Minocycline Down-regulates MHC II Expression in Microglia and Macrophages through Inhibition of IRF-1 and Protein Kinase C (PKC) \( \alpha/\beta \) II

Maria Nikodemova, Jyoti J. Watters, Samuel J. Jackson, Shaun K. Yang, and Ian D. Duncan

From the Departments of \(^\diamond\) Medical Sciences and \(\diamond\) Comparative Biosciences, School of Veterinary Medicine, University of Wisconsin, Madison, Wisconsin 53706

Experimental allergic encephalomyelitis, an autoimmune disorder mediated by T cells, results in demyelination, inflammation, and axonal loss in the central nervous system (CNS). Microglia play a critical role in major histocompatibility complex class II (MHC II)-dependent antigen presentation and in reactivation of CNS-infiltrated encephalitogenic T cells. Minocycline, a tetracycline antibiotic, has profound anti-inflammatory properties and is experimentally used for treatment of many CNS disorders; however, the mechanisms involved in minocycline effects remain unknown. We show that administration of minocycline for 2 weeks ameliorated clinical severity of experimental allergic encephalomyelitis, an effect that partially involves the down-regulation of MHC II proteins in the spinal cord. Therefore, we sought to elucidate the molecular mechanisms of minocycline inhibitory effects on MHC II expression in microglia. Although complex, the co-activator class II transactivator (CIITA) is a key regulator of MHC II expression. Here we show that minocycline inhibited interferon \( \gamma \) (IFN \( \gamma \))-induced CIITA and MHC II mRNA. Interestingly, however, it was without effect on STAT1 phosphorylation or IRF-1 expression, transcription factors that are activated by IFN \( \gamma \) and necessary for CIITA expression. Further experiments revealed that MHC II expression is down-regulated in the presence of the PKC\( \alpha/\beta \) II inhibitor Go6976. Minocycline inhibited IFN \( \gamma \)-induced PKC\( \alpha/\beta \) II phosphorylation and the nuclear translocation of both PKC\( \alpha/\beta \) II and IRF-1 that subsequently inhibits CIITA expression. Our present data delineate a molecular pathway of minocycline action that includes inhibitory effects on PKC\( \alpha/\beta \) II and transcription factors that regulate the expression of critical inflammatory genes such as MHC II. Such a fundamental mechanism may underlie the pleiotropic effects of minocycline in CNS inflammatory disorders.

Experimental allergic encephalomyelitis (EAE),\(^2\) an animal model of multiple sclerosis (MS), is an autoimmune disorder characterized by T cell-mediated inflammation, demyelination, and axonal loss in the CNS. Susceptibility to EAE and MS is associated with the major histocompatibility complex class II (MHC II) genes (1, 2), suggesting that presentation of antigens on MHC II plays an important role in CD4\(^+\) T cell activation and disease initiation. After priming of encephalitogenic T cells in the periphery, they migrate into the CNS, where they trigger inflammatory responses including microglial activation. To sustain their activation, the self-reactive T cells have to be reactivated in the CNS by encountering their cognate antigen bound to MHC II. Microglia, the resident innate immune cells of the CNS, have been shown to be the main antigen-presenting cells of the CNS (3, 4). They normally express low levels of MHC II proteins; however, in inflammatory or neurodegenerative conditions, activated microglia highly up-regulate MHC II and co-stimulatory molecules (5). It was recently shown that microglial paralysis inhibited the development and maintenance of inflammatory CNS lesions, providing the first direct evidence of microglial significance in EAE pathology (6). Microglial activation precedes the clinical onset of EAE and infiltration of peripheral myeloid cells into the CNS, suggesting that they play a critical role in the induction and progression of EAE (7). Therefore, the inhibition of microglial activation and MHC II expression may be therapeutically significant in MS.

In recent years, minocycline, a semisynthetic antibiotic of the tetracycline family, has emerged as a potent anti-inflammatory drug that has been shown to be beneficial in animal models of many CNS disorders including ischemia (8), Huntington disease (9), Parkinson disease (10), amyotrophic lateral sclerosis (11), and MS (12). We have previously shown that minocycline ameliorated the clinical course of EAE and decreased MHC II expression in the spinal cord (13); however, the exact molecular mechanisms underlying these anti-inflammatory effects of minocycline remain unknown.

Therefore, in this study, we investigated the molecular mechanisms of minocycline effects on MHC II expression both \( in vivo \) in an EAE model and \( in vitro \) using primary rat microglial and macrophage cultures. Since MHC II expression in microglia is requisite for T cell reactivation, understanding the mechanism whereby minocycline represses MHC II expression may elucidate critical pathways that can be targeted by other thera-

\(^{1}\) This work was supported by the National Multiple Sclerosis Society Grant RG3472A8. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

\(^{2}\) To whom correspondence should be addressed: Dept. of Medical Sciences, School of Veterinary Medicine, University of Wisconsin, 2015 Linden Drive, Madison, WI 53706. Tel.: 608-263-9829; Fax: 608-265-2474; E-mail: nikodemova@svm.vetmed.wisc.edu.

\(^{3}\) The abbreviations used are: EAE, experimental allergic encephalomyelitis; CNS, central nervous system; MHC, major histocompatibility complex; CIITA, class II transactivator; IFN, interferon; IRF-1, IFN \( \gamma \) regulatory factor; PKC, protein kinase C; MS, multiple sclerosis; MOG, myelin oligodendrocyte glycoprotein; MOPS, 4-morpholinepropanesulfonic acid; RT, reverse transcription; JAK, Janus kinase; STAT, signal transducers and activators of transcription; MAP, mitogen-activated protein.
Minocycline Inhibits MHC II Expression

Animals—Female Dark Agouti rats and Sprague-Dawley rats purchased from Harlan Sprague Dawley, Inc. (Madison, WI) were housed under standard conditions. All experiments were conducted with approval of Research Animal Resources Center of the University of Wisconsin.

MOG Purification—DHS5α cells transfected with pQE12 vector with rat myelin oligodendrocyte glycoprotein (MOG) insert (amino acids 1–125) with a His6 sequence at the 3’ end was a generous gift of N. Ruddle (Yale University School of Medicine). After isopropyl-1-thio-β-D-galactopyranoside induction (4 h) of protein expression, bacterial extract was prepared using BugBuster reagent (Novagen, Madison, WI). MOG protein was purified from cell extract using His-Bind columns (Novagen) under denaturing conditions following the manufacturer’s protocol. Isolated proteins were then dialyzed against acetate buffer for 24 h using Slide-A-Lyzer dialysis cassette (Pierce). To determine the purity of isolated MOG, proteins were run on 10% SDS-PAGE gel and stained with GelCode blue stain reagent (Pierce).

Induction of EAE and Minocycline Treatment—EAE was induced in 150–175 g of Dark Agouti female rats by immunization with 10 µg of MOG in complete Freund’s adjuvant. MOG protein in phosphate-buffered saline was emulsified with an equal volume of complete Freund’s adjuvant, to a final concentration of 10 µg of MOG and 50 µg of Mycobacterium tuberculosis H37Ra in 100 µl of emulsion. Rats were immunized by subcutaneous injection of a 100-µl emulsion at the base of the tail. Control animals were injected with complete Freund’s adjuvant only. Animals were weighed daily and examined for clinical signs of EAE, scored on the following scale: 0.5, partial loss of tail tone; 1, complete tail atony; 2, hind limb weakness; 3, hind limb paralysis; 4, moribund; 5, death. The first clinical signs usually occurred between 10 and 12 days after immunization. Minocycline was administered intraperitoneally twice a day at the first day of clinical signs and then once a day for 14 days at the dose of 45 mg/kg.

Immunohistochemistry—Animals underwent transcardiac perfusion with phosphate-buffered saline followed by 4% paraformaldehyde. Spinal cords were dissected and cut into ∼2-mm segments and post-fixed in 4% paraformaldehyde overnight. Blocks were cryoprotected in 15% and then 30% sucrose for 24 h followed by embedding into OCT compound (Sakura, Tokyo, Japan) and stored at −80 °C until further use. 14-µm sections were cut from the blocks with a cryostat and used for immunostaining and hematoxylin and eosin staining. The quantification of immunostaining was performed using MetaView Software. Immunopositive areas were measured at a set threshold and expressed as the percentage of the total area of the section.

Primary Microglial Cultures—Primary rat microglial cultures were prepared by the shaking method from 4-day-old Sprague-Dawley rat brain as we described previously (15). Briefly, brains were dissected, minced, and trypsinized with 0.25% trypsin-EDTA for 20 min at 37 °C. The reaction was stopped by the addition of horse serum, and the tissue was triturated with a Pasteur pipette and filtered consecutively through 70- and 45-µm pore size nylon cell strainers. Cells were resuspended in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 100 units/ml penicillin/streptomycin and plated in 80-mm2 tissue culture flasks. After 10 days, the flask was gently shaken for 1 h, and medium was harvested and centrifuged for 10 min at 1000 × g to collect microglial cells. The microglia were resuspended in the medium described above and plated in either 12-well plates at a density of 750,000 cells/well or 8-chamber slides at a density of 150,000 cells/chamber.

Isolation of Peritoneal Macrophages—Peritoneal macrophages were isolated from Sprague-Dawley rats by flushing the peritoneal cavity with 30 ml of ice-cold Hanks’ balanced salt solution. After centrifugation, the cells were resuspended in RPMI medium supplemented with 10% fetal bovine serum, 10 mM MOPS, 50 µM 2-mercaptoethanol, 100 units/ml penicillin/streptomycin and plated on a 12-well plate at a density of 800,000 cells/well. After a 1-h incubation at 37 °C, the medium was changed to remove non-adherent cells.

Western Blot—Whole cell extract was prepared by lysing cell in 2 × lysis buffer (20 mM Tris, 2 mM EDTA, 1 mM Na3VO4, 2 mM dithiothreitol, 2% SDS, 20% glycerol). Proteins (5–20 µg/lane) were separated by 10% SDS-PAGE and transferred to Immobilon-P polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA). Membranes were blocked in 5% nonfat milk for 1 h at room temperature followed by incubation with primary antibodies. Anti-phospho-STAT1 (Cell Signaling, Beverly, MA) and anti-phospho-PKCα/β (Cell Signaling) antibodies were used at a 1:1000 dilution overnight at 4 °C. OX6 antibody (anti-MHC II) and IRF-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) were used at a 1:500 dilution overnight at 4 °C. After washing, the membranes were incubated with appropriate secondary horseradish peroxidase-conjugated antibodies (anti-rabbit, anti-mouse or anti-goat, 1:8000, Santa Cruz Biotechnology) for 1 h at room temperature followed by detection with chemiluminescence. An image analysis system was used to collect and analyze data obtained from the chemiluminescent light emission (UVP Inc., Upland, CA). After analysis, membranes were stripped with Restore Western blot stripping buffer (Pierce) for 30 min at 37 °C and reprobed with anti-tubulin antibody (1:500, Santa Cruz Biotechnology), anti-STAT1 (1:1000, Cell Signaling), or anti-PKCα (1:2000, Santa Cruz Biotechnology) for 1 h at 37 °C. Although the PKCα antibody is specific for the PKCα isoform, it also has 80% cross-reactivity with the PKCβ isoform.

Immunocytochemistry—Primary microglial cells or macrophages were treated as described above. Cells were fixed with
Minocycline Inhibits MHC II Expression

cold 100% methanol for 10 min followed by a 10-min incubation in 10% normal goat serum to block nonspecific binding. Cells were stained with OX42 antibody (CD11b) and OX6 antibody (MHC II) at 1:1000 or 1:200 dilution, respectively, for 1 h at room temperature followed by a 30-min incubation with anti-mouse secondary antibodies conjugated with Alexa Fluor 488 or 595 (Molecular Probes, Eugene, OR). Nuclei were stained with 4',6-diamidino-2-phenylindole.

Nuclear Extract Preparation—Primary microglial cells were treated for 24 h as described above. Nuclear extracts were prepared using a NucBuster protein extraction kit (Novagen) according to the manufacturer’s protocol. Nuclear proteins were then separated by 10% SDS-PAGE and analyzed by Western blot.

RNA Isolation and RT-PCR—Primary microglial cells were grown on 6-well plates at the density of 1.5 \times 10^6 cells/well. After a 30-min preincubation with minocycline, cells were treated with 100 units/ml IFN{\gamma} with or without minocycline (100 \muM) for 24 h. Cells were lysed in 1 ml of TRizol reagent (Invitrogen), and total RNA was isolated according to the manufacturer’s protocol. RNA was treated with DNase I (Invitrogen) for 15 min at room temperature, and 1 \mu g of total RNA was then used for reverse transcription (RT) using oligo(dT) primers and MMLV reverse transcriptase (Retroscript kit, Ambion, Austin, TX). Following RT, 3 \mu l of RT reaction was utilized for 35 cycles of PCR using SuperTaq polymerase (Ambion). The sequences of primers were: 5’-CACAAAGGACCTTGCA-3’ and 5’-CACATCTGGGCTGTGA-3’ for CIITA; 5’-TGTGCAGACGCAGCA-3’ and 5’-TCTGCAATTTCCAGTGA-3’ for MHC II; 5’-AGACAGCCGATCTCTTGT-3’ and 5’-CCACAGTCTCTGGAGTGGCA-3’ for glyceraldehyde-3-phosphate dehydrogenase. The PCR products were separated on a 1% agarose gel and visualized by ethidium bromide staining.

Data Analysis—Data were analyzed by one-way analysis of variance and Student’s t test using SigmaStat software. All in vitro experiments were repeated at least four times, and data represent the mean \pm S.E. Differences between experimental groups were considered statistically significant at \( p < 0.05 \). The clinical scores of minocycline-treated and untreated groups were analyzed by a two-way analysis of variance.

RESULTS

Minocycline Attenuates the Clinical Course of EAE—Immunization of Dark Agouti rats with 10 \mu g of MOG resulted in a biphasic disease course with the onset occurring 10–12 days after immunization. The clinical course of disease was less severe in minocycline-treated animals when compared with the non-treated group (Fig. 1). Hematoxylin and eosin staining revealed a significant infiltration of leukocytes into the parenchyma of both the white and the gray matter of the spinal cord in EAE, an effect that was ameliorated by minocycline (Fig. 2). Both the number and the size of lesions in the spinal cord were decreased by minocycline treatment (Fig. 2).

Minocycline Decreases MHC II Expression in EAE—MOG-induced EAE in Dark Agouti rats is characterized by severe inflammatory lesions found predominantly in the spinal cord accompanied by a strong microglial response. In naive animals (control), the number of CD11b\(^+\) cells (microglial marker; Fig. 3, A and C) is 10 times greater than the number of MHC II\(^+\) cells (Fig. 3, B and C), suggesting that most microglia do not express MHC II in the resting state. Only a few MHC II-positive cells with a ramified morphology can be found localized mostly in the white matter of the spinal cord (Fig. 3B, arrowhead). At the onset of the disease, we did not observe any increase in the total number of CD11b\(^+\) cells; however, the density of CD11b\(^+\) cells was increased in lesion sites located adjacent to blood vessels (Fig. 3A, arrows, and 3C). Also, we noticed a change in morphology of microglial cells from ramified to amoeboid, suggesting phagocytic properties. There was a significant increase in the number of MHCII\(^+\) cells at the onset of the disease that correlated with the number of CD11b\(^+\) cells. 2 weeks after the
clinical onset of EAE, the number of CD11b<sup>+</sup> and MHC II<sup>+</sup> cells was three times higher than at the onset. Treatment of animals with minocycline for 2 weeks decreased the number of CD11b<sup>+</sup> cells by 24%, suggesting decreased microglial proliferation and/or decreased migration of macrophages from the periphery into the CNS parenchyma. The number of MHC II<sup>+</sup> cells was reduced by 42% in the minocycline group, although MHC II levels were still significantly higher than those observed upon disease onset when minocycline treatment was initiated.

**Minocycline Decreases MHC II and CIITA Expression in Microglia**—As was observed in vivo, primary microglial cells are CD11b<sup>+</sup> (Fig. 4A), but they do not express MHC II under...
Minocycline Inhibits MHC II Expression

---

**FIGURE 4. Minocycline and Gö6976 decrease MHC II expression in microglia.** A, the purity of primary microglial culture was more than 90% as assessed by an immunostaining for CD11b, a microglial marker (green), 4′,6-Diamidino-2-phenylindole (blue) was used to stain nuclei. MHC II expression (OX-6 antibody, red) in primary microglial cells was induced by IFNγ (100 units/ml) stimulation for 24 h. The presence of minocycline (100 μM) or Gö6976 (1.3 μM), a PKCε inhibitor, decreased MHC II expression by 55%. A graph in the lower right corner is a quantification of the MHC II+ cells counted in four random areas for each treatment. MHCII+ cells are expressed as the percentage of the total number of cells. M, minocycline; Gö, Gö6976, PKCε inhibitor; **, p < 0.01 versus IFNγ. B, primary microglial cells were exposed to IFNγ (100 units/ml) for 24 h and minocycline (Mino, 100 μM) or Gö6976 (Go, 1.3 μM) as indicated in the figure. Cells were then lysed, and the protein levels of MHC II were determined by Western blot. Minocycline decreased MHC II expression by 47%. In the presence of Gö6976, the MHC II expression was reduced by 80%. Four independent blots were quantified using LabWorks software. IFNγ treatment was considered as 100% stimulation of MHC II expression to compare band densities in different blots. **, p < 0.01 versus IFNγ.

---

resting conditions. Treatment of cells with IFNγ (100 units/ml) for 24 h induced MHC II expression in 30% of the cells, and this was decreased by 55% upon treatment with minocycline (Fig. 4A). This correlated well with the quantification of MHC II proteins by Western blot, which revealed an almost 45% decrease in MHC II proteins after minocycline treatment (Fig. 4B). Minocycline also significantly decreased MHC II mRNA levels, suggesting that the inhibitory effects of minocycline occur at the transcriptional level (Fig. 5). Because the co-activator CIITA, an inducible non-DNA-binding protein, is vital for MHC II gene expression (see Fig. 9), we investigated the ability of minocycline to affect CIITA expression as a potential mechanism of minocycline inhibitory effects on MHC II mRNA. Minocycline significantly decreased IFNγ-induced CIITA mRNA, suggesting that this drug might target signaling pathways involved in CIITA expression.

Minocycline Does Not Affect STAT1 or IRF-1—To determine the mechanism of minocycline inhibitory effects on MHC II expression, we investigated its effects on the intracellular signaling cascade involved in IFNγ-induced CIITA and MHC II expression. The JAK-STAT signaling pathway is central to IFNγ-induced responses; therefore, we first examined the effect of minocycline on the activation of STAT1 (Fig. 6A). Treatment of microglia with IFNγ for 15 min resulted in STAT1 activation, which was assessed by phospho-specific antibodies. Surprisingly, minocycline did not affect phosphorylation of STAT1, indicating that its inhibitory effect on MHC II expression likely occurs downstream of this protein. We next investigated minocycline effects on IRF-1 expression, a transcription factor essential for CIITA expression, and thus, for MHC II production. In IFNγ-activated microglia, the levels of IRF-1 protein were two times higher when compared with unstimulated cells, and this increase was not affected by minocycline (Fig. 6B).

PKCε Is Involved in MHC II Expression on Microglia—PKCε has been shown to modulate IFNγ-induced expression of MHC II in the mouse macrophage cell line RAW 264.7 (16). In agreement with this, our previous work implicated an effect of minocycline on inhibition of PKC activity in microglial BV-2

---

15212 JOURNAL OF BIOLOGICAL CHEMISTRY

VOLUME 282 • NUMBER 20 • MAY 18, 2007
Minocycline Inhibits Phosphorylation and Nuclear Translocation of PKC_α/βII and IRF-1—Minocycline reduced IFNγ-stimulated phosphorylation of PKC_α/βII, suggesting that minocycline may modulate PKC activity (Fig. 6C). Interestingly, the increase in IFNγ-induced IRF-1 levels was prevented by Gö6976, suggesting that PKC isoforms that are not targeted by minocycline might also be involved in IRF-1 expression.

Upon IFNγ stimulation, IRF-1 translocates to the nucleus, where it binds to the promoter IV region of CIITA, a transactivator critical for MHC II transcription. The recent study by Giroux et al. (16) showed that in RAW 264.7 macrophages, PKC_α translocates to the nucleus together with IRF-1, where it increases IRF-1 transcriptional activity after IFNγ stimulation. So we sought to evaluate in microglia the effects of minocycline on the nuclear transfer of PKC_α/βII and IRF-1 since the PKC inhibitor Gö6976 repressed translocation of both proteins (Fig. 7). IFNγ-induced translocation of both proteins into the nucleus was prevented by minocycline treatment (Fig. 7), implying that there is decreased IRF-1 transcriptional activity in the presence of minocycline.

Minocycline Inhibits IFNγ-independent MHC II Expression—Since our data show a potent inhibitory effect of minocycline on IFNγ-inducible MHC II expression in microglia, we were interested in determining whether minocycline could also affect IFNγ-independent MHC II expression. Peritoneal macrophages isolated from naïve adult Sprague-Dawley rats express high levels of MHC II proteins (Fig. 8A). In the presence of the PKC inhibitor Gö6976 MHC II expression was abolished, suggesting that in addition to IFNγ-inducible MHC II expression, PKC_α is also involved in IFNγ-independent MHC II production. As observed in microglia, minocycline also inhibited PKC_α phosphorylation in macrophages (Fig. 8B), suggesting that its inhibitory effect on MHC II expression is mediated via interactions with PKC_α.

**DISCUSSION**

Minocycline has profound anti-inflammatory properties quite separate from its antibiotic function. Since minocycline has the greatest permeability of all tetracyclines through the blood-brain barrier, this drug is well suited for treatment of CNS disorders. In EAE, minocycline treatment, which started at the onset of clinical symptoms, considerably decreased the severity of the clinical course of disease. The significant difference in clinical score between the treated and untreated groups was immediately observed after the first day of minocycline treatment. Therapeutically, this is important in MS because minocycline treatment would likely be initiated at the time of clinically active disease. In our study, histological examination of the spinal cord revealed that minocycline reduced both the number and the size of lesions and decreased expression of MHC II in microglia. Microglia are the primary antigen-presenting cells of the CNS, and they are the main immune effector cells mediating this demyelinating disease (4). In EAE, activated microglia differentiate into macrophage-like and dendritic-like cells, and they up-regulate levels of MHC II.
and co-stimulatory proteins that are necessary for antigen presentation to the encephalitogenic T cells (7). Moreover, activated microglia can recruit additional T cells from the periphery, thus amplifying the inflammatory process in the CNS. A number of studies suggest that the immune response induced by immunization with MOG is predominantly mediated by autoreactive CD4+ T cells, although the involvement of CD8+ T cells and B cells has also been shown (17, 18). The ability of microglia to prime naive CD4+ cells and to activate memory CD4+ cells in vitro in an antigen-specific and MHC II-restricted manner is well documented (17, 19, 20). The significance of microglial cells in EAE as well as in other CNS pathologies is apparent, and in recent years, these cells have become an attractive target for development of new therapeutic strategies for CNS disorders.

Although the exact mechanisms of minocycline anti-inflammatory effects are still poorly understood, they may include the inhibition of matrix metalloproteinase-2 activity, the inhibition of inducible nitric oxide synthase, prostaglandin E2, caspase-1, caspase-3, and COX-2 expressions and the impairment of cytokine production (9, 15, 21–23). It has been shown that some of these effects are manifested, at least in part, by the inhibition of MAP kinases (10, 15, 24), although the exact intracellular and molecular mechanisms of minocycline actions on the expression of these genes remain poorly understood. Our results show that inhibition of MHC II expression by minocycline involves the inhibition of PKC that subsequently prevents nuclear translocation of the transcription factor IRF-1, which ultimately controls MHC II expression. Because PKC activity has such pleiotropic effects in cells, the ability of minocycline to affect such a wide array of gene expression may involve the interaction with PKC.
High levels of MHC II molecules are expressed constitutively on professional antigen-presenting cells such as dendritic cells and B-cells, whereas in other cell types, including microglia, MHC II expression can be induced by IFNγ. In MS or EAE, IFNγ is produced by T cells infiltrating into the CNS parenchyma. The regulation of MHC II proteins is complex and occurs mostly at the transcriptional level, involving several transcription factors acting on the MHC II promoter (Fig. 9). However, these factors, although necessary, are not sufficient for activation of MHC II promoter on their own. Recruitment of CIITA, a transcriptional co-activator that binds to the multiprotein complex of transcription factors on the MHC II promoter to initiate its transcription, is absolutely required for both constitutive and IFNγ-inducible MHC II expression (14, 25). CIITA-deficient mice lack inducible MHC II expression and have sparse constitutive MHC II expression on subsets of thymic stromal cells (26). The expression of the CIITA gene is cell-specific and is controlled by the alternative usage of three promoters, depending on cell type (4, 27). Promoter I controls the constitutive expression of CIITA, and subsequently, MHC II in dendritic cells. Promoter III is primarily responsible for controlling constitutive expression in B lymphocytes, and promoter IV is used to regulate the IFNγ-inducible expression of CIITA in cells such as microglia (4). After binding to its membrane receptor, IFNγ activates a signaling cascade leading to the activation of STAT1. STAT1 activation is subsequently involved in the regulation of IRF-1 and CIITA expression. Both STAT1 and IRF-1 transcription factors are necessary for inducible CIITA expression from promoter IV (Fig. 9). In our experiments, IFNγ induced MHC II expression in 30% of microglial cells, which was reduced by more than 50% in the presence of minocycline. Since our results showed that minocycline decreased IFNγ-induced CIITA mRNA levels, we hypothesized that minocycline may affect the activation status of STAT1 and/or IRF-1 in primary microglial cells. However, interestingly, we observed that minocycline did not affect either IFNγ-induced STAT1 phosphorylation or IRF-1 expression, suggesting that these transcription factors are not direct targets for minocycline action.

Several studies have showed that the activation of PKC is required for MHC II expression, and the PKCα isoform specifically has been identified to regulate IFNγ-induced expression of MHC II by modulating the transcriptional activity of IRF-1 on the CIITA promoter in murine RAW264.7 macrophage cells (16). Our present data revealed that PKCα/β is involved in both the constitutive and the IFNγ-inducible expression of MHC II in macrophages and microglia, respectively. We have previously shown that minocycline inhibited PMA (a PKC activator) -induced activation of p38 and JNK1/2 MAP kinases, suggesting that minocycline interfered with PKC activity; however, we did not identify which PKC isoform was affected by minocycline (15). Since it is known that PKCα is involved in the regulation of MHC II expression in macrophages, in this study, we investigated the minocycline effect on this PKC isoform in microglia. Minocycline significantly decreased IFNγ-induced PKCα/β phosphorylation and nuclear translocation of this enzyme. It is difficult to distinguish between PKCα and PKCβ isoforms because commercially available PKCα antibodies have up to 80% cross-reactivity with PKCβ. Nuclear translocation of IRF-1 induced by IFNγ was inhibited in the presence of Gö6976, a PKCα inhibitor, suggesting that this process is PKC-dependent. However, it remains unclear how PKCα facilitates IRF-1 translocation into the nucleus. In addition, we found that minocycline also inhibited nuclear translocation of IRF-1, implicating decreased transcriptional activity of IRF-1 in the nucleus. This observation correlates well with the inhibitory effect of minocycline on CIITA mRNA expression.

Interestingly, we found that PKCα/β is involved in the regulation of both the IFNγ-dependent and the IFNγ-independent MHC II expression; however, further studies are needed to elucidate the exact mechanism of PKCα participation. Nonetheless, the ability of minocycline to generally inhibit IFNγ-independent MHC II expression suggests that this drug might also affect the antigen presentation capacity of professional antigen-presenting cells such as microglia (4). After binding to its membrane receptor, IFNγ activates the JAK-STAT signaling pathway and induces phosphorylation of PKCα/β, resulting in increased expression of IRF-1. STAT1 and IRF-1 are transcription factors that bind to CIITA promoter IV. CIITA is a non-DNA-binding protein and is a key co-activator regulating MHC II expression. Our data show that minocycline reduced IFNγ-induced PKCα/β phosphorylation and inhibited translocation of PKCα and IRF-1 into the nucleus. This resulted in decreased CIITA and MHC II expression. RFX, regulatory factor for X box; CREB, cAMP-response element-binding protein; NF-Y, nuclear factor Y.

**FIGURE 9. Regulation of MHC II expression.** A schematic illustrating signaling pathways involved in MHC II expression. The sites of minocycline inhibitory effects are demonstrated by blunt arrows. After binding to its receptor, IFNγ activates the JAK-STAT signaling pathway and induces phosphorylation of PKCα/β, resulting in increased expression of IRF-1. STAT1 and IRF-1 are transcription factors that bind to CIITA promoter IV. CIITA is a non-DNA-binding protein and is a key co-activator regulating MHC II expression. Our data show that minocycline reduced IFNγ-induced PKCα/β phosphorylation and inhibited translocation of PKCα and IRF-1 into the nucleus. This resulted in decreased CIITA and MHC II expression. RFX, regulatory factor for X box; CREB, cAMP-response element-binding protein; NF-Y, nuclear factor Y.
Minocycline Inhibits MHC II Expression

presenting cells, such as dendritic cells. This process is necessary for the activation of CD4⁺ T cells in the periphery prior to their infiltration to the CNS. In our previous study, the administration of minocycline from the day of immunization in a high dose MOG-induced EAE model resulted in a significant delay in the onset and severity of the clinical course of disease (13), suggesting that the inhibition of MHC II expression on peripheral macrophages and/or dendritic cells could be one of the mechanisms by which minocycline exerted its anti-inflammatory effects in this EAE model.

In conclusion, our data show that minocycline significantly decreased the severity of the clinical course of EAE and that these effects may be at least partially mediated by the inhibitory effect of minocycline on the MHC II expression in microglia. Our in vitro experiments on primary microglial cells revealed that the molecular mechanisms of minocycline action involve the inhibition of IFNγ-induced PKC-α/βII phosphorylation and subsequent inhibition of IRF-1 translocation to the nucleus, resulting in decreased CIITA expression. Decreased expression of this transcriptional co-activator is directly related to the expression of MHC II, providing a potential mechanistic link between the attenuated symptom severity of EAE and decreased antigen presentation capacity of CNS resident microglia.

Acknowledgment—We are grateful to Dr. Zsuzsana Fabry (University of Wisconsin) for critical reading and valuable comments on the manuscript.

REFERENCES
1. Gunther, E., and Walter, L. (2001) Immunogenetics 53, 520–542
2. de Graaf, K. L., Barth, S., Herrmann, M. M., Storch, M. K., Otto, C., Olsson, T., Melms, A., Jung, G., Wissmuller, K. H., and Weissert, R. (2004) J. Immunol. 173, 2792–2802
3. Aloisi, F. (2001) Glia 36, 165–179
4. O’Keefe, G. M., Nguyen, V. T., and Benveniste, E. N. (2002) J. Neurovirol. 8, 496–512
5. Kreutzberg, G. W. (1996) Trends Neurosci. 19, 312–318
6. Heppner, F. L., Greter, M., Marino, D., Falsig, J., Raivich, G., Hovelmeyer, N., Waisman, A., Rulicke, T., Prinz, M., Priller, J., Becher, B., and Aguzzi, A. (2005) Nat. Med. 11, 146–152
7. Ponomarev, E. D., Shriver, L. P., Maresz, K., and Dittel, B. N. (2005) J. Neurosci. Res. 81, 374–389
8. Yrjanheikki, I., Keinanen, R., Pellikka, M., Hokfelt, T., and Koistinaho, J. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 15769–15774
9. Chen, M., Ona, V. O., Li, M., Ferrante, R. J., Fink, K. B., Zhu, S., Bian, J., Guo, L., Farrell, L. A., Hersch, S. M., Hobbs, W., Vonsattel, J. P., Cha, J. H., and Friedlander, R. M. (2000) Nat. Med. 6, 797–801
10. Du, Y., Ma, Z., Lin, S., Dodel, R. C., Gao, F., Bales, K. R., Triathour, L. C., Chernet, E., Perry, K. W., Nelson, D. L., Luecke, S., Phebus, L. A., Bymaster, F. P., and Paul, S. M. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 14669–14674
11. Zhu, S., Stavrovskaya, I. G., Drozda, M., Kim, B. Y., Ona, V., Li, M., Sarang, S., Liu, A. S., Hartley, D. M., Wu du, C., Gullans, S., Ferrante, R. J., Przedborski, S., Kristal, B. S., and Friedlander, R. M. (2002) Nature 417, 74–78
12. Metz, L. M., Zhang, Y., Yeung, M., Patry, D. G., Bell, R. B., Stoian, C. A., Yong, V. W., Patten, S. B., Duquette, P., Antel, J. P., and Mitchell, J. R. (2004) Ann. Neurol. 55, 756
13. Popovic, N., Schubart, A., Goetz, B. D., Zhang, S. C., Linatingon, C., and Duncan, I. D. (2002) Ann. Neurol. 51, 215–223
14. Barton, J. A., and Ting, J. P. (2000) Mol. Cell. Biol. 20, 6185–6194
15. Nikodemova, M., Duncan, I. D., and Watters, J. J. (2006) J. Neurochem. 96, 314–323
16. Giroux, M., Schmidt, M., and Descoteaux, A. (2003) J. Immunol. 171, 4187–4194
17. Behi, M. E., Dubucquoi, S., Lefranc, D., Zephir, H., De Seze, J., Vermersch, P., and Prin, L. (2005) Immunity 23, 11–26
18. McDoole, J., Johnson, A. J., and Pirkko, I. (2006) Neurot. Res. 28, 256–261
19. Fontana, A., Frei, K., Bodmer, S., and Hofer, E. (1987) Immunity Rev. 100, 185–201
20. Frei, K., Siepfl, C., Groscurth, P., Bodmer, S., Schwerdel, C., and Fontana, A. (1987) Eur. J. Immunol. 17, 1271–1278
21. Amin, A. R., Attur, M. G., Thakker, G. D., Patel, P. D., Vyas, P. R., Patel, R. N., Patel, I. R., and Abramson, S. B. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 14014–14019
22. Kim, S. S., Kong, P. J., Kim, B. S., Sheen, D. H., Nam, S. Y., and Chun, W. (2004) Arch. Pharm. Res. (Seoul) 27, 314–318
23. Brundula, V., Newcastle, N. B., Metz, L. M., Bernard, C. C., and Yong, V. W. (2002) Brain 125, 1297–1308
24. Tikka, T. M., and Koistinaho, J. E. (2001) J. Immunol. 166, 7527–7533
25. Steimle, V., Siegrist, C. A., Mottet, A., Lisowska-Grospierre, B., and Mach, B. (1994) Science 265, 106–109
26. Chang, C. H., Guerder, S., Hong, S. C., van Ewijk, W., and Flavell, R. A. (1996) Immunity 4, 167–178
27. Zita, E., and Ting, J. P. (2005) Curr. Opin. Immunol. 17, 58–64