Minocycline attenuates T cell and microglia activity to impair cytokine production in T cell-microglia interaction

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Abstract: Minocycline, a tetracycline with anti-inflammatory properties, has been reported to down-regulate the activity of microglia, whose activation occurs in inflammatory and degenerative diseases of the central nervous system, such as multiple sclerosis and Alzheimer's disease. In these disorders, a T cell component is also evident, and we have demonstrated previously that the interaction of activated T cells with microglia led to the substantial increase in tumor necrosis factor α (TNF-α) levels. Here, we report that minocycline decreases TNF-α levels produced in human T cell-microglia interaction. This effect is mediated by a direct action of minocycline on the activated T cells and on microglia, which resulted in the decreased ability of T cells to contact microglia. In correspondence, minocycline decreased the expression on T cells of the CD40 ligand (CD40L), a key molecule regulating the contact-mediated interaction of T cells with microglia. These results demonstrate that the mechanism of action of minocycline involves not only microglia but also T cells and their subsequent activation of microglia. The capacity of minocycline to down-regulate CD40L on T cells may provide a new means to target the CD40-CD40L pathway, which regulates several inflammatory processes. *J. Leukoc. Biol.* 78: 135–143; 2005.

Key Words: *lymphocytes · neuroinflammation · neurodegeneration*

INTRODUCTION

Minocycline is a second-generation tetracycline antibiotic that has recently been found to have numerous immunomodulatory activities. Thus, minocycline inhibits the activity of matrix metalloproteinases, inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2) [1, 2] and impairs the production of cytokines such as tumor necrosis factor α (TNF-α) and interleukin (IL)-1β [3]. It is important that minocycline has been found to be beneficial in animal models of several central nervous system (CNS) diseases including ischemia [1, 4], Huntington's disease [2], amyotrophic lateral sclerosis [5], and spinal cord injury [6]. We have recently reported that minocycline attenuates the clinical and histological severity of experimental autoimmune encephalitis (EAE), an animal model of multiple sclerosis (MS) [7]; this was also observed by another group [8]. These results have generated excitement in the neurological sciences, given that minocycline is available orally, has a good safety record when used chronically, and is relatively inexpensive in comparison with many drugs currently used to treat CNS diseases.

The mechanism(s) by which minocycline alleviates multiple diseases of the CNS is unclear. In addition to the above immunomodulatory roles, minocycline can inhibit caspase-3 activity [2] or prevent the release of cytochrome c from mitochondria [5], thereby influencing the process of apoptosis. Another critical target of minocycline may be the inhibition of the proliferation and activation of microglia, the macrophage-like cell of the CNS that regulates immune reactivity within this organ [9]. In vitro, minocycline prevents the excitotoxicity of glutamate on neurons, presumably via the inhibition of N-methyl-D-aspartate-induced activation of microglia [10]. In vivo, the protection afforded by minocycline in various models of CNS diseases is correlated with a lack of the activation of microglia [4, 8, 11].

Although microglia may be a target of minocycline action, it is possible that this drug has activity on other immune cell subsets, such as T cells, with important roles in several CNS disorders including MS. A prevailing hypothesis of MS pathogenesis [12, 13] is that autoreactive T cells are activated in the periphery by unknown antigens. These T cells then cross the blood-brain barrier into the CNS parenchyma, where they are reactivated in response to antigen restimulation [14–16], resulting in the secretion of proinflammatory cytokines that induce a chain reaction with the activation of resident microglia, infiltrating macrophages, and B cells. T lymphocytes can also interact directly with microglia through cell-cell contact mechanisms to up-regulate the production of numerous pro- and anti-inflammatory cytokines, at least as demonstrated in vitro [17, 18].

The effect of minocycline on T cells has received little attention, although it has been shown that minocycline can interfere with the activation of T cells [8, 19, 20]. In the current study, we have investigated the relative capability of minocycline to affect T cells or microglia differentially. Specifically, we have examined whether minocycline can alter T cell func-

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tion and thereby down-regulate the activation of microglia by T lymphocytes. Besides microglia, we have also tested U937 cells, a myelomonoblast-like human histiocytic lymphoma cell line. Previously, we determined that U937 cells resemble human microglia in their interaction with T cells to generate various cytokines [21]. Our collective results show that although minocycline has activity on microglia cells, it also affects T cells and their subsequent ability to contact microglia or U937 cells to up-regulate various cytokines. These results have important implications for the increasing number of CNS diseases that minocycline may impact.

MATERIALS AND METHODS

Isolation of T cells

Mononuclear cells were isolated from the blood of healthy volunteers by Ficoll-Hypaque centrifugation, as described previously [17, 18, 22], and were suspended in serum-free AIM-V medium (Gibco/BRL, Burlington, Canada). To activate T cells, 1 μg/ml anti-CD3 antibody (OKT3) was added once for a period of 3 days. The floating cells were then removed from any adherent monocytes, and a fixed density was used to test cytotoxicity. Unless otherwise stated, the density of anti-CD3-activated T cells was 50,000 in 100 μl AIM-V, and this was added to 25,000 U937 cells or 15,000 microglial cells prepared previously (see below). Some mononuclear cell preparations did not receive OKT3, and the floating cells that were collected 3 days after were referred to as unactivated T cells. Flow cytometry analyses of the floating cells collected after 3 days of initiation of OKT3 treatment indicated that CD3+ T cells constituted over 90% of the total cell population; these were ~60% CD4+ and 40% CD8+ in the cell ratio. B lymphocytes (CD19+) and natural killer (NK) cells (CD56+) consisted of the rest of the floating cell population, and no monocytes (CD14+) were detected; when analyzed further, NK cells constituted less than 3% of the population. There was no significant difference in the proportion of the various cell subsets between the unactivated and activated lymphocyte populations (data not shown). Henceforth, given that the majority of cells in the floating population includes T cells, these will be referred to as T lymphocytes.

U937 cell culture

The U937 line was obtained from the American Type Culture Collection (Rockville, MD). U937 cells were cultured in RPMI 1640 containing 10% fetal bovine serum (FBS) and 10 μM β-mercaptoethanol. For all the experiments, floating U937 cells were treated with phorbol-12-myristate 13-acetate (PMA; 50 ng/mL, Sigma Chemical Co., St. Louis, MO) and were seeded into 96-well plates at a density of 2.5×10^4 cells per well. After 2 days of culture with PMA, which resulted in adherence, cells were treated with 100 IU/ml recombinant interferon-γ (IFN-γ; Genzyme, Cambridge, MA) for 24 h. Cells were used immediately after the 24-h treatment with IFN-γ. We reported previously that the regimen of PMA and IFN-γ treatment resulted in U937 cells acquiring characteristics of microglia [21].

Human fetal microglia cultures

Fetal microglia were isolated from brain tissue of human fetuses of 14–18 weeks fetal age; these were obtained following therapeutic abortion, according to guidelines approved by local institutional ethics committees. For the preparation of brain cell cultures, 5–15 g brain tissue diced into fragments of 1 mm or less with a pair of scalpels was incubated in 40 ml vol for 15 min at 37°C with 0.25% trypsin and 200 μg/ml DNase I in phosphate-buffered saline (PBS). The suspension was then washed through a filter of 130 μm pore size, and the filtrate was centrifuged at 1200 rpm for 10 min. The cell pellet containing neurons, microglia, and astrocytes was resuspended in PBS and centrifuged. Following a final washing step in feeding medium (see below), the pellet was suspended in feeding medium, and cells were plated into T-75 flasks coated with 10 μg/ml poly-ornithine. Plating density was 50 million cells in 25 ml medium. Feeding medium was minimum essential medium supplemented with 10% FBS, 20 μg/ml gentamicin, 0.1% dextrose, 1× nonessential amino acids, 10 μM glutamine, and 1 mM sodium pyruvate. All medium constituents were from Gibco/BRL. After 1 week, during which two medium changes occurred, floating cells were collected, and these were predominantly microglia (purity over 95%, as assessed using CD14 and Iba1 immunohistochemistry). Microglia were plated in a 96-well plate at a density of 1.5×10^4 cells per well.

Isolation of human adult microglia

Human microglia of over 95% purity were obtained from resected brain specimens of patients undergoing surgery to treat intractable epilepsy, as described previously [23]. Cells were plated in 96-well plates at a density of 1.5×10^4 cells per well.

Coculture and cytokine measurements

Fifty thousand T cells in AIM-V medium were incubated with 2.5×10^4 U937 cells or with 1.5×10^4 microglia in each well of a 96-well plate. Twenty four hours after, the cell-conditioned media were collected and microfuged at 10,000 rpm for 1 min, and the supernatant was then examined by enzyme-linked immunosorbent assays (ELISAs). In all cases, regardless of whether T cells or microglia were treated with minocycline, the cell-conditioned media were collected after 24 h of coculture. Protein levels of cytokines (TNF-α and IL-10) contained in the conditioned medium of microglia-T cell cocultures and IFN-γ levels in T cell-conditioned medium were measured using the appropriate ELISA kits (Biosource, Camarillo, CA). The assay was performed following detailed instructions from the manufacturer.

Flow cytometry

Untreated and treated T cells were collected for flow cytometry analysis. Staining was performed in a single-step process by incubating cells for 40 min at 4°C with anti-CD154 [CD40 ligand (CD40L)]-fluorescein isothiocyanate-conjugated antibody (Becton Dickinson, Missisagua, Ontario, Canada). For integrin staining, mouse anti-β1 (CD29) and anti-β2 (CD18) antibody (Chemicon, El Segundo, CA), followed by phycoerythrin (PE)-conjugated anti-mouse immunoglobulin G (Becton Dickinson), was used. To measure the activation state of T cells, staining was done by incubating cells for 40 min at 4°C with anti-CD25 PE-conjugated antibody (Becton Dickinson).

Assessment of T cell proliferation

T cell proliferation was measured by a colorimetric bromodeoxyuridine (BrdU) incorporation assay (Roche Diagnostics, Penzberg, Germany) in a 96-well microplate following the instructions of the manufacturer.

Cytology

To document the morphology of cells in the T cell-microglia interaction (see Fig. 7), microglia cells at a concentration of 2.5×10^4 were cultured in each chamber of an eight-well plastic slide (Lab-tek, Nalge Nunc International, Rochester, NY). Unactivated or activated T cells (5×10^4) were then added for 3 h, the medium was removed, and the coculture was washed once with PBS; the cells were then fixed for 10 min in 4% paraformaldehyde and observed under a phase-contrast microscope.

In a separate experiment, unactivated or activated T cells were prelabeled for 10 min with carboxy-fluorescein diacetate, succinimidyl ester (CFSE). These cells (5×10^4) were then plated onto plastic Lab-tek wells containing microglia. Three hours after, cultures were fixed, and microglia were stained with a rabbit anti-Iba antibody followed by a secondary antibody conjugated to rhodamine. Using an immunofluorescence microscope, the number of T cells (green) adhering onto microglia (red) was evaluated.

Statistical analysis

As all experiments involved multiple groups, statistical analyses were conducted using one-way ANOVA with Tukey's post-hoc test.

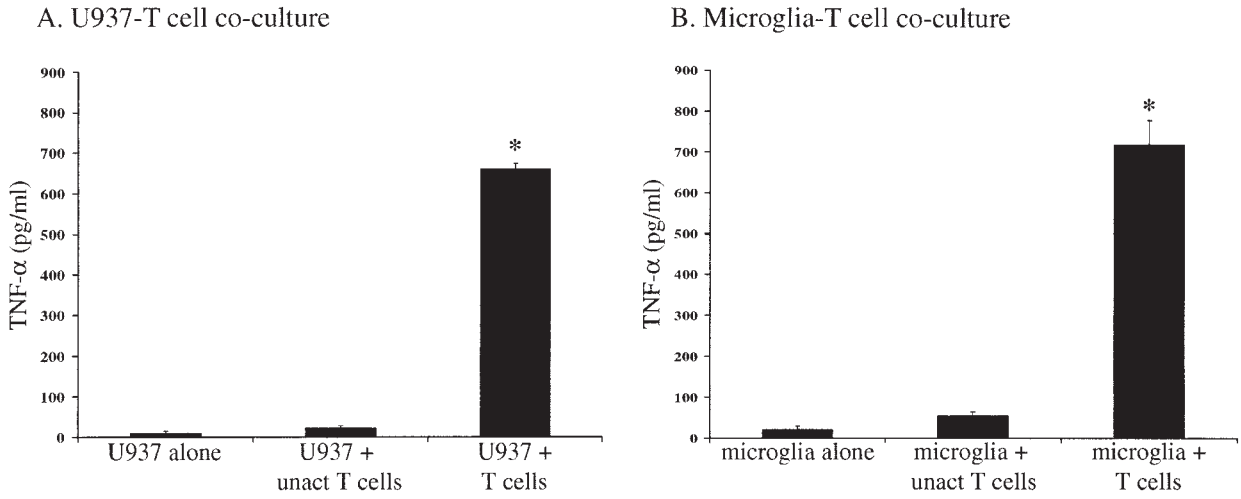

Fig. 1. The coculture of activated T cells with U937 cells (A) or fetal human microglia (B) leads to the increase of TNF-α levels in the conditioned medium. In contrast, unactivated (unact) T cells could not promote a TNF-α increase in coculture with microglia or U937 cells. Note that the conditioned medium of 5×10^4-activated T cells (in 100 μl) alone did not contain detectable TNF-α levels (data not shown). Each bar is the mean ± SD of triplicate culture, and the data have been reproduced in three different experiments. One-way ANOVA, Tukey's post-hoc test compared with U937 or microglia alone. *, $P < 0.001$.

RESULTS

Minocycline treatment of T cells impairs their ability to interact with U937 cells or microglia to increase TNF-α levels

We first confirmed previous results [17, 21] that T cells could activate U937 cells or microglia to increase TNF-α levels. T lymphocytes from adult human donors, without any apparent neurological diseases, were activated by anti-CD3 treatment and then cocultured with U937 cells or microglia derived from fetal human brains. After 24 h, the cell-conditioned medium was collected for TNF-α measurement by ELISA. When cultured in isolation, U937 cells or microglia had undetectable levels of TNF-α (**Fig. 1**), and this was not altered by coculture with unactivated T cells. In contrast, the interaction of activated T cells with U937 cells or microglia resulted in a significant increase in the TNF-α level within 24 h (Fig. 1, A and B, respectively).

We addressed the result of minocycline treatment of T cells on their subsequent interaction with U937 cells. T cells following anti-CD3 ligation were treated for 3 days with minocycline (2–100 μg/ml), and the viability was assessed by trypan-blue exclusion; this was >90% in all cases (data not shown). Then, 50,000 cells were incubated with each U937 culture. **Figure 2** demonstrates that the pretreatment of activated T cells with increasing concentrations of minocycline resulted in a dose-dependent decrease of TNF-α levels generated in the subsequent coculture. The effect of minocycline was apparent from 10 μg/ml (Fig. 2).

Figure 2 emphasizes that the level of TNF-α detected in the coculture is not the result of what the T cells produce in the absence of U937 cells or microglia, as the T cells in isolation in the experimental paradigm used (50,000 cells in 100 μl medium per well of a 96-well plate), with or without minocycline treatment, did not contain appreciable amounts of this cytokine. Finally, we exposed minocycline-pretreated T cells to microglia cultured from fetal or adult human brains. **Figure 3** shows that the pretreatment of T cells with minocycline decreased their ability to interact with microglia to increase the TNF-α level.

Minocycline treatment of T cells increases IL-10 in the coculture

We examined whether other cytokines were also affected by minocycline treatment. We focused on IL-10, as this regulatory

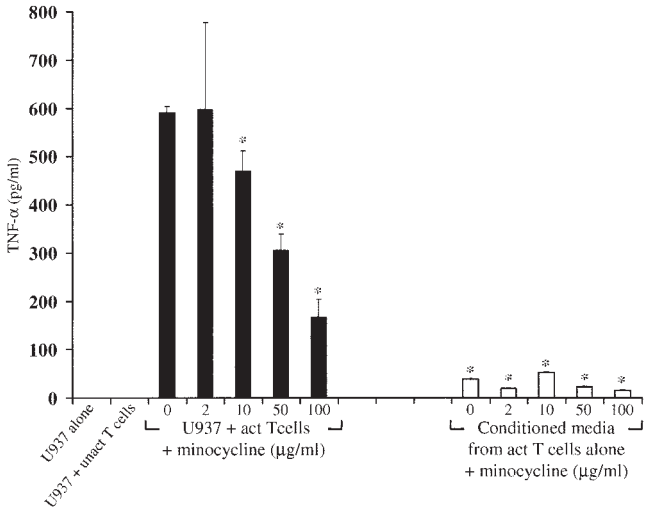

Fig. 2. TNF-α production in U937-T cell coculture is decreased by the pretreatment of T cells with minocycline. In this and subsequent figures, the 0 group refers to cocultures without minocycline treatment. Note that in the right panels, conditioned media collected from activated (act) T cells in isolation (i.e., no coculture) and treated with minocycline did not contain appreciable levels of TNF-α, indicating that the detection of TNF-α required the coculture of T cells and monocytoid cells. Each bar is the mean ± SD of triplicate culture, and the data have been reproduced in three experiments. One-way ANOVA, Tukey's post-hoc test compared with U937 plus activated T cells in the absence of minocycline. *, $P < 0.05$.

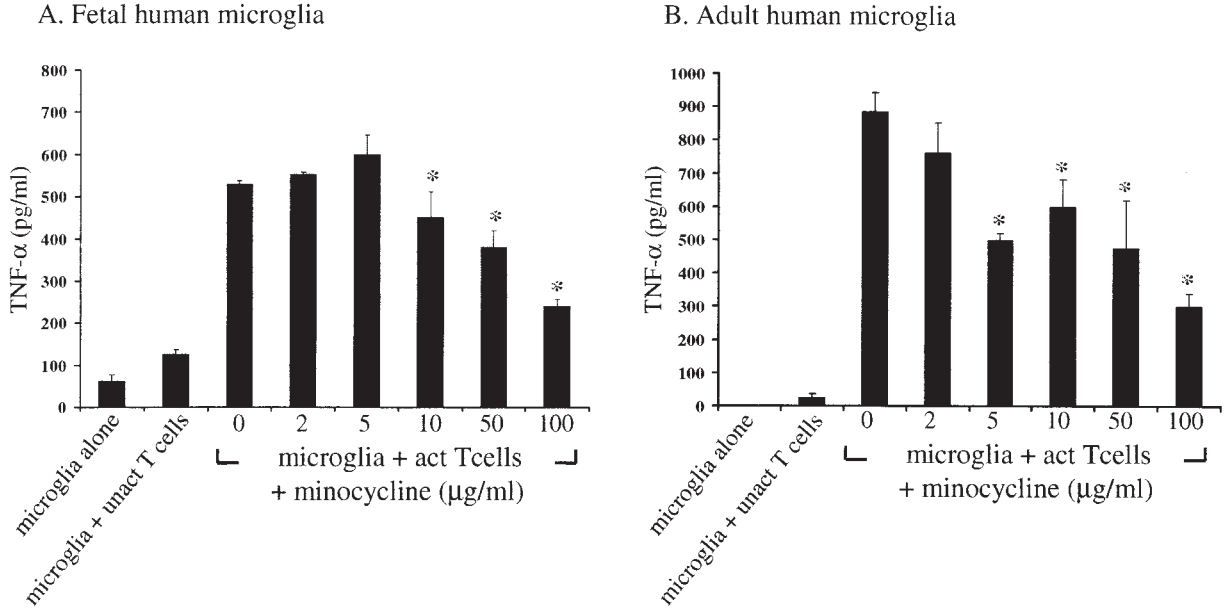

Fig. 3. Activated T cells interact with human microglia to increase TNF-α levels, and this is reduced by the pretreatment of T cells with minocycline. (A) Fetal human microglia; (B) the results for adult human microglia. Each bar is the mean ± SD of triplicate culture, and the data have been reproduced in three experiments. One-way ANOVA, Tukey's post-hoc test compared with the coculture with activated T cells. *, $P < 0.05$.

cytokine is produced during the interaction between T cells and microglia [18, 24]. Pretreatment of T cells with minocycline resulted in these cells eliciting an increase in IL-10 produced in the coculture with microglia (**Fig. 4**). The increase in IL-10 was concentration-dependent and was apparent with 10 μg/ml minocycline.

The mechanism of minocycline action is on T cells and microglia

As noted earlier, the focus of various studies in the literature has been that minocycline inhibits microglia activity. Thus, we addressed whether the treatment of microglia with minocycline might alter their ability to interact with T cells to regulate cytokine levels. **Figure 5A** documents that the pretreatment of microglia for 72 h with minocycline did reduce the level of TNF-α, which is generated in the subsequent coculture of microglia with activated T lymphocytes. In contrast, when minocycline was administered at the time of the coculture of microglia with activated T lymphocytes, the increase in TNF-α resulting from the coculture was not modulated (Fig. 5B).

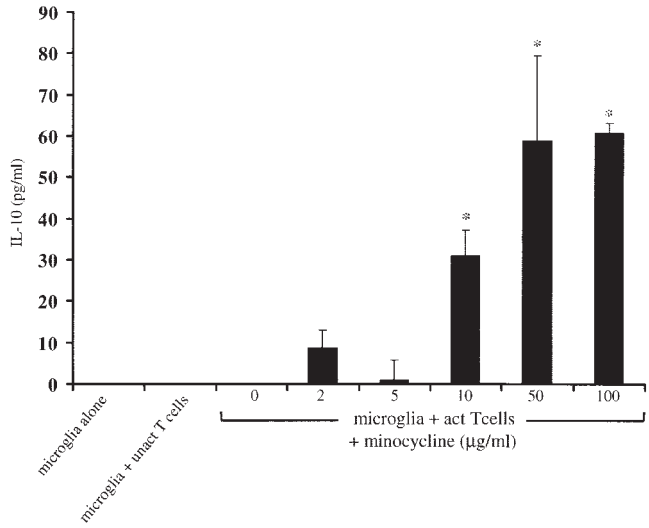

Fig. 4. IL-10 levels in microglia-T cell coculture are increased by pretreatment of T cells with minocycline. One-way ANOVA, Tukey's post-hoc test compared with microglia plus activated T cells. *, $P < 0.05$.

Collectively, our results show that the decrease of TNF-α, affected by minocycline, manifested when T cells or microglial cells were pretreated with this drug. These data confirm previous reports showing an effect of minocycline on microglial cells, and they demonstrate that this drug also has a direct activity on T cells.

To evaluate the activity of minocycline on T cells further, we measured BrdU incorporation, IFN-γ levels, and CD25 expression as markers of the activity of T cells. The proliferation of T cells, measured by BrdU incorporation in a colorimetric assay, was decreased significantly by minocycline (**Fig. 6A**). IFN-γ levels, measured in the conditioned medium of T cells, were decreased in a concentration-dependent manner by minocycline compared with the untreated, activated T cells (Fig. 6B). Finally, flow cytometry analysis of CD25 (IL-2 receptor) expression on T cells reveals that this was reduced by 30%, and the higher concentrations of minocycline (50–100 μg/ml) were compared with the untreated, activated T cells (data not shown).

In conclusion, these data suggest that minocycline acts directly on T cells to reduce their activation state and that this resulted in a decreased interaction with microglia to generate TNF-α levels. However, IL-10 was increased in the coculture by the pretreatment of T cells with minocycline.

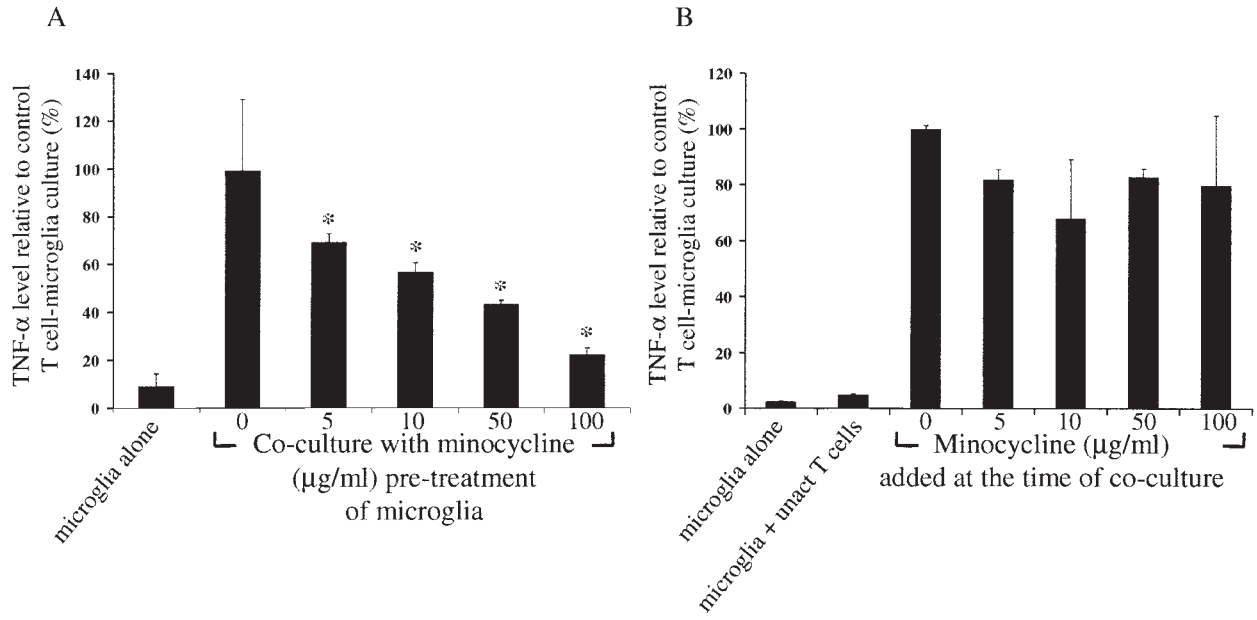

Fig. 5. The pretreatment of microglia cells with minocycline was effective in modulating TNF-α levels in subsequent coculture with activated T cells (A). In contrast, minocycline added at the time of the coculture did not attenuate TNF-α levels (B). Results are normalized to that of microglia plus activated T cells without minocycline treatment (100%). One-way ANOVA, Tukey's post-hoc test compared with microglia plus activated T cells. *, $P < 0.05$.

Minocycline down-regulates CD40L expression on activated T cells

We addressed the mechanisms by which minocycline-pretreated T cells were less able to evoke a TNF-α increase in a T cell-U937 (or microglia) interaction. We noted that when activated T cells without minocycline pretreatment were added to microglia, they clustered around the microglia cells (**Fig. 7C**). However, the pretreatment of activated T cells with minocycline decreased the number of T cells that adhered to microglia (Fig. 7D). To quantitate this effect more precisely, we have counted, using fluorescence microscopy, the number of CFSE-prelabeled T cells adherent to single Iba1-tagged microglia cells. In control T cell-microglia culture, the average number of activated T cells adhering onto a single microglia was 11 ± 4 (mean±SD). In contrast, the number of minocycline-pretreated T cells that adhered onto a single microglia was 2 ± 2 ($P<0.001$ compared with control). These observations suggest that minocycline down-regulates molecules on T cells that are involved in cell-cell contact with monocytoid cells.

Recent evidence has shown that integrins are involved in modulating microglia activation [26]. Furthermore, we previ-

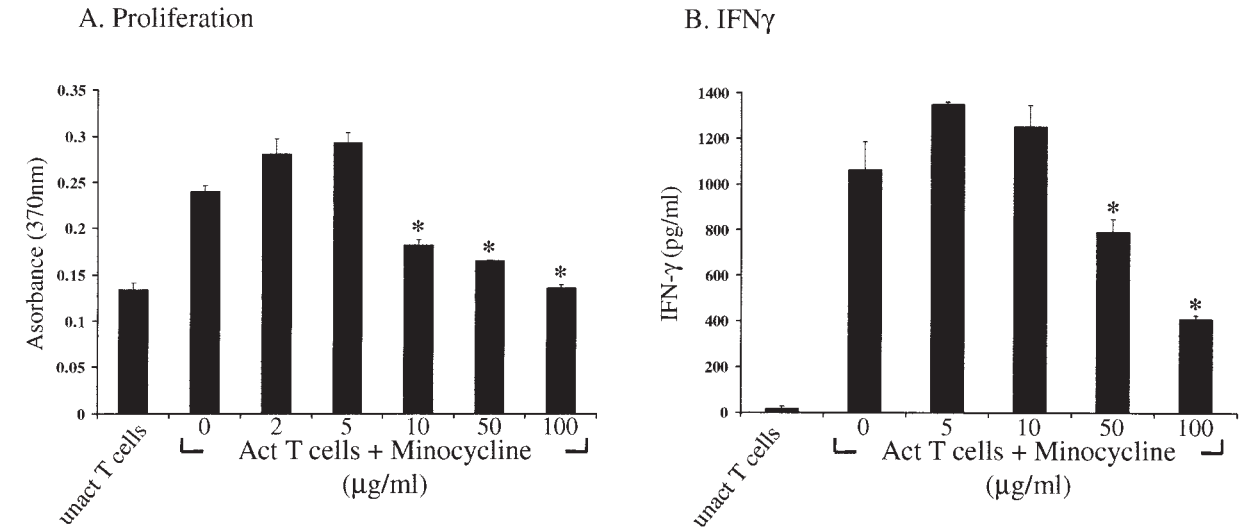

Figure 6. Minocycline decreases T cell proliferation and IFN-γ production. Proliferation was measured by a BrdU incorporation colorimetric assay (A), and IFN-γ levels in the T cell-conditioned medium were assayed by ELISA (B). Each bar is the mean ± SD of triplicate cultures, and the data have been reproduced in three experiments. One-way ANOVA, Tukey's post-hoc test compared with activated T cells without minocycline. *, $P < 0.05$.

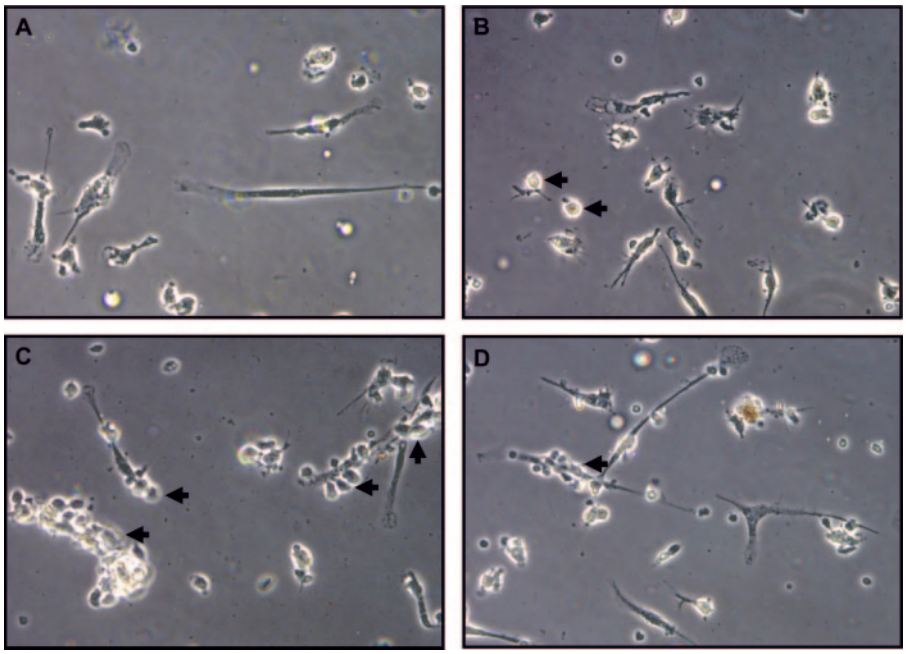

Fig. 7. Minocycline prevents the adhesion of T cells to microglia. (A) Microglia alone, displaying cells of many morphologies, including bipolar forms and rounded amoeboid cells with foot processes (top right of frame); these multiple forms have been described previously [25]. (B) Microglia plus unactivated T cells. Arrows indicate a few T cells, but most have been removed in the wash prior to fixation, as the unactivated T cells tend to have poor adhesive properties on glass. (C) Microglia plus activated T cells; many T cells could be observed adhering to microglia (arrows), and this is reduced but not abolished in microglia incubated with minocycline-pretreated T cells (D). Phase contrast microphotography. Original magnification: 400×.

ously demonstrated an involvement of the CD40-CD40L pathway in microglia activation by T cells [18]. Therefore, we tested whether minocycline modulated the expression of CD40L, β2 integrins, and β1 integrins on activated T cells. We assessed the expression of these molecules by flow-cytometry. For β2 integrins (**Fig. 8A**), the activation of T cells induced an increased expression of this integrin compared with unactivated T cells [mean fluorescence intensity (MFI): activated T cells, 1233.2; unactivated, 806.2; $P<0.05$; n=3). Minocycline, used at a concentration of 50 μg/ml, did not change the expression of these integrins on T cells (MFI: 1245), despite a decrease in the number of flourescent cells (percent of fluorescent cells: 88.9% in minocycline-treated culture compared with 95.4% in the untreated, activated T cells; $P<0.05$; n=3; Fig. 8A). In addition, fluorescein-activated cell sorter analysis for β1 integrins did not show any down-regulation of their expression on activated T cells following treatment with minocycline (Fig. 8B). In contrast, minocycline induced a significant decrease in the expression of CD40L (percent of fluorescent cells: 34.4% in minocycline, 50 μg/ml-treated culture compared with 88.5% in the untreated, activated T cell group; $P<0.001$; Fig. 8C).

Collectively, our data show that minocycline decreases the expression of CD40L on T cells and that this contributes to the reduced ability of T cells to adhere to and engage microglia to regulate TNF-α levels.

DISCUSSION

Microglia activation occurs in all diseases of the CNS. Because of their ability to react promptly to any kind of insults to the CNS, microglia have been described as the "sensor of pathology" [27]. An overactivation of microglia, however, is likely detrimental [9, 28], and reactive microglia are thought to mediate the death of neurons in the penumbra of the ischemic lesions [29] or to contribute to the senile plaques of Alzheimer's disease and the demyelinated areas of MS lesions [30–33]. Other functions of microglia include their activity as phagocytic and antigen-presenting cells and their production and release of several inflammatory mediators, including cytokines, chemokines, reactive oxygen intermediates, NO, complement proteins, coagulation proteins, and proteases [9]. Many stimuli can induce microglia activation, and these include viruses or bacteria when they invade the CNS. Lipopolysaccharide (LPS), a component of the wall of gram-negative bacteria, is a prominent stimulator of microglia activation through CD14 and Toll-like receptor interaction.

We have previously reported that activated T cells are also strong stimulators of microglia and that this occurs through a cell-cell, contact-mediated mechanism [17]. MS lesions are characterized by T cell infiltration in the acute phase, and these lymphocytes thus have the potential to further up-regulate microglia activity. The inhibition of microglia overactivation and the dampening of T cell-microglia interaction may thus offer new strategies to decrease the detrimental aspects of neuroinflammation in MS and other CNS disorders.

The tetracycline derivative, minocycline, has been found recently to have multiple immunomodulatory properties that are not related to their antimicrobial activity. These studies have generated strong interests in the mechanisms of minocycline, as its administration can attenuate the severity of a spectrum of neurological diseases, including in animal models of MS, ischemia, amyotrophic lateral sclerosis, Parkinson's disease, and Huntington's disease [1–5]. Indeed, minocycline is currently being tested in phase I/II clinical trials in MS [34] and amyotrophic lateral sclerosis [35], where it has been found to be safe and well-tolerated.

The mechanisms of action of minocycline in neurological diseases are still unclear. Although it has been postulated to have a direct effect on microglia, whereby minocycline was able to reduce the proliferation of microglia and their production of IL-1β [10], other immunomodulatory properties of it

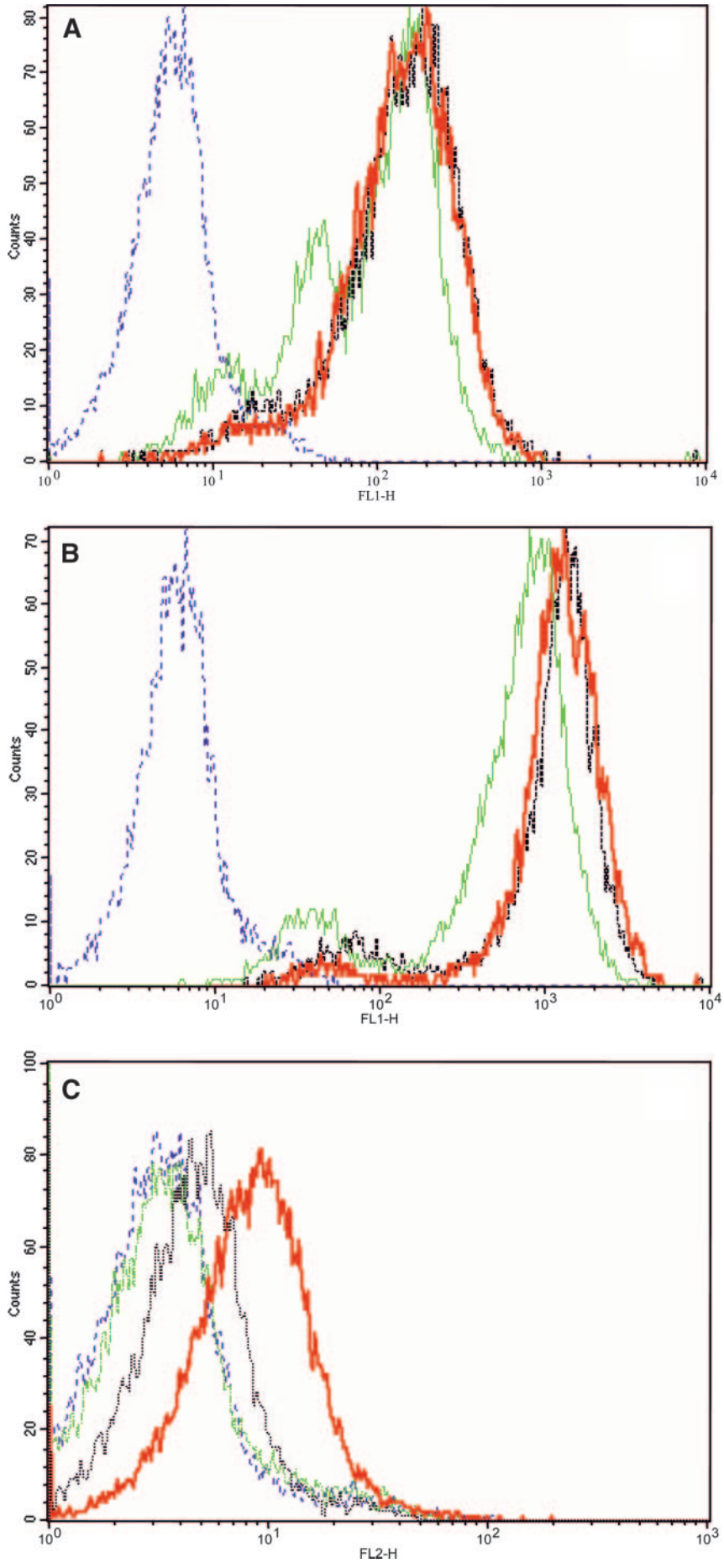

Fig. 8. Minocycline decreases the expression of CD40L but not β1 or β2 integrins on activated T cells. Single fluorescence flow cytometry with fluorescein (FL1-H) as the fluorophore. (A) β2 expression; (B) β1 expression; (C) CD40L expression. Blue: Isotype control; green: unactivated T cells; red: activated T cells; black: minocycline (50 μg/ml)-treated T cells.

may be even more critical. Specifically, we tested in this manuscript the ability of minocycline to alter T cell reactivity in T cell-microglia interactions. We demonstrate that minocycline treatment of T cells reduced their ability to engage monocytoid cells and that this resulted in a decrease in TNF-α, which normally accompanies T cell-microglia (or U937) interaction. Although this effect on TNF-α can be the result of the reaction of T cells to allogeneic microglia, the same effect has been shown previously in a syngeneic system, suggesting that it is independent from a major histocompatibility complex-mediated mechanism [36]. We confirm that minocycline can impair microglia function directly, as its pretreatment of microglia before their encounter with T cells attenuated a subsequent TNF-α generation (Fig. 5A). A pretreatment period of microglia or T cells with minocycline is necessary, as the addition of minocycline at the time of the coculture was inadequate to affect TNF-α (Fig. 5B).

The concentrations of minocycline that were used in this study deserve some attention. Our center has a cohort of 10 MS patients, who have been treated with oral minocycline, 100 mg twice a day, for at least 6 months [34], and the steady-state serum concentrations in these patients range from 3 to 8 μg/ml (unpublished observations). Thus, the effects of 5 and 10 μg/ml minocycline in this manuscript should be achievable in human subjects treated with oral minocycline. It is possible that higher concentrations of minocycline are achieved at critical immunological synapses or within the microenvironment of microglia, although this remains to be established.

Overall, this is the first demonstration that minocycline can down-regulate microglia activation by a direct effect on T cells. However, Popovic et al. [8] have shown no effect of minocycline in rodents on T cell proliferation and IFN-γ production; this disparity with our results may be a result of a different response of human T cells compared with rodent T cells or of a different action of minocycline on lymph node-derived lymphocytes versus blood-derived ones. In addition, in vitro T cell activation with anti-CD3 antibody is a much stronger stimulus compared with in vivo antigen presentation in the study by Popovic et al. [8], and this may increase the sensitivity of anti-CD3-activated T cells to minocycline action in our study.

Minocycline has recently been reported to have neuroprotective effects in models of global and focal ischemia [1, 4]. The minocycline-induced reduction in infarct size and increased survival of hippocampal neurons after focal or global ischemia, respectively, were accompanied by a reduction of IL-1β-converting enzyme, COX-2, and iNOS mRNA in affected brain regions. In vitro, activated microglia has been shown to facilitate the TNF-α-mediated motor neuron death [37] and in the presence of IFN-γ, to induce cortical neuronal injury [38]. We have recently described the high vulnerability of human neurons to T cell-mediated cytotoxicity [39]. We speculate that the decreased activation state of T cells induced by minocycline may be responsible, at least in part, for the neuroprotective effect of the drug, by reducing the direct cytotoxicity of activated T cells and by reducing T cell-induced activation of microglia. In addition, the suppression of microglia activation can be important for the remyelination processes [40].

There are other aspects of minocycline actions that are worth noting, such as the increased production of IL-10 in the microglia-T cell coculture when the T cells were pretreated with the higher concentrations of minocycline (Fig. 4). IL-10 is an immunoregulatory cytokine that can inhibit microglia activation. In vitro, the pretreatment of microglia with IL-10 prevented the production of TNF-α induced by LPS [41]. Another observation of interest is the reduced adhesion between T cells and microglia after the pretreatment of T cells with minocycline (Figs. 7 and 8). We have previously shown that integrins such as very late antigen-4 are involved in the interaction between these two cell types [17]. However, treatment of T cells with minocycline did not down-regulate β1 (Fig. 8B) or β2 integrins (Fig. 8A) but instead, reduced the expression of CD40L on activated T cells (Fig. 8C). We previously reported the CD40-CD40L interaction as a major pathway involved in microglia activation by T cells [18]. Thus, the reduction of the CD40-CD40L pathway could be responsible for the reduced microglia-T cell interaction caused by minocycline.

The decrease of CD40L expression by minocycline may have further implications apart from the attenuating T cell-microglia interaction. High levels of CD40L-expressing cells have been found to be colocalized with CD40-positive cells in active MS lesions [42], suggesting that this pathway may be a mediator of the evolution of MS lesions. Further, inhibition of the CD40-CD40L interaction in EAE retards the development of the disease [42]. CD40L-activated microglia has been postulated to promote the β-amyloid pathology in a model of Alzheimer's disease [43], suggesting a role for T cell-activated microglia in this disorder. In vitro, the addition of soluble CD40L to microglia induced TNF-α production [38]. In an animal model of MS, the treatment with anti-CD40L antibody at the peak of acute disease or during remission effectively blocked clinical disease progression and CNS inflammation [44]. In addition, considering that the CD40-CD40L pathway is involved in the neuronal death induced by cytotoxic T cells [39], the decreased expression of CD40L on T cells induced by minocycline may account, at least in part, for the neuroprotective actions of the drug in models of neurological diseases. All these observations underline the importance of the CD40-CD40L pathway in inflammatory diseases, and we propose that minocycline constitutes a novel, orally available, and inexpensive therapy to target these conditions.

In summary, this study suggests new mechanisms of minocycline activity in neurological diseases, specifically through an effect on T cell and microglia activity, and their subsequent interaction. Our findings suggest that even if T cells enter the CNS of minocycline-treated individuals, they would be less able to interact with microglia to generate proinflammatory cytokines such as TNF-α; concordantly, the increase in IL-10 may serve to decrease CNS inflammation further. Finally, the finding of modulation of CD40L expression on T cells by minocycline invites the potential application of this drug in inflammatory disorders in general.

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