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Azithromycin Attenuates Airway Inflammation in a Noninfectious Mouse Model of Allergic Asthma

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Background: Definitive conclusions regarding the antiinflammatory effects of macrolide antibiotics for treatment of asthma are difficult to formulate since their beneficial effects may be related to their antimicrobial action. We hypothesized that azithromycin possesses distinct antiinflammatory properties and tested this assumption in a noninfectious mouse model of allergic asthma.

Methods: To induce allergic airway inflammation, 7-week-old BALB/cJ mice underwent intraperitoneal ovalbumin sensitization on days 0 and 7 followed by an intranasal challenge on day 14. Mice were treated with azithromycin or phosphate-buffered saline (PBS) solution on days 13 through 16. On day 17, airway inflammation was assessed by quantifying leukocytes in the airway, expression of multiple inflammatory mediators in the BAL fluid, and mucous cell metaplasia. In a separate set of experiments, azithromycin or PBS solution treatment were initiated after the ovalbumin challenge. Each experiment was repeated 3 times (a total of 9 to 11 mice in each group).

Results: Compared to treatment with PBS solution, azithromycin attenuated the ovalbumin-dependent airway inflammation. We observed a decrease in total leukocytes in the lung tissue and BAL fluid. In addition, azithromycin attenuated the expression of cytokines (eg, interleukin [IL]-13 and IL-5) and chemokines (eg, CCL2, CCL3, and CCL4) in the BAL fluid and abrogated the extent of mucous cell metaplasia. Similar antiinflammatory effects were observed when azithromycin treatment was initiated after the ovalbumin challenge.

Conclusion: In this noninfectious mouse model of allergic asthma, azithromycin attenuated allergic airway inflammation. These findings demonstrate an antiinflammatory effect of azithromycin and suggest azithromycin may have beneficial effects in treating noninfectious airway inflammatory diseases, including asthma. (CHEST 2009; 136:498–506)

Abbreviations: ELISA = enzyme-linked immunosorbent assay; IL = interleukin; MIP = macrophage inhibitory protein; PAS = periodic acid–Schiff; PBS = phosphate-buffered saline

Asthma is a chronic inflammatory disease of the airways that affects > 22 million people in the United States.¹ Despite advanced understanding of the pathophysiology of asthma, affected patients continue to incur significant morbidity from the disease. Accordingly, there is a need for innovative antiinflammatory approaches for asthma and one potential class of medications is the macrolides antibiotics. Macrolides are bacteriostatic antibiotics that reversibly bind to 50S ribosomal subunit of susceptible microorganisms and inhibit protein synthesis. In addition to these antimicrobial effects, recent evidence suggests the macrolides also possess distinct

antiinflammatory properties.² Previous reports^{3–15} indicate that these antibiotics have been used to effectively treat chronic inflammatory airway diseases such as diffuse panbronchiolitis,³ cystic fibrosis,⁴ and asthma.^{5–15} However, it is unclear if the beneficial effects are related to their antimicrobial or antiinflammatory properties since bacteria can often be isolated from the airways in patients with these diseases.^{3,4,16}

Experimental results in infectious and noninfectious mouse airway inflammatory models support a distinct antiinflammatory role for macrolides. In a *Pseudomonas aeruginosa* infection model, azithro-

mycin treatment attenuated airway inflammation, BAL fluid neutrophilia, and expression of inflammatory mediators without altering the bacterial burden in the lungs.¹⁷ Macrolide treatment of mice also reduced the neutrophil dominated airway inflammation triggered by inhaled lipopolysaccharide.^{18,19} In addition, macrolide treatment of rats resulted in the inhibition of airway mucus production.²⁰ To the best of our knowledge, there is no available study that evaluates the effects of azithromycin treatment on allergic airway inflammation in an experimental mouse model of allergic asthma. To determine if azithromycin possessed antiinflammatory properties in a mouse model of allergic inflammation, we tested the ability of azithromycin to alter ovalbumin-dependent airway inflammation in the mouse.

MATERIALS AND METHODS

Induction of Allergic Airway Inflammation

Additional details are included in the online supplement. BALB/cJ mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and bred under specific pathogen-free conditions. Allergic airway inflammation was induced by using an ovalbumin sensitization and a challenge model as previously described.^{21,22} Briefly (Fig 1A), mice were sensitized with intraperitoneal ovalbumin plus alum on days 0 and 7, challenged twice with intranasal ovalbumin on day 14, and harvested on day 17.^{22–24} Mice were treated with subcutaneous phosphate-buffered saline (PBS) solution or azithromycin (50 mg/kg; Pfizer Pharmaceuticals; Dublin, Ireland) on days 13, 14 (1 h before the first ovalbumin challenge), 15, and 16. In a second set of experiments, PBS solution or azithromycin treatment was initiated after the ovalbumin challenge on days 14 (9 h after the first ovalbumin challenge), 15, and 16. This was done to investigate whether azithromycin treatment initiated after the allergen challenge could attenuate allergic airway inflammation. See the online supplement for pharmacokinetic and toxicology studies justifying our azithromycin dosing regimen.

Mouse Specimen Harvest

Serum, BAL fluid, and lung tissue harvest were performed as previously described.^{22–24} Lung sections were stained with hematox-

ylin-eosin and periodic acid–Schiff (PAS).²³ Quantification of mucus producing cells was done by enumerating PAS-positive cells per millimeter of lung basement membrane.²² Quantification of inflammatory cell accumulation in the lungs' parenchyma was assessed as previously described.^{25,26} Multiple inflammatory mediators were analyzed in the cell-free supernatant of BAL fluid by using a multiplex flow-cytometry based assay according to manufacturer's recommendations (Bio-Rad Laboratories; Hercules, CA) and as previously described.²³ To measure eotaxin with a more sensitive assay, an enzyme-linked immunosorbent assay (ELISA) kit with a sensitivity of 3 pg/mL (R&D Systems, Inc; Minneapolis, MN) was used. Serum specific ovalbumin IgE levels were detected by using an ELISA kit with a sensitivity of 3.8 ng/mL (MD Biosciences; St. Paul, MN).

Statistical Analysis

Means from multiple groups were analyzed for statistical significance by using a one-way analysis of variance and a *post hoc* comparison to identify significant differences between specific groups. An independent group's *t* test was used to compare means from two groups. Kruskal-Wallis and Mann-Whitney tests were used to compare the lung inflammatory score. The significance level for all tests was 0.05. Data were analyzed by using a statistical software package (SPSS 15; SPSS, Inc; Chicago, IL).

RESULTS

Azithromycin Attenuated Ovalbumin-Dependent Airway Inflammation

We first set out to determine whether the trachea or lungs from our colony of mice were actively infected or colonized with bacteria. We obtained tracheal swabs and tissue samples from both lungs of naive mice (*n* = 4) and mice that underwent ova sensitization and challenge and were treated with either azithromycin (*n* = 4) or PBS solution (*n* = 4). Bacterial cultures from all these specimens were negative. In addition, there was no serologic evidence of prior *Mycoplasma pulmonis* exposure, suggesting our colony did not have bacterial infection or colonization of the airways.

To determine whether azithromycin could confer antiinflammatory properties in a noninfectious mouse model of allergic inflammation, we sensitized and challenged mice with ovalbumin as previously described.^{21,22} Mice were sensitized to ovalbumin (days 0 and 7), and treated without or with azithromycin (50 mg/kg) for four consecutive days starting 1 day before the ovalbumin challenge (day 14) and ending 1 day before harvest (day 17) [Fig 1A]. As expected, compared with naive mice and mice that received only an ovalbumin challenge (without sensitization), lung histology revealed ovalbumin sensitized and challenged mice (treated with PBS solution only) had more intense inflammation (Fig 1B). In this model, the accumulation of immune cells occurred predominantly in the peribronchial and perivascular regions and to a lesser extent in the alveolar spaces. Compared to treatment with PBS solution, ovalbumin sensitized and challenged mice

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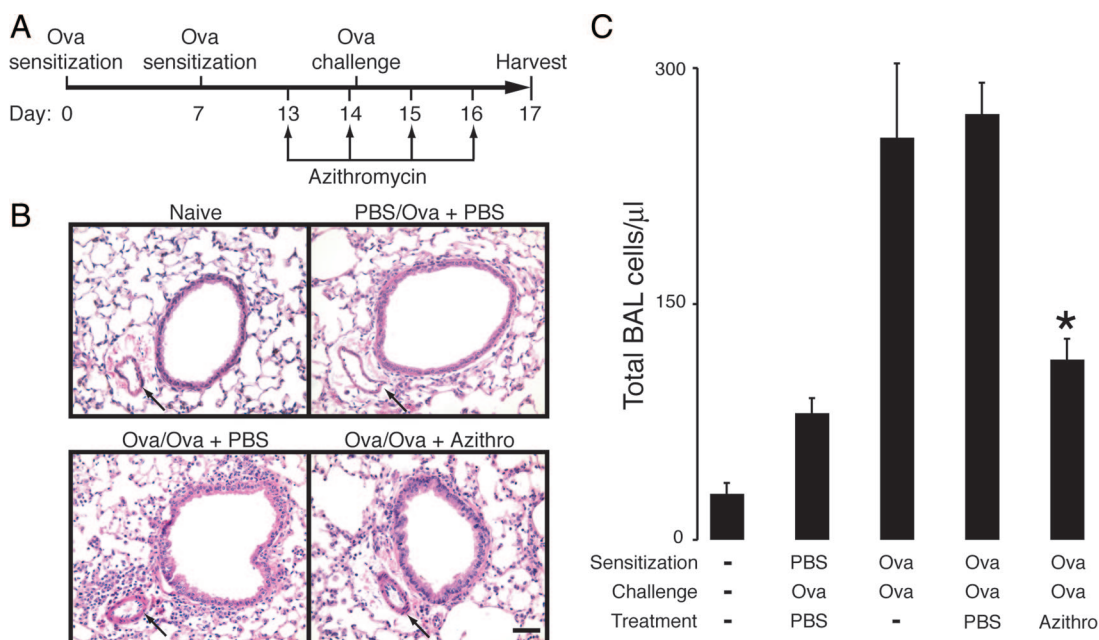


FIGURE 1. Azithromycin (Azithro) attenuated ovalbumin-dependent airway inflammation. **A:** 7-week-old BALB/cJ female mice were sensitized with ovalbumin (ova) on days 0 and 7. On day 14, mice were challenged intranasally with two doses of ova (8 h apart). Three days after the inhaled challenge (day 17), the mice were euthanized, and BAL fluid, lungs, and serum were harvested. Mice were treated with azithromycin on days 13, 14 (1 h before the first ova challenge), 15, and 16. Mice that were sensitized with PBS solution served as controls for the sensitization. Mice that were treated with PBS solution served as a control for the treatment. **B:** On day 17, lung sections were obtained from naive mice (*top left*); mice that were sensitized with PBS solution, challenged with ova, and treated with PBS solution (*top right*); mice that were sensitized and challenged with ova and treated with PBS solution (*bottom left*); and mice that were sensitized and challenged with ova and treated with azithromycin (*bottom right*). Representative photomicrographs of hematoxylin-eosin stained lung sections are shown (n = 9 to n = 11), lung vessels are marked by a black arrow, bar = 50 μm. **C:** BAL fluid was analyzed for total cell number in five groups of mice as follows: naive mice (column 1); mice that were sensitized with PBS solution, challenged with ova and treated with PBS solution (column 2); mice that were sensitized and challenged with ova (column 3); mice that were sensitized and challenged with ova and treated with PBS solution (column 4); and mice that were sensitized and challenged with ova and treated with azithromycin (column 5). Values represent mean ± SEM (n = 9 to n = 11). *Indicates a significant decrease between azithromycin-treated mice vs PBS solution-treated mice (p < 0.05).

treated with azithromycin had attenuation of the accumulation of inflammatory cells in the lung tissue (Fig 1B). Further quantification of this immune cell accumulation using an inflammatory score demonstrated that azithromycin treatment significantly attenuated accumulation of immune cells in the lung (see Fig 1A in the online supplement). In agreement with the histologic appearance of the lung tissue, azithromycin treatment attenuated the accumulation of total BAL fluid immune cells (Fig 1C, column 4 vs 5). Analysis of the BAL fluid leukocyte populations demonstrated that azithromycin treatment significantly attenuated the accumulation of eosinophils, macrophages, lymphocytes, and neutrophils, with the largest fold-reduction in eosinophils (Fig 2A–D). Taken together, we concluded that azithromycin attenuated airway inflammation in this mouse model of allergic inflammation.

Azithromycin Attenuated Ovalbumin-Dependent Airway Inflammation Is Independent of Ovalbumin-Specific IgE Production

We next quantified the serum concentration of ovalbumin-specific IgE to confirm equal allergen

sensitization in all cohorts of mice and to exclude the possibility that azithromycin attenuated the allergic inflammation by altering IgE production. Naive mice and mice that received only an ovalbumin challenge (without sensitization) produced no ovalbumin-specific IgE. Mice that were sensitized and challenged with ovalbumin had a significant increase in ovalbumin-specific IgE production (699.8 ± 178.2 ng/mL) that was not significantly changed by treatment with PBS solution (885.1 ± 193.8 ng/mL) or azithromycin (829 ± 231.1 ng/mL; p = 0.80).

Azithromycin Attenuated Ovalbumin-Dependent Airway Inflammation Is Associated With Decreased Concentrations of BAL Fluid Inflammatory Mediators

Based on the affect of azithromycin on inflammatory cell influx, we proposed that azithromycin-dependent attenuation of allergic airway inflammation would also be associated with decreased concentration of BAL fluid cytokines and chemokines. Compared to ovalbumin sensitized and challenged mice treated with PBS solution, treatment with azithromycin attenuated the expression of multiple BAL fluid cytokines, chemokines,

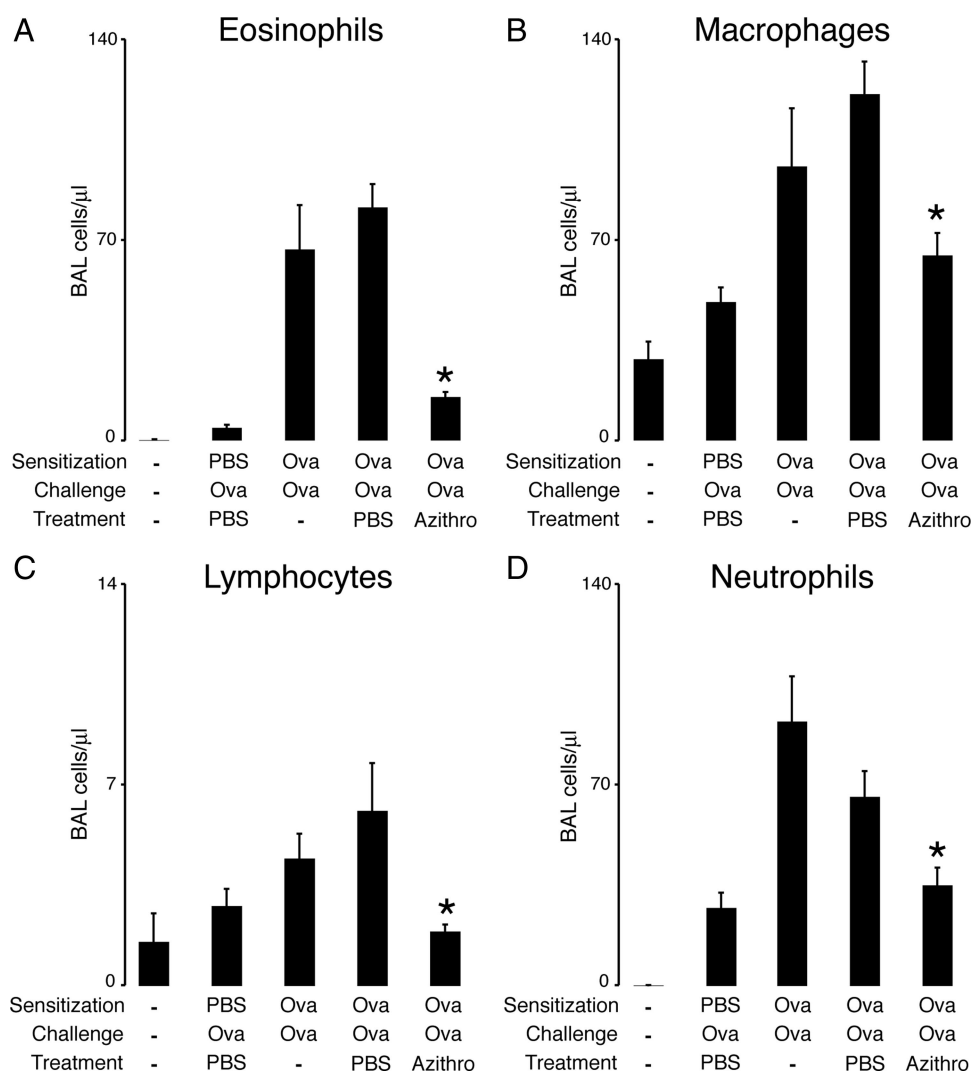


FIGURE 2. Azithromycin attenuated ovalbumin-dependent BAL accumulations of eosinophils, macrophages, lymphocytes, and neutrophils. BAL fluid was analyzed for differential cell number in five groups of mice as follows: naive mice (column 1); mice that were sensitized with PBS solution, challenged with ova, and treated with PBS solution (column 2); mice that were sensitized and challenged with ova (column 3); mice that were sensitized and challenged with ova and treated with PBS solution (column 4); and mice that were sensitized and challenged with ova and treated with azithromycin (column 5). Values represent mean $\pm$ SEM ($n = 9$ to $n = 11$) of eosinophils (A), macrophages (B), lymphocytes (C), and neutrophils (D). *Indicates a significant decrease between azithromycin-treated mice vs PBS solution-treated mice ($p < 0.05$). See Figure 1 for expansion of abbreviations.

and growth factors as measured by multiplex flow cytometry based assay (Fig 3A–F, column 4 vs 5). Importantly, we observed a statistically significant azithromycin-dependent decrease in interleukin (IL)-13 and IL-5, and a trend toward a decrease in IL-4, proteins known to mediate allergic airway inflammatory phenotypes in the airway (eg, mucous cell metaplasia and eosinophilic inflammation). In addition, azithromycin attenuated the expression of multiple other chemokines and inflammatory mediators (CCL2/JE, CCL3/macrophage inhibitory protein [MIP]-1 α , CCL4/MIP-1 β , CXCL1/KC, IL-1 α , IL-10, and granulocyte-macrophage colony-stimulating factor), but had no effect on concentration of IL-6, IL-9,

IL-1 β , IL-12 p40, IL-17, granulocyte colony-stimulating factor, or CCL5/regulated on activation, normal T-cell expressed and secreted (RANTES) [data not shown]. The levels of IL-2, IL-3, IL-12 p70, interferon- γ , tumor necrosis factor- α , and eotaxin were below levels of detection. Since high concentrations of mouse BAL fluid eotaxin were previously identified in the ovalbumin model of allergic asthma,²⁷ we measured BAL fluid eotaxin concentrations by using a more sensitive ELISA assay. Compared with naive mice, mice that underwent the ovalbumin challenge and sensitization followed by PBS solution treatment resulted in higher eotaxin BAL fluid concentrations

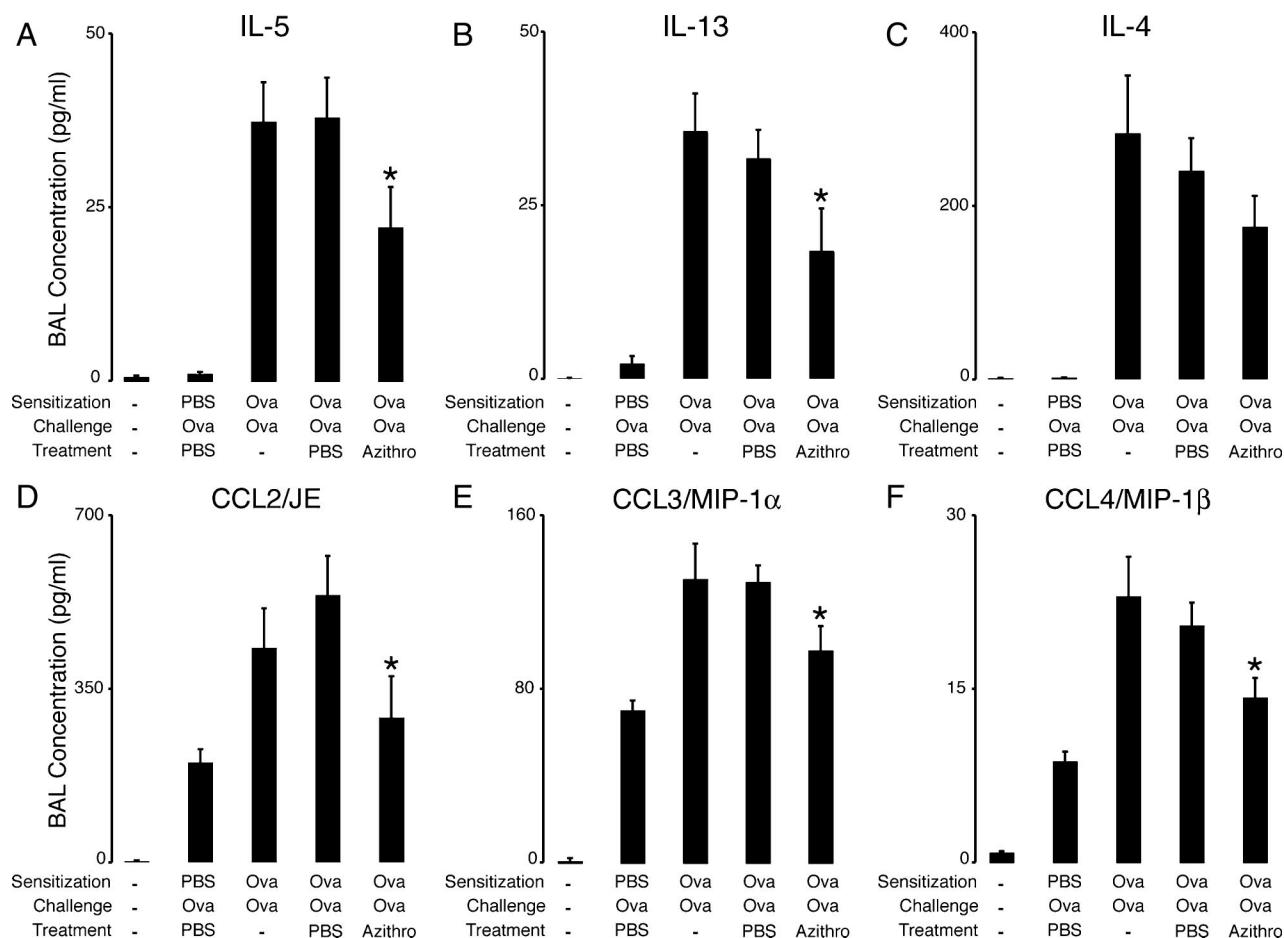


FIGURE 3. Azithromycin attenuated ovalbumin-dependent airway inflammation is associated with decreased concentrations of BAL inflammatory mediators. Chemokines and cytokines concentrations in cell-free BAL supernatant were determined by using a multiplex flow-cytometry based assay (Bio-Rad Laboratories) in five groups of mice as follows: naive mice (column 1); mice that were sensitized with PBS solution, challenged with ova, and treated with PBS solution (column 2); mice that were sensitized and challenged with ova (column 3); mice that were sensitized and challenged with ova and treated with PBS solution (column 4); and mice that were sensitized and challenged with ova and treated with azithromycin (column 5). Values represent mean $\pm$ SEM ($n = 9$ to $n = 11$) of IL-5 (A), IL-13 (B), IL-4 (C), CCL2/JE (D), CCL3/MIP-1 α (E), and CCL4/MIP-1 β (F). *Indicates a significant decrease between azithromycin-treated mice vs PBS solution-treated mice ($p < 0.05$). See Figure 1 for expansion of abbreviations.

(0.5 ± 0.1 pg/mL vs 23.1 ± 5.6 pg/mL, respectively; $p < 0.01$), but azithromycin treatment in mice had no significant effect on eotaxin BAL fluid concentration (20.4 ± 9.9 pg/mL; $p = 0.35$). Collectively, these data demonstrate that azithromycin attenuated ovalbumin-dependent expression of BAL fluid inflammatory mediators, several of which are critical for development of an allergic inflammatory response.

Azithromycin Attenuated Ovalbumin-Dependent Mucus Cell Metaplasia

Since azithromycin treatment attenuated expression of IL-13, a known mediator of mucous cell metaplasia, we proposed that azithromycin would also decrease the number of mucous cells in the airways. Mucus-containing cells in the lung identified with PAS staining were rarely present in naive mice or in mice that were sensitized with PBS solution, challenged with ovalbu-

min, and treated with PBS solution (Fig 4A: Naive and PBS/Ova + PBS). In contrast, numerous PAS-positive cells were found in mice that were sensitized with PBS solution (Fig 4A: Ova/Ova + PBS). Azithromycin treatment decreased the number of PAS-positive cells (Fig 4A: Ova/Ova + Azithro). Further quantification of the ovalbumin-dependent mucous cell metaplasia demonstrated that azithromycin significantly decreased the number of PAS-positive cells per millimeter of basement membrane, indicating azithromycin treatment significantly attenuated ovalbumin-dependent mucous cell metaplasia (Fig 4B, column 4 vs 5).

Azithromycin as a Postchallenge Treatment Attenuated Airway Inflammation

In the previous experiments, we showed that azithromycin treatment initiated before the ovalbu-

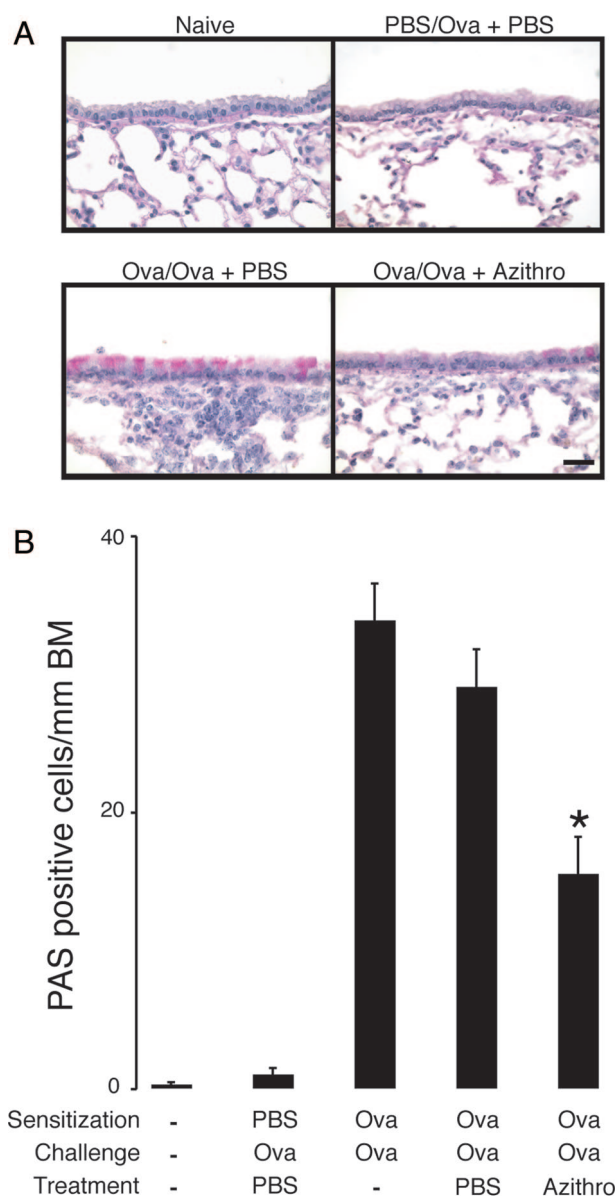


FIGURE 4. Azithromycin attenuated ovalbumin-dependent mucus cell metaplasia. **A:** lung sections were obtained from naive mice (*top left*); mice that were sensitized with PBS solution, challenged with ova, and treated with PBS solution (*top right*); mice that were sensitized and challenged with ova and treated with PBS solution (*bottom left*); and mice that were sensitized and challenged with ova and treated with azithromycin (*bottom right*). Representative photomicrographs of PAS-stained sections are shown ($n = 9$ to $n = 11$), bar = $25\ \mu\text{m}$. **B:** quantification of PAS-positive cells, represented by the number of PAS-positive cells per 1 mm of lung basement membrane (BM), in five groups of mice as follows: naive mice (column 1); mice that were sensitized with PBS solution, challenged with ova, and treated with PBS solution (column 2); mice that were sensitized and challenged with ova (column 3); mice that were sensitized and challenged with ova and treated with PBS solution (column 4); and mice that were sensitized and challenged with ova and treated with azithromycin (column 5). Values represent mean $\pm$ SEM ($n = 9$ to $n = 11$). *Indicates a significant decrease between azithromycin-treated mice vs PBS solution-treated mice ($p < 0.05$). See Figure 1 for expansion of abbreviations.

min challenge attenuated inflammation. Next, we evaluated if azithromycin delivered after an allergen challenge could also attenuate this inflammation. Accordingly, mice were sensitized and challenged with ovalbumin as in the previous experiments but treated with or without azithromycin beginning 9 h after the first challenge (1 h after the second challenge) and then on days 15 and 16 (Fig 5A). In ovalbumin sensitized and challenged mice, postchallenge azithromycin treatment compared to PBS solution treatment, reduced the number of inflammatory cells in the lung tissue (see Fig 1B in the online supplement). To quantify the accumulation of inflammatory cells in the airway, we performed BAL in these cohorts of mice. In agreement with the observations made from the lung tissue, postchallenge azithromycin treatment attenuated the accumulation of total BAL fluid immune cells (Fig 5B). Moreover, it significantly attenuated the accumulation of eosinophils, BAL fluid concentrations of IL-13 and IL-5, as well as mucous cell metaplasia (Fig 5C–E). Taken together, we concluded that azithromycin treatment initiated after the allergen challenge attenuated airway inflammation in this noninfectious mouse model of allergic inflammation.

DISCUSSION

Our study demonstrated that azithromycin has anti-inflammatory properties in a noninfectious mouse model of allergic airway inflammation. Importantly, the drug modulated many of the key allergen-associated inflammatory responses of the ovalbumin sensitization and challenge model including accumulation of eosinophils, secretion of IL-5 and IL-13, and mucus cell hyperplasia. These same antiinflammatory effects of azithromycin were noted when the mice were treated before and throughout the allergen challenge, and, although to a lesser extent, when azithromycin treatment was initiated after the challenge. This study is the first, to our knowledge, to demonstrate the beneficial effects of azithromycin in a noninfectious mouse model of allergic airway inflammation and further extends the rationale for the evaluation of this drug in other experimental models, including human studies.

In our model, azithromycin treatment attenuated the cellular influx and accumulation of lung neutrophils, a finding that is consistent with previous reports^{17–19} in nonallergic inflammatory models, in which, macrolides attenuated neutrophilic inflammation induced by inhaled lipopolysaccharide,^{18,19} or intratracheal *P. aeruginosa* infection.¹⁷ The effect of the macrolides does not appear to affect only one inflammatory cell since in our allergic inflammatory model azithromycin also attenuated the accumulation of eosinophils, macrophages, and lymphocytes.

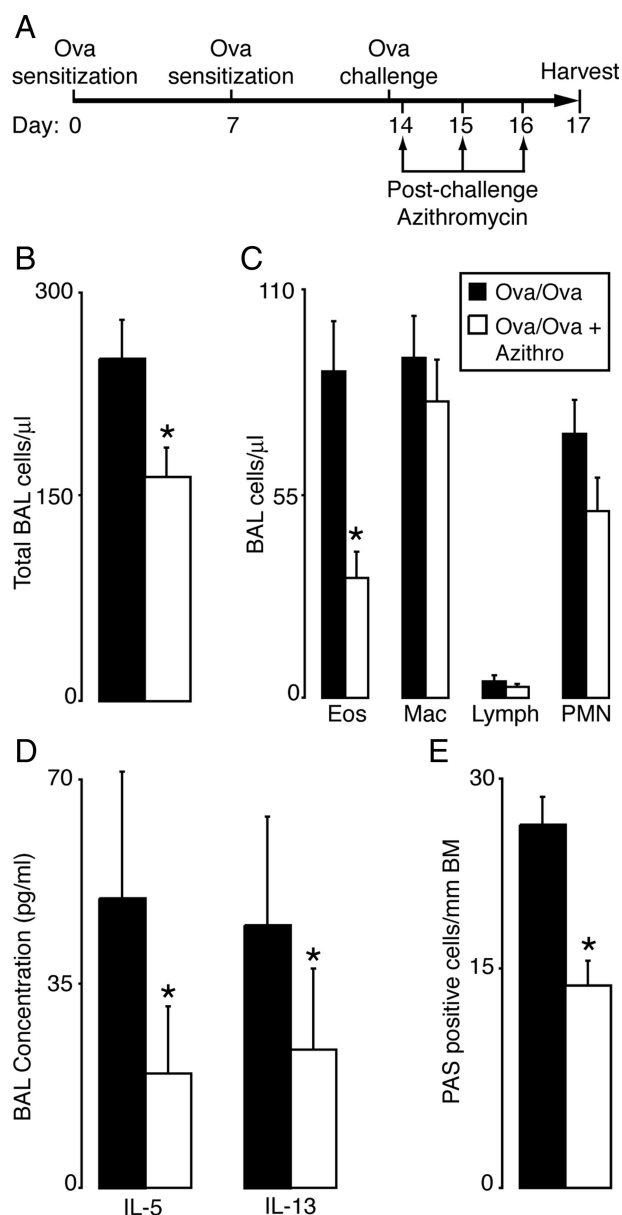


FIGURE 5. Azithromycin as a postchallenge treatment attenuated airway inflammation. **A:** 7-week-old BALB/cJ female mice were sensitized with ova on days 0 and 7. On day 14, mice were challenged intranasally with two doses of ova (8 h apart). Three days after the inhaled challenge (day 17), the mice were euthanized and BAL fluid, lungs, and serum were harvested. Mice were untreated or treated with azithromycin on days 14 (1 h after the second ova challenge), 15, and 16. **B–D:** BAL fluid analyses from mice that were not (white bars) or were treated with azithromycin (black bars). Values represent mean $\pm$ SEM ($n = 9$ to $n = 11$) of total cell number (**B**), differential cell number (**C**), concentrations of the cytokines IL-5 and IL-13 (**D**), and PAS-positive cells per 1 mm of lung BM (**E**). *Indicates a significant decrease between azithromycin-treated mice vs PBS solution-treated mice ($p < 0.05$). Eos = eosinophils; Mac = macrophages; Lymph = lymphocytes; PMN = polymorphonuclear neutrophils. See Figure 1 for expansion of abbreviations.

Accordingly, the current observations broaden azithromycin's antiinflammatory effects to airway conditions associated with neutrophils as well as all other leukocyte populations.

Although the precise biochemical mechanisms responsible for the antiinflammatory effects of macrolides are not defined, this family of drugs can inhibit multiple cellular processes involved in an inflammatory response. For example, macrolides have been shown to inhibit neutrophil chemotaxis, leukocyte-epithelial cell adhesion, cytokine secretion, and cytokine-dependent intracellular signaling. Tsai et al¹⁷ demonstrated that azithromycin has a direct inhibitory effect on neutrophil chemotaxis that was mediated by decreasing the chemokine-dependent activation of the ERK-1/2 MAPK signaling pathways. In addition, roxithromycin pretreatment of human neutrophils inhibited *in vitro* adhesion to human bronchial epithelial cells, and this was associated with a reduction of intercellular adhesion molecule-1 expression on the epithelial cells.²⁸ Another mechanism accounting for the decreased neutrophil influx may be related to our finding that azithromycin inhibited secretion of the neutrophil chemoattractant KC (the mouse homolog to human IL-8) in the BAL fluid. Other data show that macrolides may exert their antiinflammatory effects by blocking nuclear factor- κ B- and/or AP-1-dependent gene transcription of inflammatory mediators.^{19,29,30} Another possible explanation for our observations is that azithromycin may have interacted with the alum adjuvant to attenuate the ovalbumin-dependent inflammation. Kool et al³¹ have demonstrated in an ovalbumin sensitization and challenge peritonitis model, that alum enhanced dendritic cell antigen presentation, and this resulted in a more robust humoral immune response. Although we demonstrated that azithromycin did not alter ovalbumin-specific IgE, it remains possible that azithromycin may have altered other components of the humoral immune response. Additional studies will be required to further define the precise cellular mechanisms responsible for the antiinflammatory effects of macrolides in our allergic inflammatory model.

Numerous studies^{5–15} have demonstrated a beneficial effect of macrolides in treating asthma, an airway inflammatory disease associated with a variety of allergic responses.¹ In our experiments, azithromycin treatment resulted in reduced counts of all BAL fluid inflammatory cells, and the most profound effect was noted on the attenuation of eosinophils. This attenuation of eosinophil accumulation may be related to macrolide-dependent inhibition of the expression of IL-5 (an eosinophil survival factor), or the ability of macrolides to block the IL-5 intracellular signaling cascade.²⁹ Other groups of asthmatics have a mixed neutrophilic and eosinophilic inflammatory component, that may also be modulated by azithromycin, as evident by a previous report³² in which macrolides reduced secretion of the neutro-

phil chemoattractant IL-8 from eosinophils, obtained from atopic individuals.

The use of macrolides for asthma must be put in context of the apparent beneficial affects also observed in complex lung diseases with features of chronic inflammation plus infection such as cystic fibrosis and diffuse panbronchiolitis. It is difficult to determine if this improvement is related to a distinct antiinflammatory property because concurrent bacterial infection or colonization may be present, which has also been implicated in asthma.^{3,4,16} However, a previous trial¹² evaluated the role of clarithromycin in asthma patients that had no evidence of airway colonization with either *Mycoplasma pneumoniae* or *Chlamydia pneumoniae*. In this cohort, clarithromycin treatment decreased BAL fluid IL-12 and tumor necrosis factor- α expression but did not improve in FEV₁. In the future, separating the microbial and nonmicrobial properties of the macrolides would assist in determining if the nonmicrobial properties of macrolides could produce the desired antiinflammatory effects in subjects with asthma.

In summary, our results demonstrated that azithromycin possesses antiinflammatory properties in an *in vivo* noninfectious model of allergic airway inflammation. These observations suggest azithromycin may be beneficial in the treatment of inflammatory conditions, such as asthma, and support the rationale for future prospective randomized clinical trials.

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Author contributions: Dr. Beigelman: study design, performed the experiments, statistical analysis, interpretation of data, and article writing. Mr. Gunsten and Ms. Mikols: performed the experiments, revision of the article, and provided final approval of the article. Drs. Cannon and Brody: study design, revision of the article, and provided final approval of the article. Dr. Vidavsky: designed and performed the azithromycin pharmacokinetic studies, revision of the article, and provided final approval of the article. Dr. Walter: study design, performed the experiments, statistical analysis, interpretation of data, and article writing.

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Azithromycin Attenuates Airway Inflammation in a Noninfectious Mouse Model of Allergic Asthma

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Materials and Methods

Mice

BALB/cJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were bred and housed under specific pathogen-free conditions at Washington University School of Medicine where sentinel mice (pathogen free ICN-strain) exhibited no serologic or histologic evidence of exposure to 15 murine pathogens (including *Mycoplasma pulmonis*). To determine if our colony of mice were actively infected or colonized with bacteria in the trachea or lungs, we obtained tracheal swabs and tissue samples from both lungs and plated them on tryptic soy agar with 5% sheep blood and incubated for 72 hours at 37 °C. To determine if our colony of mice had serologic evidence of prior *Mycoplasma pulmonis* exposure, serum was collected for indirect ELISA using *Mycoplasma pulmonis* antigen-coated plates according to manufacturer's recommendations (Charles River Laboratories, Wilmington, MA). The Institutional Animal Use and Care Committee of Washington University School of Medicine approved all animal experiments. Each experiment was repeated 3 times with a total of 9-11 mice in each group.

Induction of allergic airway inflammation

Allergic airway inflammation was induced using an ovalbumin sensitization and challenge model as previously described.^{1,2} Briefly, 7-week-old female BALB/cJ mice were sensitized to intraperitoneal ovalbumin on days 0 and 7 with 8.0 micrograms of

24 ovalbumin (Sigma-Aldrich, catalogue #A5503, St. Louis, MO) adsorbed to 1.0 mg of
25 alum (Sigma-Aldrich, catalogue #A8222, St. Louis, MO) in 100 µl of sterile PBS. Alum
26 was used in combination with ova in order to boost the immune response as previously
27 reported.³ On the morning of day 14, mice were anesthetized with ketamine (80 mg/kg)
28 and xylazine (16 mg/kg) and challenged intranasally with 0.4 mg ovalbumin (50 µl of 2%
29 ovalbumin in sterile PBS). This same challenge was repeated 8 hours later. Mice that
30 were injected with 100 µl PBS (IP) on day 0 and day 7, served as a negative control for
31 the sensitization. On day 17, three days following inhaled challenge, the mice were
32 anesthetized, euthanized, and bronchoalveolar lavage (BAL) fluid, lungs tissue and serum
33 were collected for analyses as previously described.^{2,4,5}

35 Azithromycin treatment

36 Mice were treated with subcutaneous azithromycin (50 mg/kg, purchased from Pfizer
37 Pharmaceuticals, Ireland) dissolved in 100 µL sterile PBS, on days 13, 14 (one hour prior
38 to first OVA challenge), 15 and 16. Mice that were treated with subcutaneous sterile PBS
39 served as a control for the azithromycin treatment. In a second set of experiments
40 azithromycin treatment was initiated after ovalbumin challenge on days 14 (nine hours
41 after the first ovalbumin challenge), 15 and 16.

43 Mouse specimen harvest

44 Serum, BAL and lung tissue harvest were performed as previously described.^{2,4,5} Briefly,
45 mice underwent intraperitoneal injection with ketamine (80 mg/kg) and xylazine (16
46 mg/kg) followed by anterior neck dissection. Whole blood was collected by cardiac

puncture using a 25-gauge needle and syringe without heparin. Serum was isolated from whole blood using a serum separator tube according to manufacturer's recommendation (BD Biosciences, #365956). For BAL and lung fixation, the trachea was cannulated with a 22-gauge angiocatheter and lungs lavaged with 1 ml of PBS for BAL following by fixation with Histochoice (25-cm water pressure). Lung sections were stained with hematoxylin and eosin (H&E) and Periodic Acid–Schiff (PAS) according to standard protocols. Quantification of mucus producing cells was done by enumerating PAS positive cells per one millimeter of lung basement membrane as previously described.² BAL fluid was centrifuged and the cell-free supernatant was collected and stored at -20 °C for cytokine analysis, while the cell pellet was resuspended in 1 ml of PBS for total cell count, cytospin preparation, and Wright-Giemsa staining. Two blinded observers determined immune cell differential using standard light microscopy criteria as described previously.⁴

BAL cytokines and chemokines assays:

To quantify multiple inflammatory mediators in the BAL, we analyzed cell-free supernatant in a multiplex flow-cytometry based assay according to manufacturer's recommendations (Bio-Rad Laboratories) and as previously described.⁴ Unique beads, each conjugated with a distinct capture antibody, were incubated with 50 µl of BAL and 0.5% BSA or a serially diluted standard mix with a known concentration of all measured inflammatory mediators. The beads were then washed and sequentially incubated with a detection antibody mixture that contained cytokine specific biotin-conjugated antibodies and then streptavidin-phycoerythrin. The phycoerythrin intensity in each of the unique

bead regions from the serially diluted standards was used to generate standard curves to calculate the concentration of each inflammatory mediator in the BAL samples. The detection limit for the Bio-plex mouse cytokine 23-plex panel (Bio-Rad) is: IL-1 α – 2 pg/ml; IL-1 β – 2 pg/ml; IL-2 – 3 pg/ml; IL-3 – 2 pg/ml; IL-4 – 3 pg/ml; IL-5 – 2 pg/ml; IL-6 – 2 pg/ml; IL-9 – 15 pg/ml; IL-10 – 2 pg/ml; IL-12 (p40) – 2 pg/ml; IL-12 (p70) – 4 pg/ml; IL-13 – 9 pg/ml; IL-17 – 1 pg/ml; eotaxin – 148 pg/ml; G-CSF – 1 pg/ml; GM-CSF – 7 pg/ml; IFN- γ – 6 pg/ml; KC – 3 pg/ml; CCL2/JE – 14 pg/ml; CCL3/MIP-1 α – 24 pg/ml; CCL4/MIP-1 β – 2 pg/ml; CCL5/RANTES – 5 pg/ml; and TNF- α – 6 pg/ml. To measure eotaxin with a more sensitive assay, an ELISA kit with a sensitivity of 3 pg/ml (R&D Systems, Inc.) was used.

Quantification of inflammatory cell accumulation in the lungs:

The severity of peribronchial inflammatory cell infiltration was determined by two observers in a blinded fashion based on a 5-point scoring system as previously described.^{6,7} Ten airways per animal were scored according to the extent of peribronchial inflammation: 0, no cells; 1, a few cells; 2, a ring of cells 1 cell layer deep; 3, a ring of cells 2–4 cells deep; 4, a ring of cells > 4 cells deep.

Serum and blood analyses:

Peripheral blood leukocyte counts were performed using an automated veterinary hematologic analyzer with a pre-programmed murine calibration mode (Hemavet 950FS, Drew Scientific) as previously described.⁴ Alanine transferase (ALT), alkaline phosphatase, and creatinine measurements were performed using an automated dry

reagent chemistry analyzer (Vitros DT60 Chemistry System, Ortho Clinical Diagnostics, Johnson & Johnson).

Serum specific ovalbumin IgE levels were detected in duplicate using a commercial available ELISA kit with a sensitivity of 3.8 ng/mL according to the manufacturer's recommendation (MD Biosciences, MN USA).

Serum azithromycin levels were quantified using liquid chromatography and mass spectrometry with the Mass Spectrometry Orbitrap XL analyzer (ThermoFisher, San Jose) according to manufacture's recommendation and as previously described.⁸ Briefly, Azithromycin was identified in mouse serum by LC-HRMS (Liquid Chromatography – High Resolution Mass Spectrometry) and LC-MS/MS (Liquid Chromatography – Mass Spectrometry / Mass Spectrometry) detection using modified extraction and chromatographic methods previously described.⁸ The LC-MS system utilized a 0.075 x 150 mm self packed column with Magic C18, 5 μ , 120 Å (Michrom Bioresources, Inc., Auburn, CA) packing material. The mobile phase consisted of solvent A 0.1% formic acid in water, Solvent B 0.1% formic acid in Acetonitrile. After injection of 1 μ l the concentration of solvent B was held at 0% until 2 minutes and then raised to 60% in 35 minutes linearly. The flow rate was 260 η l/min. The detector mass Spectrometer was Orbitrap XL (ThermoFisher, San Jose). The MS scan was operated at the range of m/z 350-900, 100,000 resolving power followed by MS/MS scan of ion m/z 375.2 [M+2H]⁺⁺, 1.2 Da isolation, 30% wideband activation, scan range m/z 100-770, 60,000 resolving power. The spray voltage was 1.4 KV, no sheath gas was used. Capillary temperature was

200°C, capillary voltage was 7V, tube lens voltage was 35V. The extraction method for serum samples was: using 50 µl of sample serum, add 100 µl 0.06 M K₂CO₃ and vortex. Next, add 1 mL of HPLC grade methyl t-butyl ether (MTBE) and vortex 45 sec. Centrifuge for 10 min at 14,000 g transfer 0.9 mL of MTBE phase into clean centrifuge tubes. The MTBE was then evaporated in vacuum. The sample was reconstituted in 50 µl mobile phase A and 1 µl injected onto LC-MS system.

Statistical analysis

For continuous variables, the means from multiple groups were analyzed for statistical significance using a one-way analysis of variance (ANOVA) for a factorial experimental design. We employed one-way ANOVA analysis, and not multiple t-test comparisons, in order to avoid type I error inflation. In this analysis, each experimental condition was grouped, and the ANOVA analysis was used to determine if overall differences existed between the means of all the experimental conditions. If significant differences between the means of all the experimental conditions was achieved, we then performed post-hoc comparisons using least squares difference procedure to identify significant differences between two specific experimental conditions. The significance level for the overall comparison and the post hoc analysis corresponded to a p value of less than 0.05. For continuous variables, the means from two groups (post-challenge treatment with PBS versus azithromycin) were analyzed for statistical difference using an independent groups *t* test (or Student's *t* test). The significance level for the *t* test corresponded to a two-tailed value of 0.05. For the inflammatory score (an ordinal variable), the Kruskal-Wallis test was used to determine statistical differences between multiple groups and the Mann-

Whitney tests was used to determine significant differences between two groups. Significance was defined as a p value less than 0.05. Data was analyzed using SPSS 15 software.

Results

Pharmacokinetic and toxicology studies for azithromycin dosing regimen

To determine if azithromycin possessed anti-inflammatory properties in a non-infectious mouse model of allergic inflammation, we first set out to determine a dosing regimen in mice that would produce serum levels similar to those observed in humans treated with the recommended azithromycin dose. Overall, a higher dosage of azithromycin is required in mice than humans. This is due to faster liver metabolism in mice, resulting in an elimination half-life of 2.3 hours compared to 68 hours in humans.^{9 10} We found that a subcutaneous dose of azithromycin at 50 mg/kg every 24 hours for 4 days resulted in a trough serum level of 0.11 ± 0.08 $\mu\text{g/ml}$, similar to that reported in humans following the recommended intravenous dose of 500 mg/day for 3-5 days (0.2 ± 0.15 $\mu\text{g/ml}$)¹¹. Using this dosing regimen in mice we observed no weight loss, leucopenia, thrombocytopenia, anemia, transaminitis, or elevation in serum creatinine (data not shown).

Figure legend

Supplement Figure 1S. **Azithromycin attenuated ovalbumin-dependent airway inflammation.** In **A:** Seven-week-old BALB/cJ female mice were sensitized with ovalbumin (Ova) or PBS on days 0 and day 7. On day 14, mice were challenged intranasally with 2 doses of Ova (8 hours apart). Mice were treated with PBS or azithromycin on days 13, 14 (one hour prior to first Ova challenge), 15 and 16. On day

17, lung sections from naive mice (column 1), mice that were sensitized with PBS, challenged with Ova and treated with PBS (column 2), mice that were sensitized and challenged with Ova and treated with PBS (column 3), and mice that were sensitized and challenged with Ova and treated with azithromycin (column 4) were scored for peribronchial inflammation. Inflammation score was determined in a blinded fashion based on a 5-point scoring system as previously described.^{6,7} Briefly, the scoring system was: 0, no cells; 1, a few cells; 2, a ring of cells 1 cell layer deep; 3, a ring of cells 2–4 cells deep; 4, a ring of cells > 4 cells deep. Values represent mean $\pm$ SEM (n=10-11). * Indicates a significant decrease between azithromycin treated mice versus PBS treated mice (p<0.05). In **B**: BALB/cJ female mice were sensitized and challenged with Ova as described above. Mice were untreated or treated with azithromycin post-challenge on days 14 (one hour after second Ova challenge), 15 and 16. On day 17, lung sections from mice that were sensitized and challenged with Ova and untreated (column 1) and mice that were sensitized and challenged with Ova and treated with azithromycin post-challenge (column 2) were scored for peribronchial inflammation. Values represent mean $\pm$ SEM (n=8-10). * Indicates a significant decrease between azithromycin treated mice vs. untreated mice (p<0.05).

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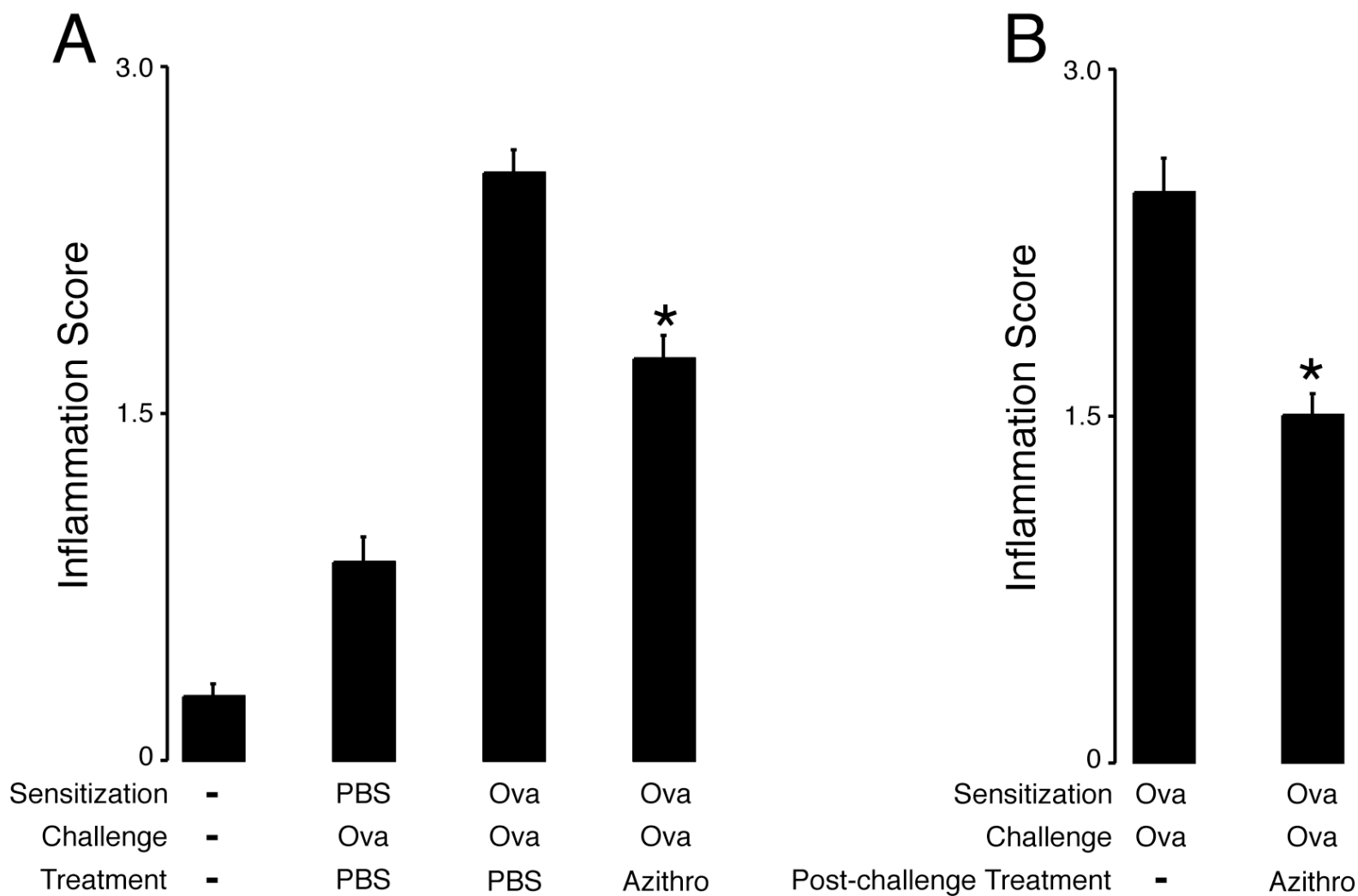


Figure 1S. Beigelman, et al.