MINOCYCLINE BLOCKS ASTHMA-ASSOCIATED INFLAMMATION IN PART BY INTERFERING WITH THE T CELL RECEPTOR-NF-κB-GATA-3-IL-4 AXIS WITHOUT A PROMINENT EFFECT ON PARP


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Running title: Modulation of allergic lung inflammation by minocycline

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Keywords: Tetracycline; PARP; Asthma; T cell receptor; IgE; NF-κB; Interleukins; Signal transduction.

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 TCR/NFAT1/IL-2 pathway without a direct effect on PARP activity.

Conclusion: The results provide new insight on the mechanism of action of minocycline.

Significance: The present results provide further support to the therapeutic potential of minocycline in reducing or preventing allergen-induced asthma symptoms.

SUMMARY

Minocycline protects against asthma independently of its antibiotic function and was recently reported as a potent 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 lungs of minocycline-treated/ovalbumin-challenged mice correlated with decreased oxidative DNA damage. Minocycline’s effect 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 suggesting. 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 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 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 be clearly delineated. 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 was associated with the ability of the drug to inhibit PARP-1 at nanomolar concentrations (9). Our group has extensively studied the role of PARP-1 in inflammatory situations. We previously reported 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. reported that minocycline also protects against simulated ischemia–reperfusion injury in cardiac myocytes by inhibiting PARP-1 (14). 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, the present 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, the 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 Laboratories, Bar Harbor, ME) were housed in a specific-pathogen free facility at LSUHSC, New Orleans, LA, and allowed unlimited access to sterilized chow and water. All experimental protocols were approved by the LSUHSC Animal Care & Use Committee. Six-week old mice (n≥6 for each experimental condition) were sensitized with i.p. injections of 100 µg Grade V chicken ovalbumin (OVA) (Sigma-Aldrich, St. Louis MO) mixed with 2 mg aluminum hydroxide in saline 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, St Louis MO) or saline i.p. 1 h prior to the OVA challenge. This dose of minocycline was selected based on studies that demonstrated its protective effect in a variety of models of inflammation (17-19). Mice were then left to recover and sacrificed 24 h or 48 h later for bronchio-alveolar lavage (BAL) or lung fixation and processing for histological analysis. Some mice received intra-tracheal administration of 10 mg/kg N-methyl-N′-nitro-N-nitroso-guanidine (MNNG) (Sigma-Aldrich) under anesthesia and sacrificed 1 hour 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 System, Falls Church, VA). In brief, the baseline readings were taken and averaged for 3 min after animals were placed in a barometric plethysmographic chamber. Normal saline or increasing concentrations (12.5 to 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 hematoxylin and eosin (H&E) or Periodic Acid-Schiff (PAS) staining using standard protocols, or to immunohistochemistry (IHC) with antibodies to poly(ADP-ribose) (PAR) or 8oxodG as described previously (20,21).

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, GMCSF and MCP-1 according to the manufacturer’s instructions and specifications. 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 standard enzymatic digestion protocol. Assays and methods were conducted as described (20) (21,22). CD4+ T cells were purified from 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) per the manufacturer’s instructions. The cells were then cultured and TCR stimulation was performed with a combination of plate bound CD3 (1µg/ml) and soluble CD28 (2µg/ml) antibodies (from BD Pharmingen, San Jose, CA) in the absence or presence of minocycline. The cell culture supernatant was then collected at 96 hours 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 immune-fluorescence 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-κB (S536), phospho-GSK3β-α (S21/9), phospho-Src (T416), phospho-ERK1/2 (T202/Y204), phospho-p38 MAPK (T180/Y182), phospho-IκBα (S32/36), phospho-IKKα/β (S176/180), total IKKα/β, total-p65 NF-κB, total ERK1/2 (all from Cell Signaling Technology, Beverly, MA), 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 co-cultured at a 2:1 ratio (2 × 10⁶ B cells: 1 × 10⁶ CD4⁺ T cells per well) in a 48-well plate in the presence of 0, 0.1, or 10 µM minocycline with/without OVA (200 µg/ml) alone or 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, Raleigh, NC) as described above.

Ferric reducing ability assay and poly(ADP-ribosylation)ation in Vitro. The ferric reducing ability of minocycline was measured essentially as previously described (22). The anti-oxidant capacity of minocycline was calculated from the linear calibration curve and expressed as mmol FeSO₄ equivalents. As for the poly(ADP-ribosylation)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 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 means ± SD 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 ANOVA 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—Figure 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 i.p. injection of minocycline 1 hour prior to challenge. Figure 1B shows that the OVA-induced increase in eosinophils in the lungs of C57BL/6J 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. 1C 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—Figure 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 BALF (Fig. 1G) and sera (Fig. 1H) 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. Figure 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. 2A and B). Poly(ADP-ribose) immunoreactivity was largely absent in lung tissues of control animals (Figure 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 \( N\)-methyl-\( N'\)-nitro-\( N\)-nitroso-guanidine (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 hour later. Lungs were removed, and protein extracts were prepared followed by immunoblot analysis with antibodies to poly(ADP-ribose). Figure 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.

We previously reported that the excessive activation of PARP-1 upon allergen exposure is the result of ROS and RNS production (11). We next tested whether minocycline inhibited PARP activation in response to direct exposure to \( \text{H}_2\text{O}_2 \), a reliable ROS generator within cells and a DNA damaging agent. Figure 3D and E show that a 10 min treatment of lung cells, in vitro, with 500 \( \mu \)M \( \text{H}_2\text{O}_2 \) induced a robust PARP activation as revealed by immunofluorescence using anti-PAR antibodies to poly(ADP-ribose). Minocycline moderately inhibited PARP, however, at a concentration 100 times higher than that of TIQ-A.

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 \( \text{Fe}^{3+}\)-to-\( \text{Fe}^{2+} \) reducing activities of minocycline in a cell-free system. Figure 2F shows that minocycline displayed antioxidant activity at doses higher than 10 \( \mu \)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. Figure 2G and 2H show that minocycline treatment markedly reduced 8oxodG immunoreactivity compared to 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 co-cultured with CD4\(^+\) T cells in the presence of 0, 0.1, or 10 \( \mu \)M minocycline. Figure 3A shows that minocycline at the 10 \( \mu \)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 alteration 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 \( \mu \)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.

8oxodG, a well-recognized marker of tissue DNA damage.
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. Figure 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 axis but rather it did so upstream of such 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).

Modulation of the TCR-NF-κB-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-κB (34). Of these factors, NF-κB appears to play a pivotal role in GATA-3 gene expression (35). Fig. 4B and 4C show that all aforementioned signaling events were activated by TCR-stimulation in our experimental system. Only NF-κB activation, assessed by the phosphorylation status of the p65 subunit of the transcription factor at serine-536 and of I-κBα at serine residues-32/36, was reduced by minocycline treatment upon TCR-stimulation. Interestingly, despite the effect on p65 NF-κB and I-κBα phosphorylation, minocycline did not effect 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-κB signal transduction. Although the phosphorylation of GSK and p38 MAPK remained unaltered in the presence of minocycline, the drug promoted a slightly higher phosphorylation of Src (Fig. 4B) and ERK1/2 (Fig. 4C). The increase in ERK1/2 phosphorylation appeared to occur even in the absence of TCR stimulation (Fig. 4C).

Minocycline blocks cytokine-induced NF-κB activation and expression of NF-κB-dependent genes without preventing nuclear translocation of the transcription factor—Untreated asthmatics exhibit greater levels of NF-κB activation in immune as well as in structural cells such as lung epithelial cells, goblet cells, smooth muscle cells, and fibroblasts compared to individuals with controlled disease (36-38). In addition to GATA-3, NF-κB 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-κB activation was restricted to TCR stimulation or can affect other
stimuli such as TNF-α. TNF-α was selected as an inducer in this experiment given its potency in inducing NF-κB-dependent genes and involvement in lung inflammation and asthma (40,41). Figure 5A shows that TNF-α treatment of lung fibroblasts induced rapid degradation of the NF-κB inhibitor I-κBα, an effect that was largely unaltered by minocycline treatment despite a moderate effect on the phosphorylation status of I-κBα. Surprisingly, however, minocycline treatment completely blocked p65 NF-κB phosphorylation at serine-536. Despite such robust inhibitory effects on NF-κB phosphorylation, the drug did not prevent the nuclear translocation of the transcription factor in response to TNF-α (Fig. 5B). Inhibition of NF-κB phosphorylation coincided, however, with a marked reduction in the expression of the NF-κB-dependent genes such as ICAM-1, VCAM-1, and iNOS (Fig. 5C). These results suggest that minocycline may inhibit NF-κB activation in response to more than one stimulus and such inhibition appeared to reside at the phosphorylation level of the protein, an event that is crucial for its transcriptional activity.

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 (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 and colleagues [reviewed (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 animal 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 the present study, a single i.p. 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’ group recently reported that oral delivery of the drug required much higher doses to drastically reduce BPO(14)-KLH-specific IgE (6). 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 provides new light on such connection and further support the broader effect of the drug but places TCR stimulation-NF-κB 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 (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 effect was associated primarily with the established inhibitory effect of tetracycline derivatives on MMPs. 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 that display p38 MAPK phosphorylation, as assessed by FACS analysis suggesting a mechanistic link between the drug’s function 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 (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 broader effect especially given the potential anti-oxidant property of these drugs and more importantly the high dosages used by the aforementioned studies. Our current 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 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 activity rather than activation 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 while 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 observation is consistent with that reported by Szeto et al 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 PMA and ionomycin (50); it is noteworthy that the concentration of minocycline used in the current 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 (Supplementary 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 (Supplementary 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 (Supplementary 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 airway AHR. The effect of minocycline on AHR is consistent with those reported by Lee et al. in the toluene diisocyanate-induced asthma model (43). 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.

References


**Footnotes:**

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The abbreviations used are: IL, interleukin; OVA, ovalbumin; TNF, tumor necrosis factor; BAL, bronchoalveolar lavage; TCR, T cell receptor; NFAT1, nuclear factor of activated T cell; STAT-6, signal transducer and activator of transcription-6; IHC, immunohistochemistry;

**Figure legends**

**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 i.p. 10 mg/kg of minocycline (Mc) 1 hour 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. (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 means ± SD of values from at least six mice per group. (C) Lung sections from the different experimental groups were subjected to PAS staining. (D) The extent of mucus production (histological mucin index) was assessed as described in the Methods. 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. Data are given as means ± SD of values obtained from at least six mice per group. *, difference from unchallenged mice, p < 0.01; #, difference from OVA-
challenged mice; \( p < 0.01 \). (K) Mice were sensitized to OVA and subjected to a challenge with aerosolized OVA with (▲) or (■) without an i.p. 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 baseline and expressed as mean ± SEM 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; bars: 4 \( \mu m \).

**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 IHC staining with antibodies to poly(ADP-ribose) moieties of PARP-1-modified proteins (PAR) and observed by light microscopy; bars: 4 \( \mu m \). (B) The extent of immunoreactivity was then assessed using Image Pro-Plus. Data is 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 intra-tracheal administration of 10 mg/kg MNNG under anesthesia and were sacrificed 1 hour later. A group of mice received 10 mg/kg minocycline 1 hour 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 \( \mu M \) \( H_2O_2 \) for 10 min in the presence of different doses of minocycline (Mc). Cells were then fixed and subjected to immunofluorescence with antibodies to PAR. Note that the cytosolic staining is non-specific 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. (F) Anti-oxidant capacity of minocycline based upon 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 ± SD 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 (Mc) treatment were subjected to IHC staining with antibodies to 8oxodG and observed by light microscopy; bars: 4 \( \mu m \). (H) the extent of immunoreactivity was then assessed using Image Pro-Plus. Data is expressed as fold increase of control. *, difference from control unchallenged mice, \( p < 0.05 \); #, difference from OVA-challenged mice, \( p < 0.05 \).

**Figure 3.** Effects of minocycline on NF-κB-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 co-cultured with CD4+ T cells and stimulated with OVA (200 \( \mu g/ml \)) in the presence of 0, 0.1, or 10 \( \mu M \) minocycline. Supernatants were collected after 96 h for the quantification of OVA-specific IgE by sandwich ELISA. Data are given as means ± SD 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\( \mu g/ml \)) and soluble CD28 (2\( \mu g/ml \)) antibodies in the absence or presence of minocycline at dose of 0.1 or 10\( \mu M \). The cell culture supernatants were then collected after 96 hours of incubation for measurement of IL-4 levels. Data are given as means ± SD 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\( \mu M \) 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 \( \beta \)-actin. Data are given as fold change of control (non-stimulated) ± SD normalized to \( \beta \)-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 hours of incubation for measurement of IL-4 levels. Data are given as means ± SD 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 μM 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 (Mc). 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 10μM 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 10μM minocycline. Cells were then fixed and subjected to immunofluorescence staining with antibodies to NFAT1.

**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 10μM 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 GATA-3 or β-actin. Data are given as fold change of control (non-stimulated) ± SD 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 10μM 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 I-κBα (S32/36), IKKα; IKKβ; the phosphorylated form of IKKα/β (S176/180); the phosphorylated form of IKKα/β (S176/177); the phosphorylated form of GSK3 β-α (S21/9), the phosphorylated form of Src (Y416), the phosphorylated form of p38 MAPK (T180/Y182), 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 T202 and Y204 or actin. Note that some of blots are the same and were stripped and re-probed but others were generated using the same samples. The actin for these blots is similar (data not shown).

**Figure 5.** Effects of minocycline on TNF-α-induced NF-κB activation and expression of NF-κB-dependent genes. (A) Lung cells were treated with 10 ng/ml TNF-α for different time intervals in the absence or presence of 10μM minocycline. Protein extracts were prepared and subjected to immunoblot analysis with antibodies to I-κBα, its serine residues-32/36 phosphorylated form ([p]I-κBα), the serine-536 phosphorylated form of p65 NF-κB ([p]p65NF-κB), 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-κB. (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.
Figure 3

A

B

C

D

E

F

G
**Figure 4**

**A**

GATA-3

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**B**

CD3/CD28 (min):

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- [p]p65 NF-κB
- Total p65 NF-κB
- [p]IkBα (S32/36)
- [p]IkBα/β (S176/180)
- [p]IkBα/β (S176/177)
- IKKα
- IKKβ
- [p]GSK3 β-α (S21/9)
- [p]Src (Y416)
- [p]p38 MAPK
- Actin

**C**

CD3/CD28 (min):

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<td>+</td>
<td>-</td>
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- [p]ERK1/2
- Total ERK1/2
- Actin
Figure 5

Panel A: Graph showing the effect of different concentrations of Mc on TNF-α (min) on I-κBα, [p]I-κBα, [p]p65 NF-κB, and Actin. The x-axis represents the concentration of Mc (μM) and the y-axis represents TNF-α (min).

Panel B: Images showing the effect of TNF-α (min) on control, No Mc, and Mc (10μM) treated cells, stained for p65 NF-κB.

Panel C: Graph showing the effect of different concentrations of Mc on TNF-α (3 h) on ICAM-1, VCAM-1, iNOS, and Actin. The x-axis represents the concentration of Mc (μM) and the y-axis represents TNF-α (3 h).
Minocycline blocks asthma-associated inflammation in part by interfering with the T Cell receptor-NF-κB-GATA-3-IL-4 axis without a prominent effect on PARP

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