

Minocycline Blocks Asthma-associated Inflammation in Part by Interfering with the T Cell Receptor-Nuclear Factor κ B-GATA-3-IL-4 Axis without a Prominent Effect on Poly(ADP-ribose) Polymerase^{*[S]}

Received for publication, September 13, 2012, and in revised form, November 18, 2012. Published, JBC Papers in Press, November 26, 2012, DOI 10.1074/jbc.M112.419580

Amarjit S. Naura^{†§1}, Hogyoung Kim^{‡¶}, Jihang Ju^{‡¶}, Paulo C. Rodriguez[‡], Joaquin Jordan^{||}, Andrew D. Catling^{‡¶}, Bashir M. Rezk^{**}, Zakaria Y. Abd Elmageed[‡], Kusma Pyakurel^{‡¶}, Abdelmetalab F. Tarhuni^{‡¶}, Mohammad Q. Abughazleh[‡], Youssef Errami^{‡¶}, Mourad Zerfaoui^{‡¶}, Augusto C. Ochoa[‡], and A. Hamid Boulares^{‡¶12}

From the [†]Stanley S. Scott Cancer Center, [¶]Department of Pharmacology and Experimental Therapeutics, [§]Department of Medicine, Louisiana State University Health Sciences Center, New Orleans, Louisiana 70112, the ^{||}Dpto Ciencias Médicas, Facultad de Medicina, Universidad Castilla-La Mancha, Albacete 02006, Spain, and the ^{**}Department of Natural Sciences, Southern University at New Orleans, New Orleans, Louisiana 70126

Background: Minocycline protects against asthma independently of its antibiotic function.

Results: Minocycline blocks asthma-associated traits, including IgE production, by modulating the TCR-NF- κ B-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 as 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- κ B 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- κ B 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- κ B 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- κ B 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)³ 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 benzylpenicilloyl(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

^{*} This work was supported in part by Grant HL072889 from National Institutes of Health and grant RSG-116608 from the American Cancer Society (to A. H. B.). This work was also supported by grant National Institutes of Health/COBRE P20GM103501 (to A. S. N., with A. C. O. as overall Program Director).

^[S] This article contains supplemental Figs. S1–S3.

¹ To whom correspondence may be addressed: 533 Bolivar St. 701#509, New Orleans, LA 70112. Fax: 504-568-6888; E-mail: anaura@lsuhsc.edu.

² To whom correspondence may be addressed: 533 Bolivar St. #509, New Orleans, LA 70112. Fax: 504-568-6888; E-mail: hboulr@lsuhsc.edu.

³ 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, I κ B 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/6J 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 ($n \geq 6$ for each experimental condition) were sensitized with intraperitoneal injections of 100 μ g of grade V 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 bronchio-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 8oxodG, 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/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 μ g/ml) and soluble CD28 (2 μ g/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 transcriptase 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- κ B (Ser-536), phospho-GSK3 β - α (Ser-21/9), phospho-Src (Thr-416), phospho-ERK1/2 (Thr-202/Tyr-204), phospho-p38 MAPK (Thr-180/Tyr-182), phospho-I- κ B α (Ser-32/36), phospho-IKK α / β (Ser-176/180), total IKK α / β , total-p65 NF- κ B, 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 (2×10^6 B cells, 1×10^6 CD4⁺ T

cells/well) in a 48-well plate in the presence of 0, 0.1, or 10 μM minocycline with or without OVA (200 $\mu\text{g}/\text{ml}$) alone or a combination of IL-4 and OVA. The cells were maintained at 37 $^{\circ}\text{C}$, 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-ribose)ylation 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 FeSO_4 equivalents. For the poly(ADP-ribose)ylation reactions, purified recombinant PARP-1 (Axxora) was incubated in a reaction mixture containing 100 mM Tris-HCl, 1 mM DTT, 10 mM MgCl_2 , 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 $^{\circ}\text{C}$. 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 $^{\circ}\text{C}$ for 5 min. Samples were then subjected to immunoblot analysis with antibodies to PAR or actin.

Data Analysis—All data are expressed as mean $\pm$ 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 chemoattractant protein 1 (MCP-1, Fig. 1F) was modest and statistically insignificant, which was rather different from that achieved on the Th2 cyto-

kines. 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-ribosyl)ated 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.

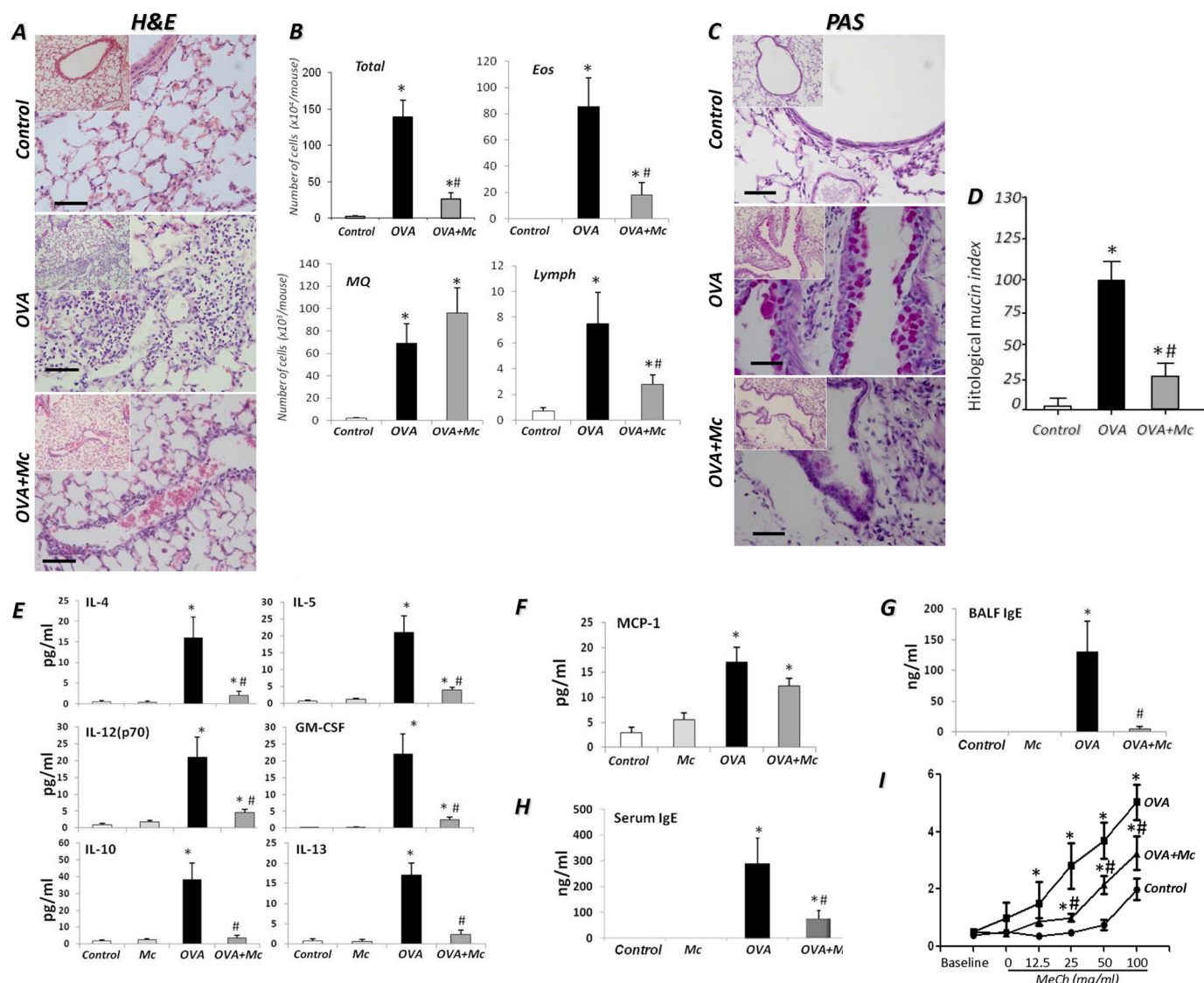


FIGURE 1. Minocycline prevents airway eosinophilia and mucus production without reducing macrophage recruitment in OVA-challenged mice and association between the protective effect of minocycline against OVA-induced airway inflammation and blockade of Th2 cytokines and IgE production. C57BL/6J mice were subjected to OVA sensitization followed by challenge or left untreated. A group of mice were administered intraperitoneal 10 mg/kg of minocycline (Mc) 1 h prior to challenge. Mice were sacrificed 48 h after the last challenge. Lungs were subjected to formalin fixation or BAL. A, H&E stain of lung sections from the different experimental groups. Scale bars = 4 μ m. B, cells of BAL fluids were differentially stained, and total, eosinophils (eos), macrophages (MQ), and lymphocytes (Lymph) were counted. Data are expressed as total number of cells per mouse. Data are mean $\pm$ S.D. of values from at least six mice per group. C, lung sections from the different experimental groups were subjected to periodic acid-Schiff (PAS) staining. Scale bars = 4 μ m. D, the extent of mucus production (histological mucin index) was assessed as described under "Experimental Procedures." Assessment of BAL or sera fluids collected from the different experimental groups 24 h after OVA challenge for IL-4, IL-5, GM-CSF, IL-12(p70), IL-10, or IL-13 (E), for MCP-1 (F), BAL IgE (G), or sera IgE (H). Assessment of cytokines was conducted using a Bio-Rad multiplex system, and quantification OVA-specific IgE was done by sandwich ELISA. For B, D, E, F, G, and H, data are given as mean $\pm$ S.D. of values obtained from at least six mice per group. *, difference from unchallenged mice, $p < 0.01$; #, difference from OVA-challenged mice, $p < 0.01$. I, mice were sensitized to OVA and subjected to a challenge with aerosolized OVA with (▲) or (■) without an intraperitoneal injection of minocycline. Control mice were not challenged (●). Enhanced pause (Penh) was recorded 24 h later using a whole body plethysmograph system before and after the indicated concentrations of aerosolized methacholine (MeCh). Results are plotted as maximal fold increase of Penh relative to base line and expressed as mean $\pm$ S.E., where $n = 5$ mice per group. *, difference from control mice; #, difference from OVA-challenged mice without minocycline treatment, $p < 0.01$. Inserts in A and C represent a lower magnification of the depicted pictures.

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 H_2O_2 , 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 μ M H_2O_2 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 Fe^{3+} -to- Fe^{2+} reducing activities of minocycline in a cell-free

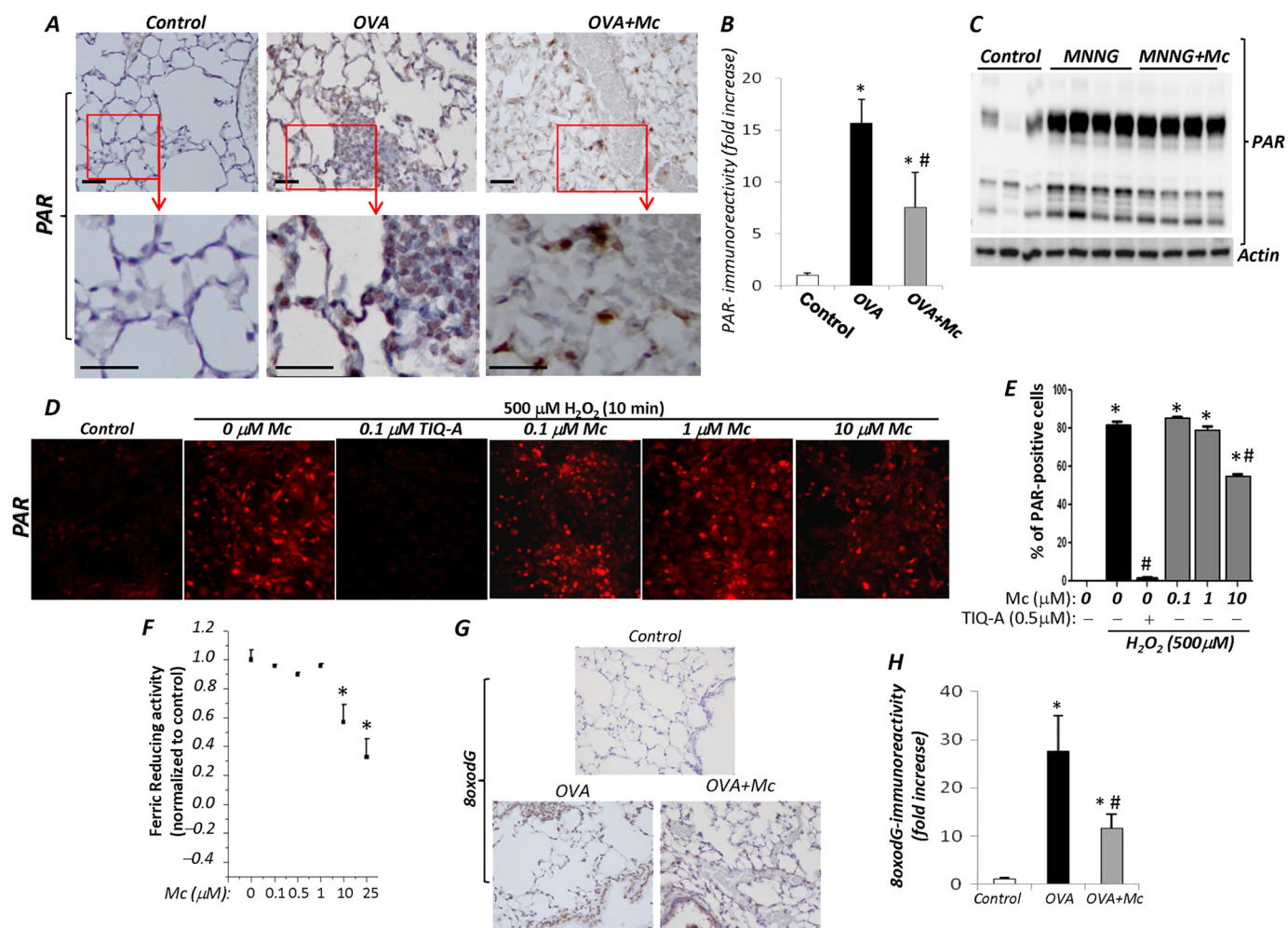


FIGURE 2. Lack of a strong association between minocycline-mediated reduction in OVA-induced airway eosinophilia in mice and PARP inhibition. A, lung sections from OVA-challenged mice with or without minocycline (Mc) treatment were subjected to immunohistochemistry staining with antibodies to poly(ADP-ribose) moieties of PARP-1-modified proteins (PAR) and observed by light microscopy. Scale bars = 4 μ m. B, the extent of immunoreactivity was then assessed using Image Pro-Plus. Data are expressed as fold increase of control. *, difference from control unchallenged mice, $p < 0.05$; #, difference from OVA-challenged mice, $p < 0.05$. C, mice received intratracheal administration of 10 mg/kg MNNG under anesthesia and were sacrificed 1 h later. A group of mice received 10 mg/kg minocycline 1 h prior to MNNG exposure. Lungs were removed, and protein extracts were prepared for immunoblot analysis with antibodies to PAR or actin. D, lung cells were treated with 500 μ M H_2O_2 for 10 min in the presence or absence of different doses of minocycline. Cells were then fixed and subjected to immunofluorescence with antibodies to PAR. Note that the cytosolic staining is nonspecific, as it appears in all cells. E, PAR-positive cells were counted and expressed as percent positive cells in a given area. *, difference from untreated cells; #, difference from H_2O_2 -treated cells. TIQ-A, thieno[2,3-c]isoquinolin-5-one. F, antioxidant capacity of minocycline on the basis of its ability to reduce Fe^{3+} to Fe^{2+} . Data were calculated from the linear calibration curve and expressed as mmol FeSO₄ equivalents. The data represent mean $\pm$ S.D. of at least three independent experiments. *, difference from control conditions without minocycline; $p < 0.05$. G, lung sections from OVA-challenged mice with or without minocycline treatment were subjected to immunohistochemistry staining with antibodies to 8oxodG and observed by light microscopy. Scale bars = 4 μ m. H, the extent of immunoreactivity was then assessed using Image Pro-Plus. Data are expressed as fold increase of control. *, difference from control unchallenged mice, $p < 0.05$; #, difference from OVA-challenged mice, $p < 0.05$.

system. Fig. 2F shows that minocycline displayed antioxidant activity at doses higher than 10 μ M. 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. 2, G 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 0, 0.1, or 10 μ M minocycline. Fig. 3A shows that minocycline at the 10 μ M 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-

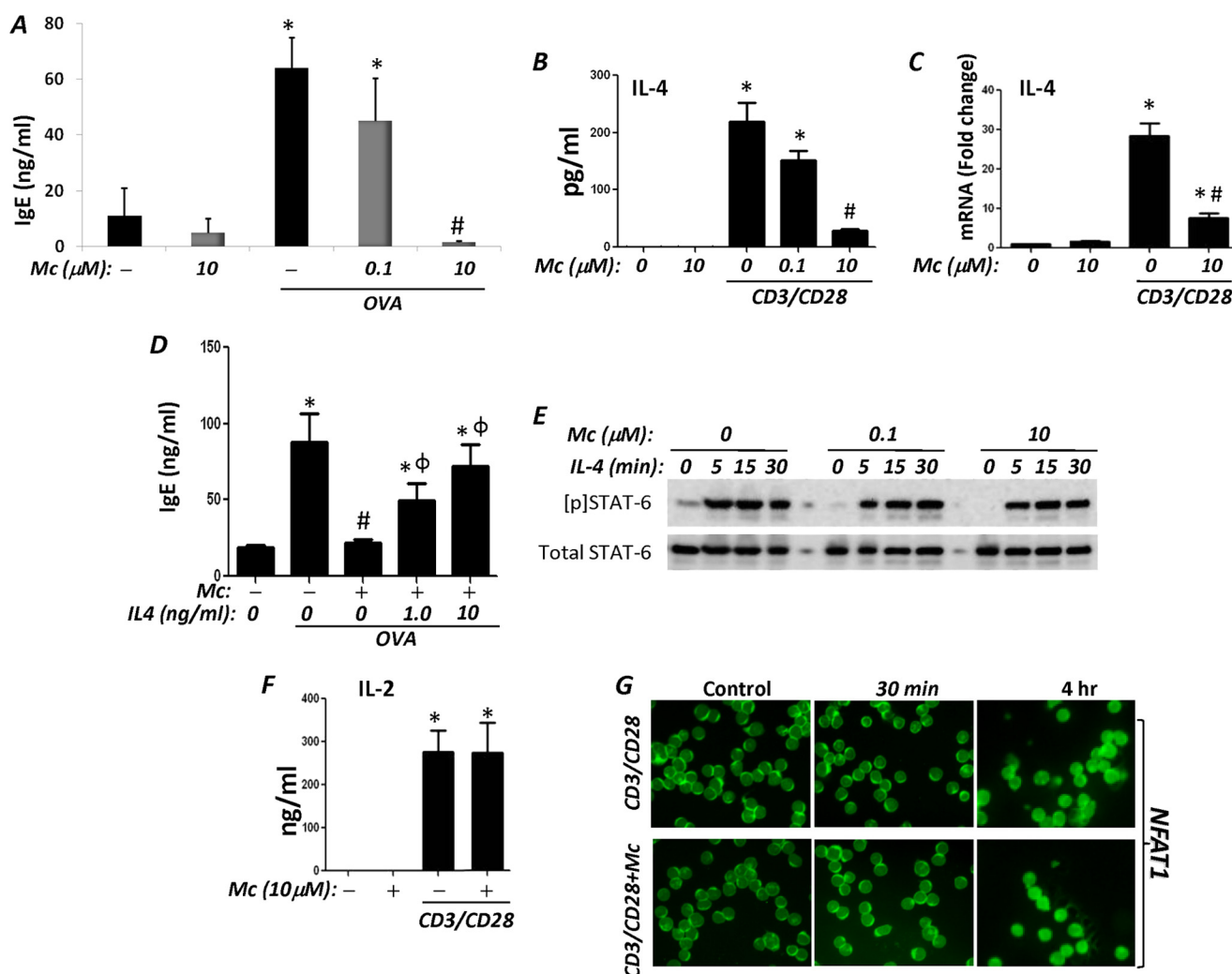


FIGURE 3. Effects of minocycline on the NF- κ B-GATA3 axis for production of IL-4 following TCR stimulation in mouse primary CD4⁺ T cells. A, B cells derived from OVA-sensitized mice were cocultured with CD4⁺ T cells and stimulated with OVA (200 μ g/ml) in the presence of 0, 0.1, or 10 μ M minocycline (Mc). Supernatants were collected after 96 h for the quantification of OVA-specific IgE by sandwich ELISA. Data are given as means $\pm$ S.D. of values obtained from at least six mice per group. *, difference from unchallenged mice, $p < 0.01$; #, difference from OVA-challenged mice; $p < 0.01$. B, purified mouse CD4⁺ T cells were stimulated in triplicates with a combination of plate-bound CD3 (1 μ g/ml) and soluble CD28 (2 μ g/ml) antibodies in the absence or presence of minocycline at a dose of 0.1 or 10 μ M. The cell culture supernatants were then collected after 96 h of incubation for measurement of IL-4 levels. Data are given as means $\pm$ S.D. of values obtained from duplicates of the triplicates. *, difference from non-stimulated cells, $p < 0.01$; #, difference from CD3/CD28-stimulated cells; $p < 0.01$. C, CD3/CD28-stimulated CD4⁺ T-cells in the absence or presence of 10 μ M minocycline 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 IL-4 or β -actin. Data are given as fold change of control (non-stimulated) $\pm$ S.D. normalized to β -actin levels. *, difference from non-stimulated cells, $p < 0.01$; #, difference from CD3/CD28-stimulated cells; $p < 0.01$. D, CD3/CD28-stimulated CD4⁺ T-cells were treated with minocycline but in the presence of 1 or 10 ng/ml of mouse IL-4. The cell culture supernatants were then collected after 96 h of incubation for measurement of IL-4 levels. Data are given as means $\pm$ S.D. of values obtained from duplicates of the triplicates. *, difference from non-stimulated cells, $p < 0.01$; #, difference from CD3/CD28-stimulated cells; $p < 0.01$; Φ , difference from CD3/CD28-stimulated cells in the presence of 10 μ M minocycline; $p < 0.01$. E, splenocytes, freshly isolated from untreated C57BL/6 mice, were treated with 10 ng/ml IL-4 for different time intervals in the absence or presence of the indicated concentrations of minocycline. Protein extracts were prepared and subjected to immunoblot analysis with antibodies to STAT-6 or its tyrosine 641-phosphorylated form ([p]STAT-6). F, culture supernatants of CD3/CD28-stimulated CD4⁺ T-cells in the absence or presence of 10 μ M minocycline were collected after 96 h of incubation and assessed for IL-2 as described above. G, CD4⁺ T-cells, grown in chamber slides, were stimulated with CD3/CD28 for the indicated times in the absence or presence of 10 μ M minocycline. Cells were then fixed and subjected to immunofluorescence staining with antibodies to NFAT1.

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 μ M 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

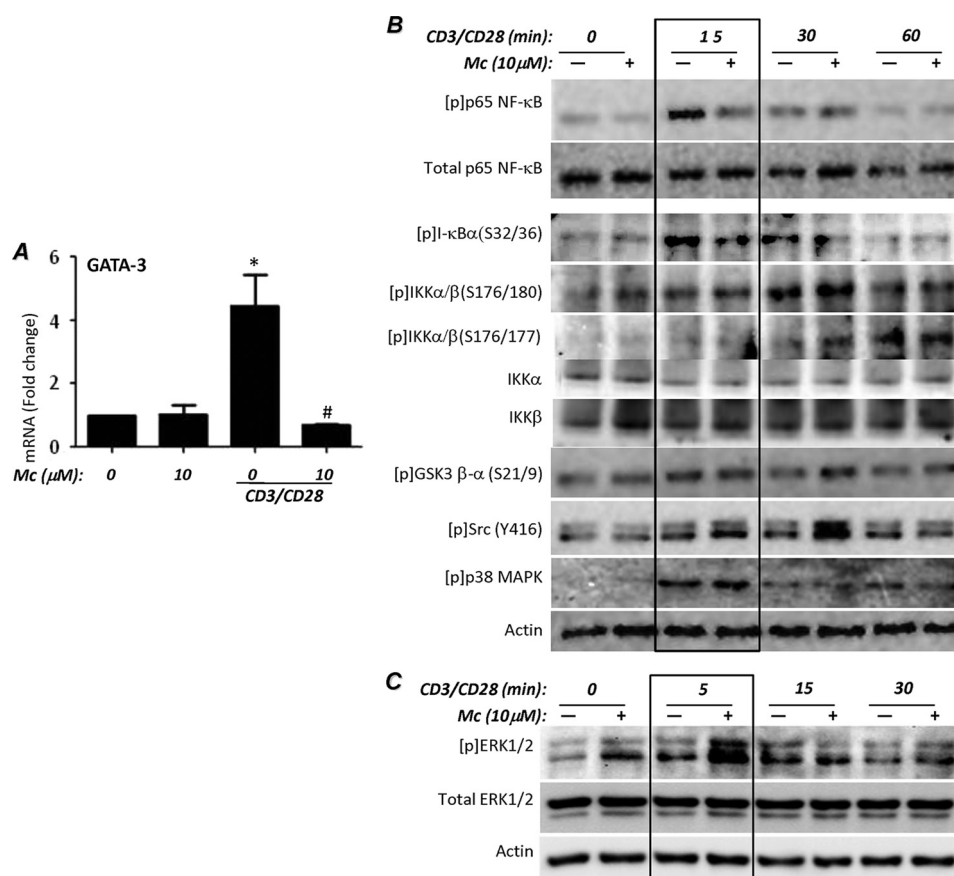


FIGURE 4. Effect of minocycline on GATA-3 expression and stimulation of TCR-associated signaling in CD4⁺ T cells. A, CD3/CD28-stimulated CD4⁺ T cells in the absence or presence of 10 μ M minocycline (Mc) were collected after 24 h of incubation for total RNA preparation. cDNAs were then generated and 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) $\pm$ 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 μ M 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- κ B, its phosphorylated form at serine 536, the phosphorylated form of I- κ B α (Ser-32/36), IKK α , IKK β , the phosphorylated form of IKK α / β (Ser-176/180), the phosphorylated form of IKK α / β (Ser-176/177), the phosphorylated form of GSK3 β - α (Ser-21/9), the phosphorylated form of 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).

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 devel-

opment 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).

Modulation of the TCR-NF- κ B-GATA3-IL4 Axis in Primary CD4⁺ T Cells as an Underlying Mechanism for the Anti-inflammatory Effects of Minocycline and Inhibition of IgE Production by B Cells—One of the most prominent transcription factors involved in IL-4 gene expression is GATA-3, a TCR-induced transcription factor (33). Fig. 4A shows that TCR-stimulated GATA-3 mRNA expression was completely blocked by minocycline. This led us to speculate that the drug may be interfering

with TCR-mediated signal transduction, leading to the expression of the GATA-3 gene. TCR stimulation promotes activation of numerous signaling pathways primarily through phosphorylation of GSK3, Src, p38 MAPK, ERK1/2, IKK, and NF- κ B (34). Of these factors, NF- κ B appears to play a pivotal role in GATA-3 gene expression (35). Fig. 4, B and C, show that all aforementioned signaling events were activated by TCR stimulation in our experimental system. Only NF- κ B activation, assessed by the phosphorylation status of the p65 subunit of the transcription factor at serine 536 and of I- κ B α at serine residues 32/36, was reduced by minocycline treatment upon TCR stimulation. Interestingly, despite the effect on p65 NF- κ B and I- κ B α phosphorylation, minocycline did not affect IKK activation as assessed by its phosphorylation at multiples sites. These results suggest that minocycline may be inhibiting IKK activity rather than its activation.

Overall, these above results suggest that the drug may be inhibiting GATA-3 and subsequent IL-4 gene expression by blocking NF- κ B signal transduction. Although the phosphorylation of glycogen synthase kinase and p38 MAPK remained unaltered in the presence of minocycline, the drug promoted a slightly higher phosphorylation of Src (Fig. 4B) and ERK1/2 (C). The increase in ERK1/2 phosphorylation appeared to occur even in the absence of TCR stimulation (Fig. 4C).

Minocycline Blocks Cytokine-induced NF- κ B Activation and Expression of NF- κ B-dependent Genes without Preventing Nuclear Translocation of the Transcription Factor—Untreated asthmatics exhibit greater levels of NF- κ B activation in immune as well as in structural cells such as lung epithelial cells, goblet cells, smooth muscle cells, and fibroblasts compared with individuals with controlled disease (36–38). In addition to GATA-3, NF- κ B is crucial for the expression of a number of asthma-associated genes, including, iNOS, adhesion molecules, IL-6, IL-8, GM-CSF, and many others (2, 39). Thus, we tested whether the effect of minocycline on NF- κ B activation was restricted to TCR stimulation or whether it can affect other stimuli such as TNF- α . TNF- α was selected as an inducer in this experiment, given its potency in inducing NF- κ B-dependent genes and involvement in lung inflammation and asthma (40, 41). Fig. 5A shows that TNF- α treatment of lung fibroblasts induced rapid degradation of the NF- κ B inhibitor I- κ B α , an effect that was largely unaltered by minocycline treatment despite a moderate effect on the phosphorylation status of I- κ B α . Surprisingly, however, minocycline treatment completely blocked p65 NF- κ B phosphorylation at serine 536. Despite such robust inhibitory effects on NF- κ B phosphorylation, the drug did not prevent the nuclear translocation of the transcription factor in response to TNF- α (Fig. 5B). Inhibition of NF- κ B phosphorylation coincided, however, with a marked reduction in the expression of NF- κ B-dependent genes such as ICAM-1, VCAM-1, and iNOS (Fig. 5C). These results suggest that minocycline may inhibit NF- κ B activation in response to more than one stimulus and that such inhibition appeared to reside at the phosphorylation level of the protein, an event that is crucial for its transcriptional activity.

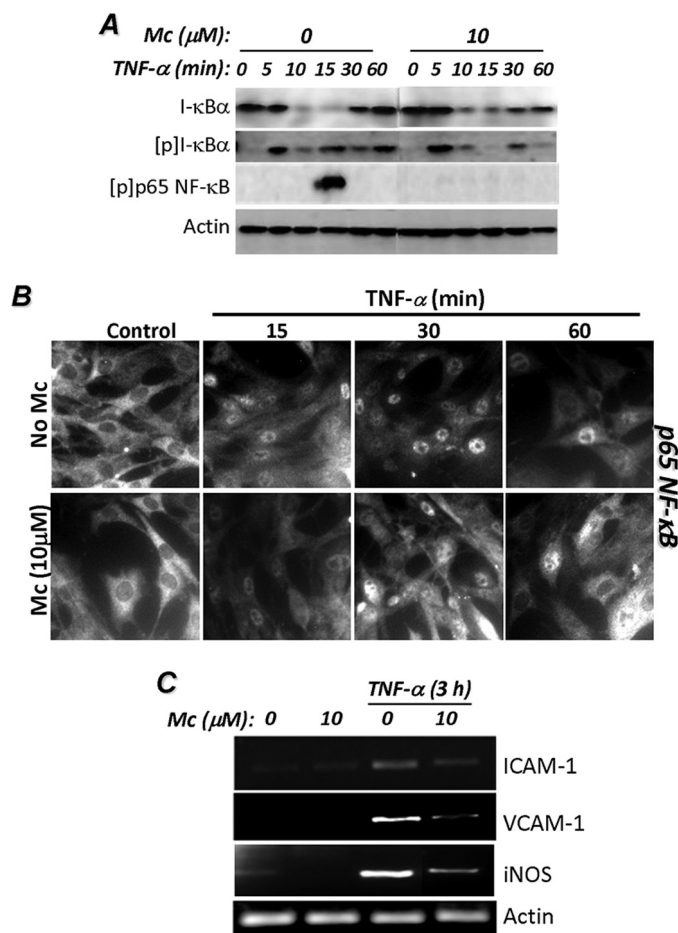


FIGURE 5. Effects of minocycline on TNF- α -induced NF- κ B activation and expression of NF- κ B-dependent genes. A, lung cells were treated with 10 ng/ml TNF- α for different time intervals in the absence or presence of 10 μ M minocycline (Mc). Protein extracts were prepared and subjected to immunoblot analysis with antibodies to I- κ B α , its serine residue 32/36-phosphorylated form ([p]I- κ B α), the serine 536-phosphorylated form of p65 NF- κ B ([p]p65NF- κ B), or actin. B, cells grown in chamber slides were treated with TNF- α for the indicated times in the absence or presence of the indicated concentrations of minocycline. Cells were then fixed and subjected to immunofluorescence staining with antibodies to p65 NF- κ B. C, cells were treated with TNF- α for 6 h, after which total RNA was prepared and subjected to cDNA generation followed by conventional PCR with primers specific to mouse ICAM-1, VCAM-1, iNOS, or β -actin.

DISCUSSION

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-

mal 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- κ B activation-GATA-3 expression-IL-4 production axis as an important site at which the drugs achieves 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 μ M, 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- κ B activation as measured by the phosphorylation of the p65 subunit at serine 536 and its inhibitor I- κ B α , 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- κ B α , 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- κ B 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- κ B 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 μ M (*i.e.* 10 μ g/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 μ M (*i.e.* 5 μ g/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 H₂O₂-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 diisocyanate-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.

Acknowledgments—We thank Chris Fehrenbach for helping with the editing of the manuscript. We also thank Jordy Saravia for assistance with AHR measurements and Dr. Stephania Cormier for access to the plethysmograph.

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Fig. S1. *Minocycline reduces dendritic cell (DCs) differentiation in vitro.* Bone marrow cells were collected from sacrificed C57BL/6 mice, and single-cell suspensions were prepared by passing through a stainless mesh. Erythrocytes were lysed by treatment with RBC lysis buffer. Cells were plated in RPMI culture medium supplemented with 20 ng/ml GM-CSF and 10 ng/ml IL-4 (R&D systems, Minneapolis, MN) in the absence or presence of 10 μ M minocycline for a period of 7 days. Cultures were fed with fresh medium every 3 days. After one week, cells were stained with FITC/PE labeled CD11b and CD11c antibodies (eBioscience, Inc, San Diego, CA) and analyzed by FACS. Data are given as means $\pm$ SD of values obtained from three replicates. *, difference from unstimulated cells at day 0, $p < 0.01$; #, difference from GM-CSF/IL-4-stimulated cells; $p < 0.01$.

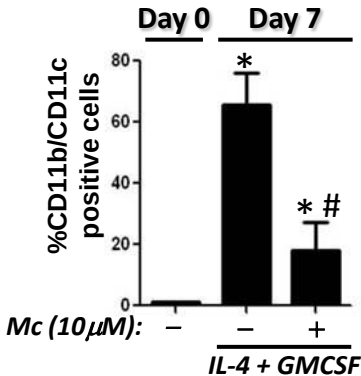


Fig. S2. *Minocycline treatment reduces the efficiency of dendritic cell to induce T cell proliferation.* DCs were generated in the presence (■) or absence (●) of 10 μ M minocycline as described in Fig. S1. DCs, at different densities, were then co-cultured in the absence of minocycline with CD8⁺ or CD4⁺ T cells isolated from OT-I or OT-II transgenic mice, respectively. Proliferation of CD8⁺ and CD4⁺ T cells was stimulated with specific OVA peptides. Cells were pulsed with [³H]thymidine for 18 h before harvest and counted on a beta counter. *, difference from stimulated cells co-cultured with DCs that were not treated with minocycline; $p < 0.01$.

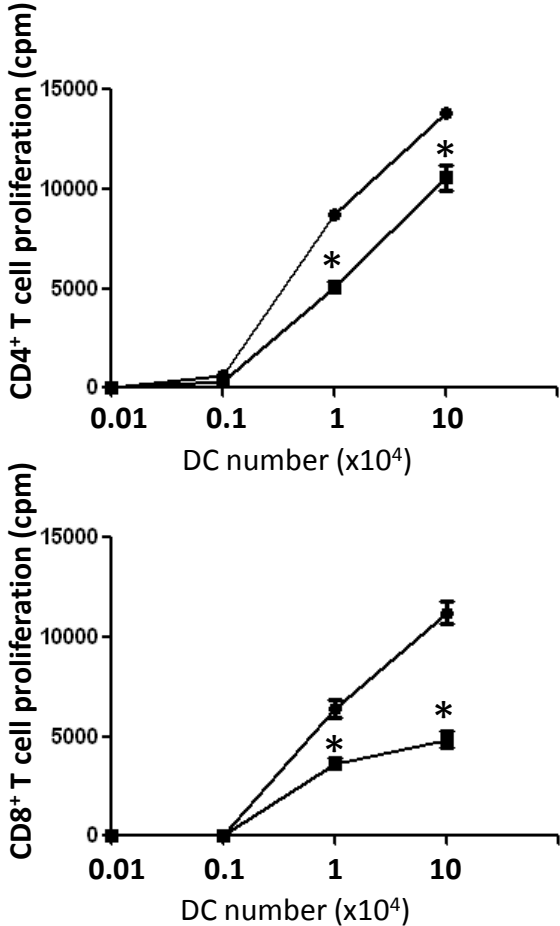


Fig. S3. *Minocycline does not prevent NAD⁺ depletion upon H₂O₂ exposure in the monocytic cell line U937.* Cells, cultured in 24 well-plates, were treated with 0, 0.1, 1, or 10 μ M minocycline 10 min prior exposure to 500 μ M H₂O₂. Cells were then collected after an additional 30 min incubation in the presence of the drug. Cell pellets were subjected to NAD⁺ extraction followed by an assessment of NAD⁺ levels using a cycling enzyme assay as described in our previous publications. Data are given as means $\pm$ SD of values obtained from quadruplicates. *, difference from control untreated cells, $p < 0.05$; #, difference from H₂O₂-treated cells, $p < 0.05$.

