Minocycline Blocks Asthma-associated Inflammation in Part by Interfering with the T Cell Receptor-Nuclear Factor κB-GATA-3-IL-4 Axis without a Prominent Effect on Poly(ADP-ribose) Polymerase

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Background: Minocycline protects against asthma independently of its antibiotic function.
Results: Minocycline blocks asthma-associated traits, including IgE production, by modulating the TCR-NF-κB-GATA-3-IL-4 axis but not the TCR/NFAT1/IL-2 pathway without a direct effect on PARP activity.
Conclusion: These results provide new insight into the mechanism of action of minocycline.
Significance: These results provide further support to the therapeutic potential of minocycline in reducing or preventing allergen-induced asthma symptoms.

Minocycline protects against asthma independently of its antibiotic function and was recently reported to be a potent poly-(ADP-ribose) polymerase (PARP) inhibitor. In an animal model of asthma, a single administration of minocycline conferred excellent protection against ovalbumin-induced airway eosinophilia, mucus hypersecretion, and Th2 cytokine production (IL-4/IL-5/IL-12(p70)/IL-13/GM-CSF) and a partial protection against airway hyperresponsiveness. These effects correlated with pronounced reduction in lung and sera allergen-specific IgE. A reduction in poly(ADP-ribose) immunoreactivity in the lungs of minocycline-treated/ovalbumin-challenged mice correlated with decreased oxidative DNA damage. The effect of minocycline on PARP may be indirect, as the drug failed to efficiently block direct PARP activation in lungs of N-methyl-N'-nitro-N-nitroso-guanidine-treated mice or H2O2-treated cells. Minocycline blocked allergen-specific IgE production in B cells potentially by modulating T cell receptor (TCR)-linked IL-4 production at the mRNA level but not through a modulation of the IL-4-JAK-STAT-6 axis, IL-2 production, or NFAT1 activation. Restoration of IL-4, ex vivo, rescued IgE production by minocycline-treated/ovalbumin-stimulated B cells. IL-4 blockade correlated with a preferential inhibition of the NF-κB activation arm of TCR but not GSK3, Src, p38 MAPK, or ERK1/2. Interestingly, the drug promoted a slightly higher Src and ERK1/2 phosphorylation. Inhibition of NF-κB was linked to a complete blockade of TCR-stimulated GATA-3 expression, a pivotal transcription factor for IL-4 expression. Minocycline also reduced TNF-α-mediated NF-κB activation and expression of dependent genes. These results show a potentially broad effect of minocycline but that it may block IgE production in part by modulating TCR function, particularly by inhibiting the signaling pathway, leading to NF-κB activation, GATA-3 expression, and subsequent IL-4 production.

Asthma is, in part, a Th2 lymphocyte-mediated inflammatory airway disease that is characterized by pulmonary eosinophilia, production of Th2 cytokines, mucus hypersecretion by goblet cells, expression of inflammatory factors such as inducible nitric oxide synthase (iNOS) and adhesion molecules, and airway hyperresponsiveness (AHR) (1, 2). The concomitant high production of allergen-specific IgE is an obvious indication of an aberrant Th2 immune response (3, 4). Recently, minocycline was reported to harbor oral steroid-sparing properties beyond its inherent antibiotic function in a cohort of human subjects with both moderate persistent and severe persistent asthma (5). Furthermore, minocycline was shown to suppress IgE production in human subjects and in a benzylpenicillloyl(14)-keyhole limpet hemocyanin (BPO(14)-KLH) mouse model of asthma (6). The mechanisms by which minocycline interferes with the process of inflammation have yet to be elucidated.

The abbreviations used are: iNOS, inducible nitric oxide synthase; AHR, airway hyperresponsiveness; PARP, poly(ADP-ribose) polymerase; TCR, T cell receptor; OVA, ovalbumin; BAL, broncho-alveolar lavage; MNNG, N-methyl-N'-nitro-N-nitroso-guanidine; IKK, IκB kinase; TNF, tumor necrosis factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; GSK, Glycogen Synthase Kinase.
be delineated clearly. Minocycline has been suggested to block several signal transduction pathways that are critical for the expression of inflammatory genes (7, 8).

Interestingly, in an in vitro system, it was recently reported that the neuroprotective and anti-inflammatory effects of minocycline were associated with the ability of the drug to inhibit PARP-1 at nanomolar concentrations (9). We have extensively studied the role of PARP-1 in inflammatory situations. We reported previously that PARP-1 inhibition, pharmacologically or by gene knockout, blocks important inflammatory traits that result from allergen exposure; specifically, the production of Th2 cytokines, eosinophilia, mucus production, and airway hyperresponsiveness (10–12). Very recently, Huang et al. (13) reported that minocycline might protect mice from 5-fluorouracil-induced intestinal mucositis, in part through inhibition of PARP-1. Additionally, Tao et al. (14) reported that minocycline also protects against simulated ischemia reperfusion injury in cardiac myocytes by inhibiting PARP-1. The relationship between minocycline and PARP-1 is of great interest, as a great deal of effort has been made to take PARP-1 inhibitors to the clinic to treat both inflammatory diseases and a number of different types of cancer (15, 16). Accordingly, firstly, this study was designed to test the hypothesis that minocycline blocks allergen-induced airway inflammation in an animal model of asthma by a direct modulation of PARP enzymatic activity. Secondly, this study examined how minocycline blocks allergen-specific IgE production by B cells by focusing on the signaling events that could be modulated by the drug after T cell receptor (TCR) stimulation in immune cells.

**EXPERIMENTAL PROCEDURES**

*Animals, Protocols for Sensitization and Challenge, and Measurement of AHR—C57BL/6* male mice (Jackson Laboratory, Bar Harbor, ME) were housed in a specific-pathogen free facility at Louisiana State University Health Sciences Center, New Orleans, LA, and allowed unlimited access to sterilized chow and water. All experimental protocols were approved by the LSUHSC Animal Care and Use Committee. Six-week-old mice (*n* ≥ 6 for each experimental condition) were sensitized with intraperitoneal injections of 100 μg of mucus 5 chicken ovalbumin (OVA) (Sigma-Aldrich, St. Louis, MO), mixed with 2 mg of aluminum hydroxide in saline, and then challenged with aerosolized OVA (3% OVA in saline) as described (10). The control groups were not sensitized or challenged. Additional groups of mice received 10 mg/kg minocycline (Sigma-Aldrich) or saline intraperitoneally 1 h prior to the OVA challenge. This dose of minocycline was selected on the basis of studies that demonstrated its protective effect in a variety of models of inflammation (17–19). Mice were then left to recover and were sacrificed 24 h or 48 h later for broncho-alveolar lavage (BAL) or lung fixation and processing for histological analysis. Some mice received intratracheal administration of 10 mg/kg N-methyl-N’-nitro-N-nitroso-guanidine (MNNG) (Sigma-Aldrich) under anesthesia and sacrificed 1 h later. Lungs were removed, and protein extracts were prepared for immunoblot analysis.

AHR to inhaled methacholine was measured in unrestrained, conscious mice 24 h after the OVA challenge by recording “enhanced pause” (Penh) by whole body plethysmography (EMKA Systems, Falls Church, VA). In brief, the base-line readings were taken and averaged for 3 min after animals were placed in a barometric plethysmographic chamber. Normal saline or increasing concentrations (12.5–100 mg/ml) of aerosolized methacholine were nebulized, and readings were taken and averaged for 3 min after each nebulization and enhanced pause (Penh) representing AHR was calculated.

**Organ Recovery, Staining, and Th2 Cytokine and OVA-specific IgE Assessments—**Animals were killed by CO₂ asphyxiation, and lungs were fixed with formalin for histological analysis or subjected to BAL. Formalin-fixed lungs were sectioned and subjected to H&E or periodic acid-Schiff staining using standard protocols or to immunohistochemistry with antibodies to poly(ADP-ribose) (PAR) or 8oxoG, as described previously (20, 21). The histological mucin index was assessed essentially as described (21). Collected BAL fluids were subjected to cyto- spin and stained with H&E for the assessment of inflammatory cells. The cytokine assessment was conducted using the Bio-Rad Bioplex system for mouse IL-2, IL-4, IL-5, IL-10, IL-12 (p70), IL-13, GM-CSF, and MCP-1 according to the instructions and specifications of the manufacturer. OVA-specific IgE was quantified by a sandwich ELISA (Serotec, Raleigh, NC) essentially as described (10–12).

**Cell Culture, RT-PCR, Immunofluorescence Microscopy, Immunoblot Analysis, and TCR Stimulation of CD4⁺ T Cells—**Lung fibroblasts and smooth muscle cells were isolated from C57BL/6 mouse using a standard enzymatic digestion protocol. Assays and methods were conducted as described (20–22). CD4⁺ T cells were purified from a single cell suspension procured from spleens and lymph nodes of C57BL/6 mice by negative selection with the Easy Sep kit from Stem Cell Technologies (Vancouver, Canada) according to the instructions of the manufacturer. The cells were then cultured, and TCR stimulation was performed with a combination of plate-bound CD3 (1 μg/ml) and soluble CD28 (2 μg/ml) antibodies (BD Biosciences) in the absence or presence of minocycline. The cell culture supernatant was then collected at 96 h to measure cytokine product. A subset of similarly treated cells was collected at different time points for extraction of either mRNA or proteins or fixed for immunofluorescence with antibodies to NFAT1 (Cell Signaling Technology, Beverly, MA). The extracted total RNA was used for the generation of cDNA using reverse transcription III (Invitrogen) and analyzed by real-time PCR using primers specific for mouse GATA-3 (23), IL-4 (24), or β-actin (25). Protein extracts were subjected to immunoblot analysis using antibodies against phospho-p65 NF-κB (Ser-536), phospho-GSK3β-α (Ser-21/9), phospho-Src (Thr-416), phospho-ERK1/2 (Thr-202/Tyr-204), phospho-p38 MAPK (Thr-180/Tyr-182), phospho-IκBα (Ser-32/36), phospho-IKKα/β (Ser-176/180), total IKKα/β, total-p65 NF-κB, total ERK1/2 (all from Cell Signaling Technology), or actin (Santa Cruz Biotechnology).

**Production of OVA-specific IgE by B Cells—**OVA-sensitized B cells and CD4⁺ T cells were purified from spleens/lymph nodes of OVA-sensitized mice by negative selection as described above. The purified B and CD4⁺ T cells were then cocultured at a 2:1 ratio (2 × 10⁶ B cells, 1 × 10⁶ CD4⁺ T cells) with OVA protein (3.5 μg/ml) and cultured for 3 days in the presence of IL-4 (10 ng/ml). The purified B cells were then labeled with 125I, and purified CD4⁺ T cells were cultured for 3 days with anti-CD49d antibody and interferon (IFN)-γ (20 units/ml) as described (24). Supernatant was collected and assessed by gamma counter. 

**Immunoblot Analysis, and TCR Stimulation of CD4⁺ T Cells—**C57BL/6J mice using a standard enzymatic digestion protocol. Assays and methods were conducted as described (20–22). CD4⁺ T cells were purified from a single cell suspension procured from spleens and lymph nodes of C57BL/6J mice by negative selection with the Easy Sep kit from Stem Cell Technologies (Vancouver, Canada) according to the instructions of the manufacturer. The cells were then cultured, and TCR stimulation was performed with a combination of plate-bound CD3 (1 μg/ml) and soluble CD28 (2 μg/ml) antibodies (BD Biosciences) in the absence or presence of minocycline. The cell culture supernatant was then collected at 96 h to measure cytokine product. A subset of similarly treated cells was collected at different time points for extraction of either mRNA or proteins or fixed for immunofluorescence with antibodies to NFAT1 (Cell Signaling Technology, Beverly, MA). The extracted total RNA was used for the generation of cDNA using reverse transcription III (Invitrogen) and analyzed by real-time PCR using primers specific for mouse GATA-3 (23), IL-4 (24), or β-actin (25). Protein extracts were subjected to immunoblot analysis using antibodies against phospho-p65 NF-κB (Ser-536), phospho-GSK3β-α (Ser-21/9), phospho-Src (Thr-416), phospho-ERK1/2 (Thr-202/Tyr-204), phospho-p38 MAPK (Thr-180/Tyr-182), phospho-IκBα (Ser-32/36), phospho-IKKα/β (Ser-176/180), total IKKα/β, total-p65 NF-κB, total ERK1/2 (all from Cell Signaling Technology), or actin (Santa Cruz Biotechnology).

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cells/well) in a 48-well plate in the presence of 0, 0.1, or 10 μM minocycline with or without OVA (200 μg/ml) alone or a combination of IL-4 and OVA. The cells were maintained at 37 °C, and culture supernatants were collected after 96 h for the quantification of OVA-specific IgE by sandwich ELISA (Serotec) as described above.

Ferric Reducing Ability Assay and Poly(ADP-ribosyl)ation in Vitro—The ferric reducing ability of minocycline was measured essentially as described previously (22). The antioxidant capacity of minocycline was calculated from the linear calibration curve and expressed as mmol FeSO₄ equivalents. For the poly(ADP-ribosyl)ation reactions, purified recombinant PARP-1 (Axxora) was incubated in a reaction mixture containing 100 mM Tris-HCl, 1 mM DTT, 10 mM MgCl₂, 40 μg of protein extracts prepared from PARP-1−/− smooth muscle cells, sonicated (activated) salmon sperm DNA, and 2 mM NAD (Sigma-Aldrich) in the presence of different minocycline doses for 30 min at 37 °C. PARP-1 was preincubated with minocycline before the addition of NAD and activated DNA. The reaction was terminated by the addition of SDS sample buffer and heating at 95 °C for 5 min. Samples were then subjected to immunoblot analysis with antibodies to PAR or actin.

Data Analysis—All data are expressed as mean ± S.D. of values from at least six mice per group unless stated otherwise or triplicate conditions when cells are used. Prism software (GraphPad, San Diego, CA) was used to analyze the differences between experimental groups by one-way analysis of variance followed by Dunnett’s multiple comparison test.

RESULTS

Minocycline Prevents OVA-induced Airway Eosinophilia and Mucus Production without a Clear Effect on Macrophage Recruitment in OVA-challenged Mice—Fig. 1A shows that OVA sensitization and challenge induced a clear and marked perivascular and peribronchial infiltration of eosinophils into the lungs of C57BL/6 mice. Such inflammatory cell infiltration was greatly reduced in mice that received a single intraperitoneal injection of minocycline 1 h prior to challenge. Fig. 1B shows that the OVA-induced increase in eosinophils in the lungs of C57BL/6 mice was significantly reduced by treatment with minocycline prior to OVA challenge. Similarly, the number of lymphocytes recruited to lungs of OVA-challenged mice was reduced by drug treatment. Interestingly, minocycline did not appear to exert any modulatory effects on OVA-induced macrophage recruitment. The marked reduction in eosinophilia achieved by minocycline treatment was mirrored by a significant reduction in mucus production upon OVA challenge (Fig. 1, C and D).

The Protective Effect of Minocycline against OVA-induced Airway Inflammation is Associated with a Marked Blockade of Th2 Cytokines, OVA-specific IgE and AHR in OVA-challenged Mice—Fig. 1E shows that minocycline severely reduced expression levels of IL-4, IL-5, IL-12 (p70), IL-13, and GM-CSF. Minocycline also severely reduced the expression levels of the anti-inflammatory cytokine IL-10. Interestingly, the effect of minocycline treatment on monocyte chemotactic protein 1 (MCP-1, Fig. 1F) was modest and statistically insignificant, which was rather different from that achieved on the Th2 cytokines. The marked reduction in OVA-induced airway eosinophilia and Th2 cytokine production caused by minocycline treatment was associated with a severe reduction in OVA-specific IgE levels in the BAL fluids (Fig. 1G) and sera (H) of the treated animals as assessed by ELISA.

We next assessed whether the protective effects of minocycline against eosinophilia, Th2 cytokine, IgE, and mucus production coincided with prevention of AHR, a major hallmark of human asthma. AHR to increasing doses of methacholine was assessed using whole body barometric plethysmography, a non-invasive technique for PenH measurements. Fig. 1K shows that administration of minocycline significantly reduced AHR to inhaled methacholine 24 h after OVA exposure.

Overall, these results support the notion that minocycline harbors an anti-inflammatory effect that may interfere with recruitment of eosinophils into airways and AHR in response to allergen exposure.

Lack of an Association between Minocycline-mediated Reduction in OVA-induced Airway Eosinophilia in mice and PARP Inhibition—Recently, minocycline was suggested to inhibit PARP-1 at nanomolar concentrations (9). Our laboratory previously identified an important role for PARP-1 in both OVA-induced eosinophilia and the production of Th2 cytokines (10–12, 20). To test whether the anti-inflammatory effects of minocycline in our animal model were the result of an inhibition or reduction in PARP enzymatic activation, we examined the effects of the drug on OVA-induced PARP activation in the lung. Immunohistochemical analysis revealed that OVA challenge induced marked PARP enzymatic activity, as indicated by the large increase in poly(ADP-ribose) immunoreactivity, in the airways of sensitized mice (Fig. 2A), consistent with our previous reports (11, 20) and confirmed by others (26). In marked contrast to its effects on Th2 cytokines, minocycline treatment was associated with a statistically significant but modest reduction in poly(ADP-ribose) immunoreactivity in lung sections from OVA-challenged mice (Fig. 2, A and B). Poly(ADP-ribose) immunoreactivity was largely absent in lung tissues of control animals (Fig. 3A) and OVA-challenged PARP-1−/− mice (data not shown). These results suggest that the anti-inflammatory effects of minocycline may involve a partial inhibition of PARP activity.

To conduct a direct assessment of the potential relationship between PARP and minocycline in a lung injury setting, we examined whether treatment with minocycline blocked PARP activation mediated by the potent DNA alkylating agent MNNG (27, 28). Mice, pretreated with the same dose of minocycline used to achieve the anti-inflammatory effects described above, received an intratracheal administration of 10 mg/kg MNNG and were sacrificed 1 h later. Lungs were removed, and protein extracts were prepared followed by immunoblot analysis with antibodies to poly(ADP-ribose). Fig. 3C shows that MNNG exposure caused a substantial activation of PARP, as evidenced by the number of poly(ADP-riboseylated proteins apparent on the blot. Surprisingly, however, minocycline exerted little to no inhibitory effects on MNNG-induced PARP activation, suggesting that in the lung, minocycline may not inhibit PARP.
We previously reported that the excessive activation of PARP-1 upon allergen exposure is the result of reactive oxygen species and reactive nitrogen species production (11). We next tested whether minocycline inhibited PARP activation in response to direct exposure to H2O2, a reliable reactive oxygen species generator within cells and a DNA-damaging agent. Fig. 3, D and E, shows that a 10 min treatment of lung cells, in vitro, with 500 μM H2O2 induced a robust PARP activation, as revealed by immunofluorescence using anti-PAR antibodies. This effect was completely blocked by the PARP inhibitor thieno[2,3-c]isoquinolin-5-one at nanomolar concentrations. Minocycline moderately inhibited PARP, however, at a concentration 100 times higher than that of thieno[2,3-c]isoquinolin-5-one.

The above results led us to speculate that the effects of minocycline on PARP activity at higher doses may be linked to the anti-oxidant activity of the drug (29). To this end, we tested the Fe3+-to-Fe2+ reducing activities of minocycline in a cell-free system.
system. Fig. 2F shows that minocycline displayed antioxidant activity at doses higher than 10 μM. We next examined whether the reduction in PARP activation achieved with minocycline treatment in the lungs of OVA-challenged mice correlated with a decrease in the generation of oxidative DNA damage. Lung sections were therefore subjected to immunohistochemistry with antibodies to 8oxodG, a well recognized marker of tissue DNA damage. Fig. 2G and H shows that minocycline treatment markedly reduced 8oxodG immunoreactivity compared with the levels observed in the experimental group that did not receive the drug. Collectively, these results suggest that the anti-inflammatory function of minocycline may stem in part from its ability to interfere with oxidative stress.

**Minocycline Blocks Allergen-specific IgE in B Cells Potentially as a Direct Effect on IL-4 Production but not through a Modulation of the IL-4-JAK-STAT-6 Axis, Production of IL-2, or Activation of NFAT1**—To determine whether minocycline modulated the production of IgE directly in our experimental model, we assessed the ability of the drug to block the production of the immunoglobulin by B cells. To this end, B cells derived from OVA-sensitized mice were cocultured with CD4+ T cells in the presence of OVA. Fig. 3A shows that minocycline at the 10 μM concentration completely blocked production of OVA-specific IgE by allergen-stimulated B cells. These results suggest that the effects of minocycline in the animal model may be associated with an alter-
ation of the immunoglobulin production by B cells upon allergen exposure.

As shown above, IgE-reduced production by minocycline treatment was associated with a reduction in IL-4 upon allergen challenge in our mouse model. It is noteworthy that IL-4 is a requisite for IgE production and is the result of T cell receptor (TCR) function (30). To determine whether this effect was directly associated with an interference with TCR function, we utilized an in vitro system with primary mouse CD4+ T cells in which TCR was stimulated by a combination of CD3 and CD28 antibodies. Stimulation of TCR induced a substantial level of IL-4, which was substantially reduced by minocycline at the 10 μM concentration (Fig. 3B) suggesting that the drug may be achieving its anti-inflammatory effect in part by interfering with TCR-mediated IL-4 production. Such a large decrease in the cytokine level was accompanied with a similar decrease in IL-5 production (data not shown). Minocycline treatment appeared to affect IL-4 production at the mRNA level as the drug significantly blocked TCR stimulation-mediated increase in IL-4 mRNA levels as assessed by quantitative...
RT-PCR (Fig. 3C). To confirm the link between the effect of minocycline on IL-4 and IgE production, we examined whether replenishment of IL-4 in our ex vivo system reversed production of IgE by B cells. Fig. 3D shows that, indeed, the inhibitory effect of the drug was bypassed by the replenishment of IL-4. These results provide additional support for the link between the effect of minocycline on IgE and IL-4 production by CD4⁺ T cells.

Signal transduction through the IL-4 receptor and subsequent phosphorylation of STAT-6 are crucial for T cell-mediated pathogenesis of asthma (31). IL-4 stimulation of freshly isolated splenocytes from naïve mice induced a rapid and robust phosphorylation of STAT-6 (Fig. 3E). Minocycline treatment exerted no effect on such phosphorylation, suggesting that the drug did not achieve its anti-inflammatory effects through inhibition of the IL-4/JAK/STAT pathway, but rather it did so upstream of such an axis and chiefly by blocking the expression of IL-4.

IL-2 plays a central role in Th2 differentiation and is one of the first cytokines to be produced in response to TCR stimulation (30). Additionally, IL-2 is required for the efficient development of IL-4-producing T cells and production of IL-4 (30). Thus, we examined the effect of minocycline treatment on IL-2 production in response to TCR stimulation to determine, first, whether the drug blocked all TCR-mediated signaling and second, whether the effect on IL-4 was connected to a blockade in IL-2 production. Fig. 3F shows that stimulation of TCR in CD4⁺ T cells resulted in a substantial increase in IL-2 production. Interestingly, minocycline failed to suppress such IL-2 production after TCR stimulation. The insensitivity of TCR-mediated IL-2 production to minocycline correlated well with a failure of the drug to interfere with the nuclear translocation of NFAT1 (Fig. 3G), a transcription factor that is crucial for IL-2 gene expression (32).

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**FIGURE 4. Effect of minocycline on GATA-3 expression and stimulation of TCR-associated signaling in CD4⁺ T cells.** A, CD3/CD28-stimulated CD4⁺ T cells in the absence or presence of 10 µM minocycline (Mc) were collected after 24 h of incubation for total RNA preparation. cDNAs were then generated and subjected to real-time PCR with primers specific to mouse GATA-3 or β-actin. Data are given as fold change of control (non-stimulated) = S.D. normalized to β-actin levels. *, difference from non-stimulated cells, p < 0.01; #, difference from CD3/CD28-stimulated cells; p < 0.01. B, CD3/CD28-stimulated CD4⁺ T-cells were incubated in the absence or presence of 10 µM minocycline and collected at the indicated time intervals for total protein preparation. The proteins extracted were then subjected to immunoblot analysis with antibodies to p65 NF-κB, its phosphorylated form at serine 536, the phosphorylated form of IκBα (Ser-22/23), IKBα, IKKβ, the phosphorylated form of IKKα/β (Ser-176/180), the phosphorylated form of IKKα/β (Ser-176/177), the phosphorylated form of GSK3 β-α (Ser-21/9), Src-Tyr-416, the phosphorylated form of p38 MAPK (Thr-180/Tyr-182), or actin. C, CD4⁺ T-cells were treated as in B except that an earlier time point was added, given the fast activation of ERK1/2 in our experimental system. Protein extracts were subjected to immunoblot analysis with antibodies to total ERK1/2, its phosphorylated form at Thr-202 and Tyr-204, or actin. Note that some of the immunoblots are the same and were stripped and reprobed but that others were generated using the same samples. The actin for these immunoblots is similar (data not shown).
Despite such robust inhibitory effects on NF-κB gene expression by minocycline, the results suggest that minocycline may be inhibiting NF-κB activity, rather than its activation.

Our results confirm such properties of minocycline in an animal model of allergic lung inflammation. Minocycline and other tetracycline derivatives have been increasingly investigated for their anti-inflammatory functions independently of their original, broad-spectrum antibiotic traits against bacterial infections (reviewed in Ref. 8). Interest in this drug stems from its modulatory effects against a number of inflammatory conditions both in human subjects and animal models (8). Joks and Durkin (reviewed in Ref. 8), in a pioneering study, showed that minocycline harbors oral steroid-sparing properties in a cohort of human subjects with both moderate persistent and severe persistent asthma (5). Minocycline was shown to be efficacious in reducing IgE production in an in vitro system as well as in response to BPO(14)-KLH (6, 42). Doxycycline, another member of the tetracyclines, was shown to reduce airway inflammation and hyperresponsiveness in a mouse model of toluene diisocyanate-induced asthma (43). Our results confirm such properties of minocycline in an ani-
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imal model of allergen (OVA)-induced asthma. It is important to note that the mode of drug delivery may alter the efficacy of minocycline in reducing the level of IgE. In the mouse model used in this study, a single intraperitoneal injection of 10 mg/kg minocycline was sufficient to severely reduce antigen-specific IgE levels in the BALF and sera collected from treated animals. In a very different model of antigen (BPO(14)-KLH) sensitization, Joks et al. (6) recently reported that oral delivery of the drug required much higher doses to drastically reduce BPO(14)-KLH-specific IgE. All reports indicate a beneficial role for minocycline in blocking lung inflammation in response to allergen and may prove to be an efficacious therapy in such cases. Despite the established connection between the beneficial effect of minocycline (and other tetracycline derivatives) against human asthma and suppression of allergen-induced IgE (8), the underlying molecular mechanism(s) by which these drugs achieved such effects remains unclear. Our study sheds new light on such a connection and further supports the broader effect of the drug but identifies the TCR stimulation-NF-κB activation-GATA-3 expression-IL-4 production axis as an important site at which the drugs achieve its blockade of IgE production and subsequent effects on the manifestation of asthma traits.

A very small number of studies have investigated the mechanism by which tetracycline derivatives interfere with IgE production (for a comprehensive review, see Ref. 8). Doxycycline, for instance, was shown a decade ago, in an in vitro system, to inhibit immunoglobulin secretion and class switching in B cells in response to LPS or anti-CD40 antibodies in combination with IL-4 (44). However, such an effect was associated primarily with the established inhibitory effect of tetracycline derivatives on matrix metalloproteinases. The role of p38 MAPK is increasingly being recognized in a number of aspects of allergic responses, and the kinase is becoming a viable therapeutic target for the treatment of asthma symptoms (45). Minocycline was suggested to reduce the number of CD4+ and CD8+ T cells but not B cells or monocytes from asthmatic individuals who display p38 MAPK phosphorylation, as assessed by FACS analysis, suggesting a mechanistic link between the function of the drug and IgE suppression (8). In our experimental model and using direct CD4+ T cells stimulation, minocycline exerted no effect on TCR-mediated p38 MAPK, suggesting, perhaps, that the effect observed in asthmatic individuals may not be associated with TCR stimulation or is the result of an indirect effect. Our results also suggest that the effect of minocycline in our experimental system may be upstream of p38 MAPK. The effect of minocycline (or any of the tetracycline derivatives) on the function of p38 MAPK remains uncertain given the additional conflicting reports showing that the phosphorylation of the kinase could be modulated by the drug in response to LPS but not in response to photoreceptor stimulation (46). It is noteworthy that tetracycline derivatives have been reported to inhibit several kinases, including PKC (47), JNK (48), and AKT (43). These reports may suggest a general broader effect, especially given the potential antioxidant property of these drugs and, more importantly, the high dosages used by the aforementioned studies. Our results and those recently published by some of us (49) clearly show that at concentrations higher than 10 μM, minocycline harbors anti-oxidant properties. Accordingly, it is plausible that some of the effects observed with minocycline or other tetracycline derivatives may be attributed to their anti-oxidant functions.

The confidence in the effects of minocycline on TCR-stimulated signal transduction, primarily NF-κB activation as measured by the phosphorylation of the p65 subunit at serine 536 and its inhibitor I-κBα, stems from the fact that other signaling events remained unaltered or even slightly stimulated by the drug in our experimental system. Interestingly, IKK activation was also unaltered, although phosphorylation of I-κBα, a substrate of IKK, was partially blocked. These results may suggest that minocycline inhibits rather than activates activity of IKK.

However, a better explanation of these effects required additional experimentation. Additionally, phosphorylation of GSK and, as stated above, p38 MAPK remained unaffected by the drug, whereas phosphorylation of Src and ERK1/2 increased. At this juncture, we are unable to explain the mechanism(s) by which these latter signaling events were enhanced by minocycline. The effect on NF-κB activation correlated well with the complete blockade of GATA-3 expression, which may partially explain the modulatory effect of the drug on IL-4 production in vivo and in our in vitro system. The effect on NF-κB activation does not appear to be restricted to TCR stimulation but could also be observed in response to TNF-α, further supporting the broader effect of the drug. Interestingly, minocycline did not exert any modulatory effect on NFAT1 activation as assessed by its nuclear translocation, which correlated with the failure of the drug to modulate IL-2 production. Such an observation is consistent with that reported by Szeto et al. (50), where minocycline at 20 μM (i.e. 10 μg/ml) failed to block NFAT1 activation, assessed by the dephosphorylation of the transcription factor, in human CD4+ T cells in response to a combination of phorbol ester 12-myristate 13-acetate and ionomycin. It is noteworthy that the concentration of minocycline used in this study was 10 μM (i.e. 5 μg/ml). The modulatory effects of minocycline on NFAT1 activation observed by Szeto et al. (50) was only observed at much higher concentrations than those tested in our experimental system.

Additionally, minocycline appears to interfere with dendritic cell differentiation. Minocycline significantly reduced the maturation of myeloid dendritic cells upon treatment with a combination of IL-4 and GM-CSF for 7 days, as assessed by FACS analysis conducted with fluorescently labeled anti-CD11b and anti-CD11c antibodies (supplemental Fig. S1). Such effects on dendritic cell differentiation hampered CD4+ and CD8+ T-cell proliferation when these minocycline-treated dendritic cells were cocultured with T cells in the absence of the drug (supplemental Fig. S2). The modulation of dendritic cell differentiation by minocycline may have important indirect effects on Th2 cytokine and IgE production when the drug is used in a continuous manner for a sustained duration.

Despite the excellent inhibitory effects of minocycline against OVA-induced lung inflammation, the persistence of macrophages was rather curious. It is plausible that the failure of minocycline treatment to reduce or block the increase in the macrophage population in the lung may be associated with the lack of an effect on MCP-1 expression. Interestingly, these
macrophages appeared to be active, as they displayed PARP-1 activation (Fig. 2A) and expression of iNOS (data not shown). The partial effect of minocycline on PARP-1 activation in macrophages was consistent with the inability of the drug to prevent H$_2$O$_2$-induced NAD$^+$ depletion, a measure of PARP activation, in a monocytic cell line in vitro (supplemental Fig. S3). Whether these effects are detrimental or protective to the lung is not clear. It is noteworthy that a moderate increase in iNOS, leading to a reciprocal increase in NO, may be beneficial and could explain the protective effects of minocycline against AHR. The effect of minocycline on AHR is consistent with those reported by Lee et al. (43) in the toluene disocyanate-induced asthma model. Our interest in minocycline was triggered by its potential inhibitory effects against PARP-1 enzymatic activity (5), bolstered by our long interest in the role of PARP-1 in asthma pathogenesis. Our results show that although minocycline is an excellent modulator of allergen (OVA)-induced airway eosinophilia, mucus hypersecretion, and production of Th2 cytokines and IgE, these effects may not be strictly related to a direct inhibition of PARP. The reduction in the generation of poly(ADP-ribose), a marker of PARP function, in vivo and in vitro may be linked in part to a modulation of oxidative DNA damage. However, this does not rule out the possibility that the drug affects PARP activation through other, yet unidentified, means.

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