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Cytokine production in T lymphocyte–microglia interaction is attenuated by glatiramer acetate: a mechanism for therapeutic efficacy in multiple sclerosis

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The efficacy of glatiramer acetate in multiple sclerosis (MS) is thought to involve the production of Th2 regulatory lymphocytes that secrete anti-inflammatory cytokines; however, other mechanisms cannot be excluded. Given that activated T lymphocytes infiltrate into the CNS and become in close proximity to microglia, we evaluated whether glatiramer acetate affects the potential interaction between T cells and microglia. We report that the co-culture of activated T lymphocytes with microglia led to the induction of several cytokines, and that these were reduced by glatiramer acetate treatment. Morphological transformation of bipolar/ramified microglia into an activated ameboid form was attenuated by glatiramer acetate. These results reveal a novel mechanism for glatiramer acetate: the impairment of activated T cells to effectively interact with microglia to produce cytokines. The net result of a non-inflammatory milieu within the CNS, in spite of T cell infiltration, may help account for the amelioration of disease activity in MS patients on glatiramer acetate therapy.
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Introduction

Glatiramer acetate (Copaxone[R]) is a polymer of four amino acids (L-alanine, L-glutamate, L-lysine and L-tyrosine) with efficacy in the treatment of patients with relapsing-remitting multiple sclerosis (MS).[1–4] In an animal model of MS, experimental autoimmune encephalomyelitis (EAE), glatiramer acetate suppresses both the acute and chronic disease induced by several myelin proteins.[5–7] The principal mechanism of action of glatiramer acetate in MS is thought to involve antigen presentation (reviewed in Refs. [8,9]). In this regard, glatiramer acetate has been shown to compete with, or displace, myelin peptides from binding to major histocompatibility complex (MHC) molecules; to act as an altered peptide ligand of immunogenic epitopes of myelin basic protein; or as an antigen itself [10–15] Glatiramer acetate has also been described as an antagonist at the T-cell receptor to the immunodominant epitope of MBP.[16] Consequences of affecting antigen presentation include the apoptosis or functional inactivation (anergy) of encephalitogenic T helper 1 (Th1) lymphocytes that mediate the disease, and the formation of glatiramer acetate-specific Th2 suppressor/regulatory cells that produce anti-inflammatory cytokines. The latter are thought to traffic into the CNS and participate in the attenuation of disease through bystander suppression.[14,15,17,18] In support of these concepts, mononuclear cells from MS subjects treated with glatiramer acetate show evidence of a shift from a pro-inflammatory Th1 cytokine profile to a Th2 bias.[15,19–23] Furthermore, copaxone reactive Th2 cells are found to accumulate in the CNS of experimental animals.[14]

It is possible that other mechanisms may also account for the efficacy of glatiramer acetate in MS. When T lymphocytes enter the parenchyma of the CNS, they become in close proximity to microglia. Although it is thought that T cells become reactivated when they re-encounter antigen presented by microglia, direct interaction between activated T cells and microglia, independent of antigen presentation, may also occur. In recent studies, we demonstrated that activated human T cells and microglia can interact in a contact-dependent manner, whether these were syngeneic or allogeneic, in an apparently non-antigen-dependent fashion. This interaction yields substantial amounts of tumour necrosis factor-α (TNF-α),[24] IL-10,[25] IL-Iβ, IL-4, IL-6, IL-12 and IL-13.[26] Given that TNF-α can be toxic to oligodendrocytes,[27–29] the encounter of activated T lymphocytes with microglia can lead potentially to the oligodendrocyte/myelin pathology that is characteristic of MS. The upregulation of a variety of cytokines would further propagate an inflammatory milieu within the CNS.

In this manuscript, we have addressed whether the production of cytokines in T cell–microglia interaction would be affected by glatiramer acetate. If so, this would represent a novel mechanism of glatiramer acetate in MS.

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Materials and methods

Isolation of mononuclear cells and treatment with glatiramer acetate

Heparinized blood was collected from normal volunteers, and subjected to Ficoll-Hypaque (Pharmacia Biotech, Mississauga, Ontario, Canada) centrifugation to obtain peripheral blood mononuclear cells as described previously.[24] After two washes, cells were suspended at a density of 1–2 million/ml in horizontal T-25 flasks (Nunc, Becton Dickinson, Mississauga, Ontario, Canada) in the serum-free medium, AIM V (Gibco BRL, Burlington, Ontario, Canada), to which 1 μg/ml of an anti-CD3 antibody (OKT3) was added. Three hours after the anti-CD3 addition, the T-25 flasks were stood upright from their horizontal position in order to kill monocytes that had adhered. Floating cells, which were mostly lymphocytes, were left for a period of 72 h at 37°C. Flow cytometry analysis of the mononuclear cell population at this 72-h period indicated that CD3+ cells constituted about 90% of the total cell population, with approximately 60% CD4+ and 30% CD8+ ratio. B lymphocytes (CD19+) and NK cells (CD56+) consisted of 5–6% of the total mononuclear cell population, and no monocytes (CD14+) were detected. Henceforth, given that the majority of cells in the mononuclear population are T cells, these will be referred to as T lymphocytes.

In experiments where glatiramer acetate (from TEVA Pharmaceutical Industries, Petach-Tikva, Israel; batches 242993097 and 242994498) was used, this drug (5–50 μg/ml), diluted in phosphate-buffered saline, was added to cultures 3 h after the initiation of CD3 ligation, at the time that the T-25 flasks were altered from the horizontal to upright positions. Cells were left for 69 h at 37°C, then collected, counted and resuspended in fresh AIM-V at a density of 500,000 cells/ml. Following a second treatment with glatiramer acetate, cells were left at 37°C for an additional 3 h. Thereafter, 100 μl (thus, 50,000 cells) of cell suspension was added to individual wells of a 96-well plate already containing microglia or U937 monocytoid cells (see below). Thus, unless otherwise stated, most experiments involved 72-h treatment of T cells with glatiramer acetate, administered at two time points. The purity of T cells after 72 h of glatiramer acetate treatment was not different from that of non-treated controls (unpublished results).

We have pretreated T cells with glatiramer acetate as this simulates the exposure of leukocytes to this drug at sites of subcutaneous injection and at draining lymph nodes. The literature does not provide evidence that glatiramer acetate itself can enter the CNS. Note also that the concentrations of glatiramer acetate that are used here are comparable to those used by other laboratories in tissue culture studies.[15,20–23]

Microglia—T cell interactions

Adult human microglia of over 95% purity was isolated from the resected brain tissue of patients undergoing surgical resection to treat intractable epilepsy, as in previous reports.[24,25] A total of 2.5×10^4 microglia were plated per well of a 96-well plate. Microglia culture medium was minimum essential medium supplemented with 5% FCS, 0.1% dextrose and 20 μg/ml gentamicin (all constituents from Gibco BRL). In microglia–T cell interaction assays, 100 μl containing 50,000 T cells in AIM-V (as described above) was added to individual wells of a 96-well plate already containing 25,000 microglia (or U937 monocytoid cells — see below) in 100 μl microglia culture medium. Twenty-four hours after, conditioned medium was collected for cytokine quantifications by ELISA.

Fetal human microglia was isolated from brains obtained at legal and therapeutic abortions using a protocol described by Lee *et al*.[30] Specimens ranged in gestational age from 14 to 20 weeks. The use of these and all other human materials has been approved by our local institutional ethics committee. Cells were used for interactions with T cells in a manner identical to that described for their adult counterparts.

To complement the studies of primary human microglia cultures, we have used a human pro-monocytoid cell line. Cells in this U937 line become microglia-like, as assessed by morphology and expression of cell surface molecules, when treated sequentially with 50 ng/ml phorbol-12-myristate-13-acetate (PMA) (time 0–48 h) and 100 U/ml interferon-γ (IFNγ) (from 48 to 72 h).[31] Cells were used 1–3 days after the IFNγ treatment. As with microglia, 50,000 T cells in AIM-V was added to individual wells of a 96-well plate already containing 25,000 PMA/IFNγ-treated U937 cells, and conditioned medium was collected after 24 h of co-cultures.

Cytokine protein quantification

Cytokine protein levels in the conditioned medium of microglia–T cell co-cultures were measured using enzyme-linked immunoabsorbent assay (ELISA) kits bought from BioSource International (Montreal, Quebec, Canada). Assays were performed following manufacturer instructions.

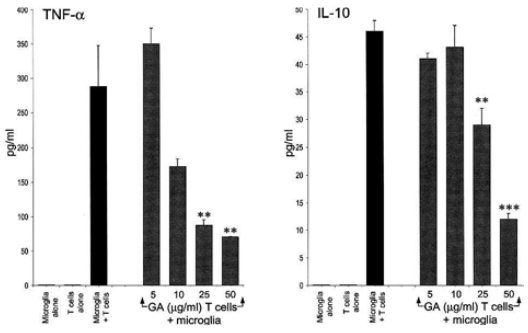

Figure 1 *Glatiramer acetate pretreatment of T cells suppresses TNF α and IL 10 production that is generated in T cell adult human microglia interactions. T cells or adult microglia in isolation produce undetectable TNF α or IL 10. In co culture, both IL 10 and TNF α levels are significantly elevated and this is reduced dose dependently by glatiramer acetate (GA) pretreatment of T cells. Values are mean±SEM of triplicate analyses; results have been reproduced in three series of experiments involving different microglia and blood donors. **p<0.01, ***p<0.001, one way ANOVA with Bonferroni post hoc comparisons*

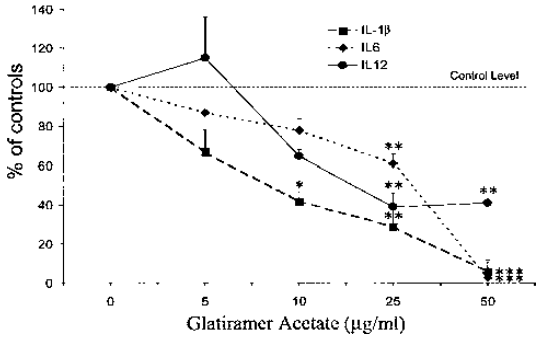

Figure 2 *Glatiramer acetate treatment of T cells also lead to the suppression of IL 1β, IL 6 and IL 12 in T cell adult human microglia interactions. Both activated T cells and adult human microglia do not secrete detectable amounts of IL 1β and IL 12 into their culture medium. With co culture, levels of IL 1β and IL 12 are increase, to 17 and 114 pg/ml, respectively. These inducible cytokines are dose dependently reduced by glatiramer acetate pretreatment of T cells. IL 6 is a constitutively expressed cytokine that can be readily detected in culture medium of T cells or microglia in isolation. Glatiramer acetate treatment of T cells also attenuated the IL 6 levels found in T cell microglia co cultures (level of IL 6 in control T cell microglia co culture in this example: 368 pg/ml). *p<0.05, **p<0.01, ***p<0.001, one way ANOVA with Bonferroni post hoc comparisons*

Adhesion assays

Wells in 96-well plates were coated with 20 μg/ml fibronectin (Sigma, St. Louis, MO, USA), a β1-integrin substrate, or with 0.2% gelatin, which evaluates β2-integrin adhesive function.[32] Coating was performed with 100 μl per well at 37°C overnight. The fluid was then removed, the wells were washed once with saline, and 50,000 T cells in 100 μl AIM-V medium were then added. These T cells had been pretreated for 3 days with glatiramer acetate after the anti-CD3 ligation as described above, or were untreated controls. After 1 h at 37°C, the fluid from each well was removed and processed through a Coulter Z1 counter to enumerate the number of unadhered cells. Adherent cells were floated by 0.25% trypsin and similarly counted. Thus, the total number of T cells was elucidated from each well and the proportion that remained floating was tabulated.

Results

Glatiramer acetate decreases cytokine levels in co-cultures of T cells and adult human microglia

When cultured in isolation, the medium of anti-CD3 activated human T cells or adult human microglia contained undetectable amounts of TNF-α or IL-10 as assayed by ELISAs. In co-culture of T cells and adult human microglia, however, substantial levels of TNF-α and IL-10 were elicited (Figure 1). Significantly, the pretreatment of T cells with glatiramer acetate prior to their encounter with adult microglia reduced, in a concentration-dependent manner, the levels of both cytokines (Figure 1). The effect of glatiramer acetate was principally on T cells since the pretreatment of microglia with glatiramer acetate (25 μg/ml,

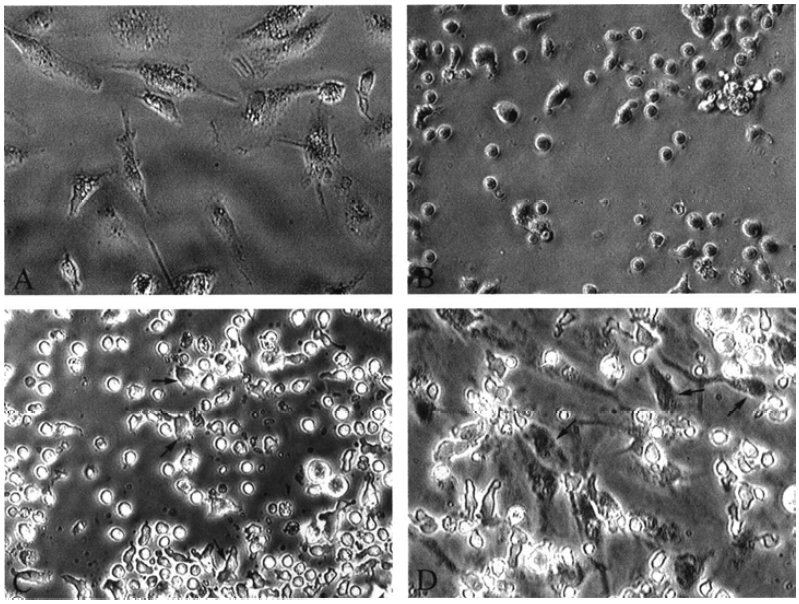

Figure 3 *Morphology of microglia in T cell adult microglia co cultures. Adult human microglia are mostly bipolar in morphology in culture (A). T cells are present as single cells or clumps when plated into wells of a 96 well plate (B). When T cells are co cultured with microglia in the absence of glatiramer acetate, bipolar microglia become rounded/ameboid in morphology (C, some microglia are shown by arrows). This morphological transformation is prevented by glatiramer acetate pretreatment of T cells (D, with some bipolar microglia indicated by arrows). All frames are of the same original magnification, ×400*

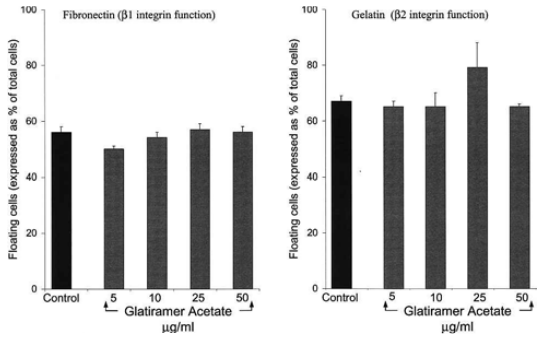

Figure 4 *The adhesive property of activated T cells is not altered by glatiramer acetate. Following 3 days of treatment of activated T cells with glatiramer acetate, 50,000 T cells were plated onto fibronectin (to evaluate β1 integrin function) or gelatin (β2 integrin) for 1 h. The proportion of cells that remained floating was enumerated and expressed as a percentage of the total adherent and floating cell population. None of the concentrations of glatiramer acetate affected adhesion when compared to controls (mean±SEM of triplicates). This result was reproduced in three different sets of experiments*

for 1–3 d) did not influence cytokine production in subsequent T cell–microglia interactions (results not shown).

The effect of glatiramer acetate in reducing cytokine production in T cell–adult microglia co-cultures is not the result of a decrease in the proliferation or survival of T cells. When the total number of T cells was counted after 72 h of glatiramer acetate treatment, cell numbers were comparable in control ($24\pm2\times10^3$) versus glatiramer acetate (5, 25 and 50 μg/ml) groups (26 ± 1, 26 ± 2 and 22 ± 1, respectively, $\times10^3$). It should be noted that while glatiramer acetate does affect the proliferation of lymphocytes, this generally requires a longer term of exposure (over 5 days) to drug *in vitro*.[15,21,33] Furthermore, dye exclusion assays revealed no overt toxicity to T cells after 3 days of glatiramer acetate treatment (unpublished observations). Finally, equal number of T cells was added to microglia in all test situations.

We have addressed other features that could be important determinants for understanding the mechanisms by which glatiramer acetate affects T cell–microglia interactions. First, T cells had to be activated with anti-CD3 antibody since co-cultures of unactivated T cells (even in the presence of 50 U/ml IL-2) with microglia did not result in increased production of TNF-α. Moreover, it was necessary for T cells to be pretreated with glatiramer acetate since its reducing effect on cytokine production did not occur when added at the time of co-culture. Indeed, our current results indicate that the pretreatment period of T cells should be at least 24 h before these cells could alter cytokine production in T cell–microglia interaction (unpublished observations).

We evaluated further the expression of other cytokines implicated in MS, specifically IL-1β, IL-6 and IL-12.[28,34,35] Levels of IL-1β and IL-12 were undetectable in the conditioned media of T cells and adult microglia in isolation, while IL-6 was present in substantial amounts (between 200 to 1000 pg/ml in six different sets of cultures). Following the co-culture of anti-CD3 activated T cells with adult microglia, IL-1β and IL-12 became detectable while levels of IL-6 remained unaltered from their high basal amounts. Glatiramer acetate pretreatment of T cells dose dependently attenuated the induction of IL-1β and IL-12 in T cell–microglia co-cultures, and also suppressed the amounts of the constitutively expressed cytokine, IL-6 (Figure 2).

We noted that when microglia encounter activated T cells, the morphology of microglia transforms from a ramified/bipolar morphology to an amoeboid rounded form (Figure 3). This is reminiscent of an activated microglia *in vivo*, which transforms progressively from a ramified resting morphology to an amoeboid form.[36] However, when T cells were pretreated with glatiramer acetate (25 μg/ml), the morphological transformation of microglia in T cell–microglia co-culture was attenuated. Overall, the lack of a morphological transformation of microglia is another indication that glatiramer acetate pretreatment of T cells results in their decreased ability to interact with microglia to produce cytokines.

Adhesion of T cells is not altered by glatiramer acetate

A possible explanation of the reduction of cytokine production by glatiramer acetate in T cell–microglia co-culture is that glatiramer-acetate-treated T cells were less able to adhere onto microglia compared to control T cells. Although this did not appear qualitatively to be the case as observed by examination of live cultures (Figure 3), we utilized an adhesion assay to test this possibility. Figure 4 demonstrates that glatiramer-acetate-pretreated T cells did not have a reduced tendency to adhere onto fibronectin or gelatin, which evaluated β1- and β2-integrin interaction, respectively.[32] Similarly, the adhesion of T cells onto a monolayer of PMA/IFNγ-treated U937 cells, a model of microglia (see next section), was not affected by glatiramer acetate (unpublished observations).

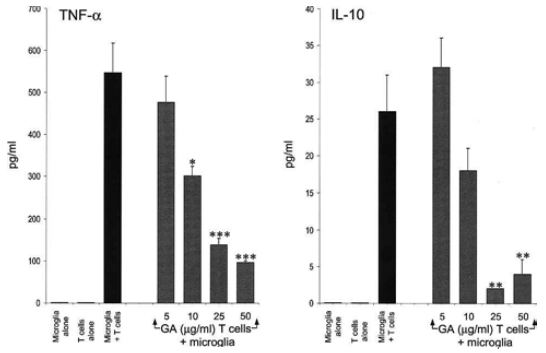

Figure 5 *Cytokine production in co cultures of T cells with fetal human microglia is reduced by glatiramer acetate. As with their adult counterparts, fetal human microglia in isolation produce negligible quantities of IL 10 and TNF α. In co cultures of T cells with microglia, increases of IL 10 and TNF α are elicited and these are reduced by the pretreatment of T cells with glatiramer acetate. Values are mean±SEM of three analyses. *p<0.05, **p<0.01, ***p<0.001, 1 way ANOVA with Bonferroni post hoc comparisons*

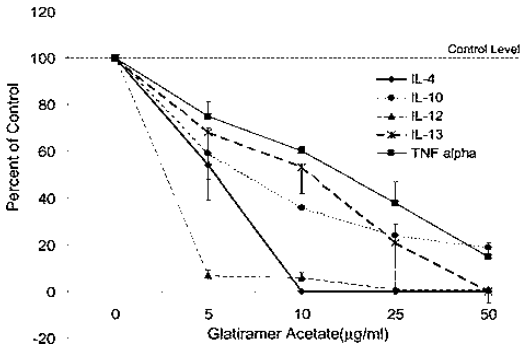

Figure 6 *Glatiramer acetate on cytokine production in co cultures of T lymphocytes with PMA/IFNγ treated U937 cells. In co culture of PMA/IFNγ treated U937 cells with T lymphocytes, the levels of cytokines induced after 24 h were as follows: 969 pg/ml for TNF α, 13 pg/ml for IL 4, 667 pg/ml for IL 10, 20 pg/ml for IL 12, and 51 pg/ml for IL 13. Values are mean±SEM of triplicate cultures, and all results have been expressed as a percentage of control T cell microglia cultures (i.e., without glatiramer acetate)*

Glatiramer acetate in other models of T cell–microglia/macrophage interactions

As with their adult counterparts, fetal human microglia in isolation do not secrete detectable amounts of IL-10 or TNF-α into the culture medium. When fetal human microglia were co-cultured with activated T cells, however, significant amounts of IL-10 and TNF-α were induced. With glatiramer acetate pretreatment of T cells, the resultant IL-10 and TNF-α produced in T cell–microglia co-cultures was reduced in a concentration-dependent manner (Figure 5).

U937 is a human pro-monocytoid cell line that assumes a bipolar microglia/macrophage-like morphology when sequentially treated with PMA and IFNγ. Indeed the PMA/IFNγ-treated U937 assumes several characteristics of microglia particularly with respect to cytokine production.[31] In this manuscript, we demonstrate that the PMA/IFNγ-treated U937 cells have low to negligible amounts of IL-10, IL-12 and TNF-α, but that their co-culture with anti-CD3 activated T cells resulted in the substantial increase in levels of these cytokines. Similarly, two Th2 cytokines, IL-4 and IL-13, were also induced in co-cultures of T cells with PMA/IFNγ-treated U937 cells. Significantly, the pretreatment of T cells with glatiramer acetate prior to their encounter with PMA/IFNγ-treated U937 cells reduced, in a concentration-dependent manner, the levels of all these cytokines (Figure 6).

Discussion

The treatment of patients with MS has improved significantly in the past few years, with drugs such as glatiramer acetate and IFNβ having a favourable impact on the clinical course of the disease. Despite this, the mechanisms of how these drugs work in MS are not fully elucidated.[29,37] Understanding the modes of action of current MS drugs and uncovering their critical targets will lead to a more rational approach to design better therapeutics.

A spate of recent papers[15,16,20,22,23,38] has provided support to an older literature[6,10,11,13,17,18] on the proposed mechanism of action of glatiramer acetate in MS. By an effect on antigen presentation, glatiramer acetate is thought to cause the production of Th2 suppressor/regulatory cells, which then migrate into the CNS. Within the CNS, and upon antigen restimulation, the glatiramer acetate-specific cells produce anti-inflammatory Th2 cytokines, including IL-5, IL-10 and IL-13, which are thought to inhibit the expansion of autoreactive Th1 cells, through a process termed bystander suppression (Figure 7).

The experiments of this manuscript were aimed at uncovering other mechanisms by which glatiramer acetate may work in MS.

Activated T cells traffic into the CNS as part of the immunological surveillance mechanism of the CNS. It is thought that those that recognize antigens are expanded and retained within the CNS while those that do not either undergo apoptosis or leave the CNS. In MS, increased numbers of activated T cells transit into the CNS parenchyma.[34,37] EAE experiments have revealed that while the initial wave of T cells that enters the brain is auto-antigen specific, the majority (over 98%) of the later arriving activated T cells have many different specificities;[39,40] similar kinetics are thought to occur in MS. In view of the large number of activated T cells of many specificities that enters the CNS, one might consider whether these can interact with CNS constituents in a promiscuous non-antigen-dependent manner to produce cytokines. In support, it was noted that in a facial nerve resection model in mouse, T cells infiltrated into the CNS and aggregated around microglia, and this was correspondent with an increase in IL-1β and TNF-α.[41] In graft-versus-host disease, activated microglia cell clusters are invariably associated with infiltrating T cell blasts.[42] Futhermore, microglia isolated from the brains of healthy adult mice stimulated the differentiation of naïve T cells into Th1 effector cells without affecting T-cell proliferation.[43]

The current model that polyclonally activated human T cells can interact with human microglia to upregulate a variety of inflammatory cytokines further sheds a new perspective on immune propagation within the CNS. Cytokine production in this T cell–microglia interaction occurs in the absence of any identifiable antigen, whether or not the microglia and T cells are MHC matched or mismatched.[24] TNF-α is produced predominantly by microglia, whereas IL-10 is produced by both cell types in the co-culture.[25] Ligand pairs that modulate IL-10 and TNF-α production in T cell–microglia interaction were found to include B7/CTLA-4:CD28, VLA-4:VCAM-1, CD40:CD40L and CD23:CD21/CD11.[25]

Thus, when activated T cells enter the CNS, they have the capacity to engage microglia to produce a variety of cytokines to promote CNS inflammation. In this manuscript, we have expanded the disease relevance of the T cell–microglia interaction by demonstrating that T cells pretreated with glatiramer acetate have a decreased capacity to evoke cytokine production in T cell–microglia co-culture. Indeed, all cytokines tested were reduced in a dose-dependent manner by glatiramer acetate.

At present, the mechanisms by which glatiramer acetate affect cytokine production in T cell–microglia interactions are unknown. Some clues may be found in

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