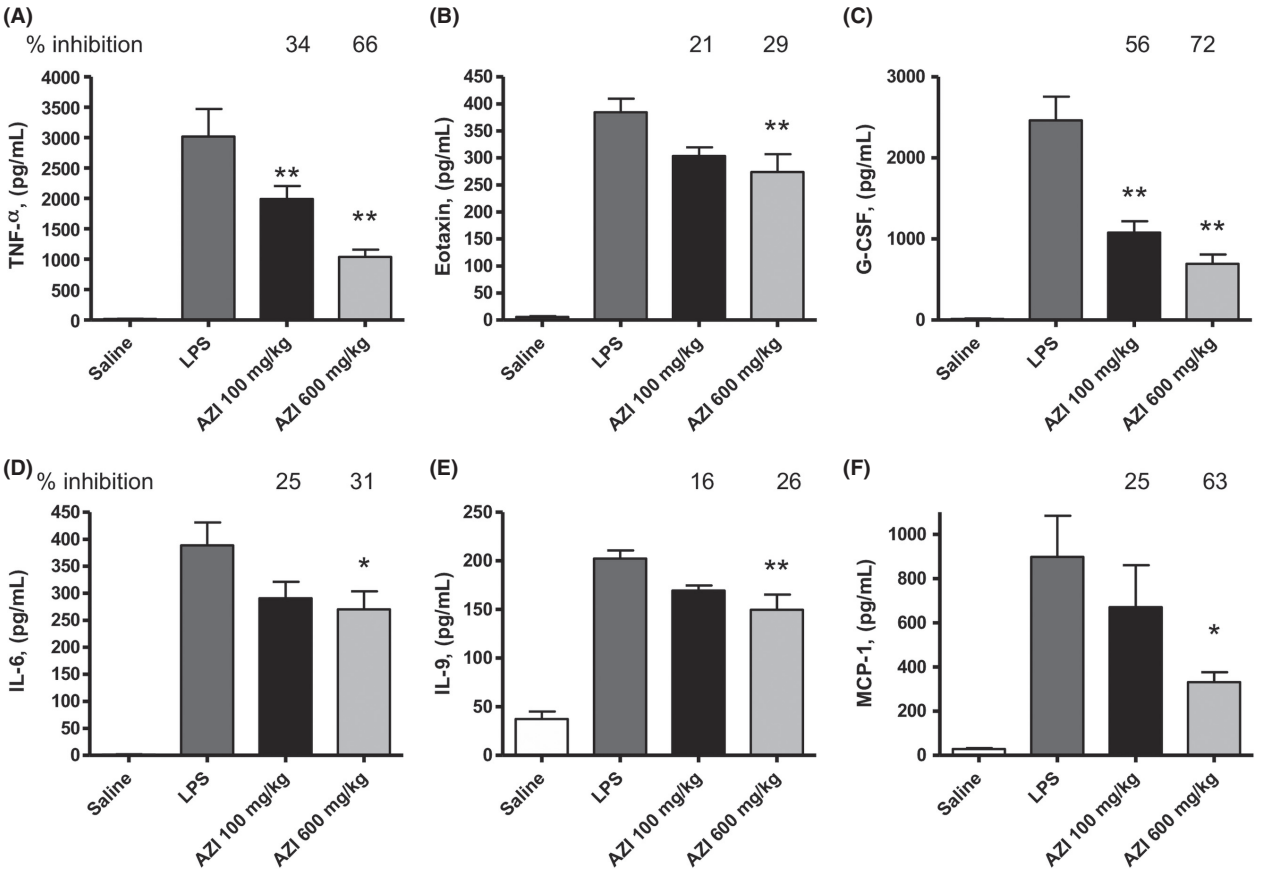

Figure 7. (A–F) LPS-induced cytokines in bronchoalveolar lavage fluid (BALF): effect of azithromycin. Transient NF-κB-luc transgenic mice were pretreated with saline or azithromycin (AZI, 100–600 mg/kg, *per os)* 4 h prior to LPS and sacrificed 24 h after tracheal instillation of LPS. A: TNF-α. B: eotaxin. C: G.CSF. D: IL-6. E: IL-9. F: MCP-1. Values are shown as mean ± SEM, $n = 6$ for each group and% inhibition versus LPS group is reported. $P < 0.05$, $^{**}P < 0.01$ versus LPS group (Dunnett's t test)

Not much is known on the molecular mechanism of the inhibition of NF-κB by azithomycin, but the possibility that the anti-inflammatory activity of macrolide antibiotics such as azythromycin or erythromycin may relate uniquely to their antibiotic activity has been ruled out by the identification of an erythromycin analog devoid of the antibiotic activity but retaining the anti-inflammatory action (Desaki et al. 2004). Conversely, it has been shown that azithromycin inhibits the binding of activator protein-1, nuclear factor of activated T cells, and interferon consensus sequence binding protein to the DNA-binding site in the IL-12p40 promoter (Yamauchi et al. 2009), and down regulation of TLR-4 receptor by azithromycin has also been reported (Iwamoto et al. 2011), and these could represent important mechanisms contributing to the anti-inflammatory effects of azithromycin.

In consideration of the striking correlation of NF-κB activation as assessed by BLI, and downstream airway inflammatory responses, monitoring of BLI in this relatively simple model of transient transgenic mouse may represent a suitable approach for in vivo assessment of NF-κB-associated inflammatory responses, and possess significant potential for the elucidation of molecular mechanisms in the pathobiology of lung diseases.

In conclusion, taking advantage of a transient transgenic mice expressing luciferase under the control of NF-κB-responsive elements, we provide evidence that azithromycin inhibits NF-κB activation in vivo, further supporting the important contribution of this mechanism of action to its anti-inflammatory activity.

Acknowledgments

The Authors would like to thank Fabrizio Facchinetti and Gessica Marchini for their skillful technical assistance.

Disclosures

F. F. Stellari, P. Caruso, T. M. Topini, C. Carnini, M. Civelli, and G. Villetti are employees of Chiesi Farmaceutici

that does not sell any of the drugs mentioned in the article.

K. P. Francis and X. Li are employees of Perkin Elmer, that sells imaging devices.

References

Aghai ZH, Kode A, Saslow JG, Nakhla T, Farhath S, Stahl GE, et al. (2007). Azithromycin suppresses activation of nuclear factor-kappa B and synthesis of pro-inflammatory cytokines in tracheal aspirate cells from premature infants. Pediatr Res 62: 483–488.

Albert RK, Connett J, Bailey WC, Casaburi R, Cooper JA Jr, Criner GJ, et al. (2011). Azithromycin for prevention of exacerbations of COPD. N Engl J Med 365: 689–698.

Alcamo E, Mizgerd JP, Horwitz BH, Bronson R, Beg AA, Scott M, et al. (2001). Targeted mutation of TNF receptor I rescues the RelA-deficient mouse and reveals a critical role for NF-kappa B in leukocyte recruitment. J Immunol 167: 1592–1600.

Ansaldi D, Hod EA, Stellari F, Kim JB, Lim E, Roskey M, et al. (2011). Imaging pulmonary NF-kappaB activation and therapeutic effects of MLN120B and TDZD-8. PLoS ONE 6: e25093.

Bar H, Bea F, Blessing E, Watson L, Wende P, Kreuzer J, et al. (2001). Phosphorylation of cytokeratin 8 and 18 in human vascular smooth muscle cells of atherosclerotic lesions and umbilical cord vessels. Basic Res Cardiol 96: 50–58.

Blackwell TS, Holden EP, Blackwell TR, DeLarco JE, Christman JW (1994). Cytokine-induced neutrophil chemoattractant mediates neutrophilic alveolitis in rats: association with nuclear factor kappa B activation. Am J Respir Cell Mol Biol 11: 464–472.

Blackwell TS, Yull FE, Chen CL, Venkatakrishnan A, Blackwell TR, Hicks DJ, et al. (2000). Multiorgan nuclear factor kappa B activation in a transgenic mouse model of systemic inflammation. Am J Respir Crit Care Med 162: 1095–1101.

Bosnar M, Bosnjak B, Cuzic S, Hrvacic B, Marjanovic N, Glojnaric I, et al. (2009). Azithromycin and clarithromycin inhibit lipopolysaccharide-induced murine pulmonary neutrophilia mainly through effects on macrophage-derived granulocyte-macrophage colony-stimulating factor and interleukin-1beta. J Pharmacol Exp Ther 331: 104–113.

Bosnar M, Cuzic S, Bosnjak B, Nujic K, Ergovic G, Marjanovic N, et al. (2011). Azithromycin inhibits macrophage interleukin-1beta production through inhibition of activator protein-1 in lipopolysaccharide-induced murine pulmonary neutrophilia. Int Immunopharmacol 11: 424–434.

Bours V, Bentires-Alj M, Hellin AC, Viatour P, Robe P, Delhalle S, et al. (2000). Nuclear factor-kappa B, cancer, and apoptosis. Biochem Pharmacol 60: 1085–1089.

Carlsen H, Moskaug JO, Fromm SH, Blomhoff R (2002). In vivo imaging of NF-kappa B activity. J Immunol 168: 1441–1446.

Cigana C, Assael BM, Melotti P (2007). Azithromycin selectively reduces tumor necrosis factor alpha levels in cystic fibrosis airway epithelial cells. Antimicrob Agents Chemother 51: 975–981.

Culic O, Erakovic V, Parnham MJ (2001). Anti-inflammatory effects of macrolide antibiotics. Eur J Pharmacol 429: 209–229.

Dagia NM, Goetz DJ (2003). A proteasome inhibitor reduces concurrent, sequential, and long-term IL-1 beta- and TNF-alpha-induced ECAM expression and adhesion. Am J Physiol Cell Physiol 285: C813–C822.

Desaki M, Okazaki H, Sunazuka T, Omura S, Yamamoto K, Takizawa H (2004). Molecular mechanisms of anti-inflammatory action of erythromycin in human bronchial epithelial cells: possible role in the signaling pathway that regulates nuclear factor-kappaB activation. Antimicrob Agents Chemother 48: 1581–1585.

Donovan CE, Mark DA, He HZ, Liou HC, Kobzik L, Wang Y, et al. (1999). NF-kappa B/Rel transcription factors: c-Rel promotes airway hyperresponsiveness and allergic pulmonary inflammation. J Immunol 163: 6827–6833.

Doyle TC, Burns SM, Contag CH (2004). In vivo bioluminescence imaging for integrated studies of infection. Cell Microbiol 6: 303–317.

Everhart MB, Han W, Sherrill TP, Arutiunov M, Polosukhin VV, Burke JR, et al. (2006). Duration and intensity of NF-kappaB activity determine the severity of endotoxin-induced acute lung injury. J Immunol 176: 4995–5005.

Guide for the Care and Use of Laboratory Animals. 1996. Guide for the care and use of laboratory animals. National Academy Press, Washington, DC.

Ianaro A, Ialenti A, Maffia P, Sautebin L, Rombola L, Carnuccio R, et al. (2000). Anti-inflammatory activity of macrolide antibiotics. J Pharmacol Exp Ther 292: 156–163.

Iwamoto S, Kumamoto T, Azuma E, Hirayama M, Ito M, Amano K, et al. (2011). The effect of azithromycin on the maturation and function of murine bone marrow-derived dendritic cells. Clin Exp Immunol 166: 385–392.

Kalogeris TJ, Laroux FS, Cockrell A, Ichikawa H, Okayama N, Phifer TJ, et al. (1999). Effect of selective proteasome inhibitors on TNF-induced activation of primary and transformed endothelial cells. Am J Physiol 276: C856–C864.

Kanoh S, Rubin BK (2010). Mechanisms of action and clinical application of macrolides as immunomodulatory medications. Clin Microbiol Rev 23: 590–615.

Kielland A, Carlsen H (2010). Molecular imaging of transcriptional regulation during inflammation. J Inflamm (Lond) 7: 20.

Lauzurica P, Martinez-Martinez S, Marazuela M, Gomez del Arco P, Martinez C, Sanchez-Madrid F, et al. (1999). Pyrrolidine dithiocarbamate protects mice from lethal shock induced by LPS or TNF-alpha. Eur J Immunol 29: 1890–1900.

Lawrence T, Gilroy DW, Colville-Nash PR, Willoughby DA (2001). Possible new role for NF-kappaB in the resolution of inflammation. Nat Med 7: 1291–1297.

Malazdrewich C, Ames TR, Abrahamsen MS, Maheswaran SK (2001). Pulmonary expression of tumor necrosis factor alpha, interleukin-1 beta, and interleukin-8 in the acute phase of bovine pneumonic pasteurellosis. Vet Pathol 38: 297–310.

Martinez FJ, Curtis JL, Albert R (2008). Role of macrolide therapy in chronic obstructive pulmonary disease. Int J Chron Obstruct Pulmon Dis 3: 331–350.

Matsumura Y, Mitani A, Suga T, Kamiya Y, Kikuchi T, Tanaka S, et al. (2011). Azithromycin may inhibit interleukin-8 through suppression of Rac1 and a nuclear factor-kappa B pathway in KB cells stimulated with lipopolysaccharide. J Periodontol 82: 1623–1631.

McGrath JC, Drummond GB, McLachlan EM, Kilkenny C, Wainwright CL (2010). Guidelines for reporting experiments involving animals: the ARRIVE guidelines. Br J Pharmacol 160: 1573–1576.

Mukaida N (2003). Pathophysiological roles of interleukin-8/CXCL8 in pulmonary diseases. Am J Physiol Lung Cell Mol Physiol 284: L566–L577.

Murphy DM, Forrest IA, Corris PA, Johnson GE, Small T, Jones D, et al. (2008). Azithromycin attenuates effects of lipopolysaccharide on lung allograft bronchial epithelial cells. J Heart Lung Transplant 27: 1210–1216.

Murugan V, Peck MJ (2009). Signal transduction pathways linking the activation of alveolar macrophages with the recruitment of neutrophils to lungs in chronic obstructive pulmonary disease. Exp Lung Res 35: 439–485.

Nassini R, Pedretti P, Moretto N, Fusi C, Carnini C, Facchinetti F, et al. (2012). Transient receptor potential ankyrin 1 channel localized to non-neuronal airway cells promotes non-neurogenic inflammation. PLoS ONE 7: e42454.

Oh HJ, W. Hwang do, H. Youn, D. D. Lee. (2013). In vivo bioluminescence reporter gene imaging for the activation of neuronal differentiation induced by the neuronal activator neurogenin 1 (Ngn1) in neuronal precursor cells. Eur J Nucl Med Mol Imaging 40: 1607–1617.

Psallidas I, Karabela SP, Moschos C, Sherrill TP, Kollintza A, Magkouta S, et al. (2010). Specific effects of bortezomib against experimental malignant pleural effusion: a preclinical study. Mol Cancer 9: 56.

Read MA, Neish AS, Luscinskas FW, Palombella VJ, Maniatis T, Collins T (1995). The proteasome pathway is required for cytokine-induced endothelial-leukocyte adhesion molecule expression. Immunity 2: 493–506.

Salminen A, Huuskonen J, Ojala J, Kauppinen A, Kaarniranta K, Suuronen T (2008). Activation of innate immunity system during aging: NF-kB signaling is the molecular culprit of inflamm-aging. Ageing Res Rev 7: 83–105.

Sen R, Baltimore D (1986). Inducibility of kappa immunoglobulin enhancer-binding protein Nf-kappa B by a posttranslational mechanism. Cell 47: 921–928.

Teramoto S, Kume H (2001). The role of nuclear factor-kappa B activation in airway inflammation following adenovirus infection and COPD. Chest 119: 1294–1295.

Tornatore L, Thotakura AK, Bennett J, Moretti M, Franzoso G (2012). The nuclear factor kappa B signaling pathway: integrating metabolism with inflammation. Trends Cell Biol 22: 557–566.

Vrancic M, Banjanac M, Nujic K, Bosnar M, Murati T, Munic V, et al. (2012). Azithromycin distinctively modulates classical activation of human monocytes in vitro. Br J Pharmacol 165: 1348–1360.

Wong C, Jayaram L, Karalus N, Eaton T, Tong C, Hockey H, et al. (2012). Azithromycin for prevention of exacerbations in non-cystic fibrosis bronchiectasis (EMBRACE): a randomised, double-blind, placebo-controlled trial. Lancet 380: 660–667.

Wu K, Meyers CA, Bennett JA, King MA, Meyer EM, Hughes JA (2004). Polyethylenimine-mediated NGF gene delivery protects transected septal cholinergic neurons. Brain Res 1008: 284–287.

Yamasawa H, Oshikawa K, Ohno S, Sugiyama Y (2004). Macrolides inhibit epithelial cell-mediated neutrophil survival by modulating granulocyte macrophage colony-stimulating factor release. Am J Respir Cell Mol Biol 30: 569–575.

Yamauchi K, Shibata Y, Kimura T, Abe S, Inoue S, Osaka D, et al. (2009). Azithromycin suppresses interleukin-12p40 expression in lipopolysaccharide and interferon-gamma stimulated macrophages. Int J Biol Sci 5: 667–678.

Yamaya M, Azuma A, Takizawa H, Kadota J, Tamaoki J, Kudoh S (2012). Macrolide effects on the prevention of COPD exacerbations. Eur Respir J 40: 485–494.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Double immunofluorescence staining of mice lungs. Duoplex immunofluorescence staining of luciferase/CK18 (epithelial cell marker) or luciferase/CD31(endothelial cell marker) were performed on paraformaldehyde-fixed lung sections, as described in Materials and Methods.

Double staining of lung obtained from saline-challenged NF- κB-luc mice showed normal staining of epithelial cells (A) and endothelial cells (C) for CK18 and CD31, respectively. On the contrary, no staining for luciferase was observed (B and D). Original magnification: 210×.

Figure S2. (A–E). LPS-induced cytokines in bronchoalveolar lavage fluid (BALF): effect of azithromycin. Transient NF-κB-luc transgenic mice were pretreated with saline or azithromycin (AZI, 100–600 mg/kg, *per os)* 4 h prior to LPS and sacrificed 24 h after tracheal instillation of LPS. A: IL1-α. B: IL1-b. C: IL-12. D: IL-12(p70). E: KC. Values are shown as mean ± SEM, $n = 6$ for each group.

Table S1. Cytokines analyred in BAL of LPS-challenged mice. Cytokines modulated by LPS showed a statistically significant increase in BAL concentrations obtained after LPS challenge.