Myelin basic protein-primed T cells induce nitric oxide synthase in microglial cells: Implications to Multiple Sclerosis

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Running title: MBP-primed T cells induces iNOS in microglia

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Abstract:

The presence of autoreactive T cells recognizing self myelin antigens is necessary

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for the development of CNS autoimmune disease like Multiple Sclerosis (MS). The present study was undertaken to investigate the role of myelin basic protein (MBP)-primed T cells in the expression of inducible nitric oxide synthase (iNOS) in microglial cells. MBP-primed T cells alone markedly induced the production of NO and the expression of iNOS protein and mRNA in mouse BV-2 microglial cells. Similarly, MBP-primed T cells also induced the production of NO in mouse primary microglia. This induction of NO production was primarily dependent on the contact between MBP-primed T cells and microglia. The expression of very late antigen-4 (VLA-4) on the surface of MBP-primed T cells and inhibition of MBP-primed T cell-induced microglial NO production by functional blocking antibodies to α4 chain of VLA-4 (CD49d) suggest that VLA-4 integrin on MBP-primed T cells plays an important role in contact-mediated induction of iNOS. Since IFN-β has been used to treat MS patients, we examined the effect of IFN-β on MBP-primed T cell-induced production of NO. Surprisingly, IFN-β alone induced the production of NO in microglial cells. However, the pretreatment of MBP-primed T cells with IFN-β inhibited the expression of VLA-4 integrin on the surface of MBP-primed T cells and thereby inhibited the ability of those T cells to induce the production of NO in microglial cells. This study illustrates a novel role of neuroantigen-primed T cells in inducing contact-mediated expression of iNOS in microglial cells which may participate in the pathogenesis of MS.

Key words: T cells, Microglia, iNOS, IFN-β, VLA-4

Introduction:

Nitric oxide (NO), a short-lived and bioactive free radical, has been recognized to have many functions in normal physiological and pathophysiological conditions (1,2). At low concentration, nitric oxide (NO) has been shown to play an unique role in neurotransmission and vasodilation, however, at higher concentration, it is a potent neurotoxin (1,2). Consistently, NO, derived in excessive amount from the activation of inducible nitric oxide synthase (iNOS) in glial cells (microglia and astrocytes), is assumed to contribute to oligodendrocyte degeneration in demyelinating diseases and neuronal death during neurodegenerative diseases (3-7). Evidence from several laboratories emphasizes the involvement of NO in the pathophysiology of multiple sclerosis (MS) and experimental allergic encephalomyelitis (EAE), the animal model of MS (8-10). Analysis of cerebrospinal fluid (CSF) from MS patients has shown increased levels of nitrite

and nitrate compared with normal control (11). The reaction of NO with O_2^- forms peroxynitrite, $ONOO^-$, a strong nitrosating agent capable of nitrosating tyrosine residues of a protein to nitrotyrosine. Increased levels of nitrotyrosine have been found in demyelinating lesions of MS brains as well as in spinal cords of mice with EAE (12,13). Consistently, uric acid, a scavenger of peroxynitrite, markedly inhibits the appearance of EAE in mice, and the incidence of MS has been found very rare among gout patients having higher levels of uric acid suggesting the critical involvement of peroxynitrite in the disease process of EAE and MS. Subsequently, semiquantitative RT-PCR for iNOS mRNA in MS brains also shows markedly higher expression of iNOS mRNA in MS brains than in normal brains (14,15).

However, the mechanism by which NO is generated in the MS brain is unclear, but it is known that the resident CNS macrophage, the microglia, is an important source. MS is the most common human autoimmune disease of the CNS of unknown etiology. The infiltration of neuroantigen-specific T cells into the CNS is considered a key event in the pathogenesis of MS or EAE (16,17). However, the mechanisms through which neuroantigen-specific T cells play an etiologic role in MS remain unclear. Since NO produced in excessive amount from the activation of iNOS participates in the pathogenesis of MS, we investigated the role of neuroantigen-specific T cells in the expression of iNOS in microglial cells.

We herein report that MBP-primed T cells induce the expression of iNOS in mouse microglial cells through cell-cell contact and that VLA-4 antigen on T cell surface plays an important role in this process. Interestingly, IFN-β inhibited the expression of VLA-4 and thereby blocked the ability of MBP-primed T cells to induce microglial iNOS.

Materials and Methods:

Reagents: Fetal bovine serum (FBS), Hanks balanced salt solution (HBSS), DMEM/F-12, RPMI 1640, L-glutamine and β-mercaptoethanol (β-ME) were from Mediatech. L-N^G-Monomethylarginine (L-NMA) and D-N^G-monomethylarginine (D-NMA) were purchased from Biomol. Arginase was purchased from Sigma. Antibodies against mouse macrophage iNOS were obtained from Calbiochem. ^{125}I-labeled protein A and [α-^{32}P]dCTP were obtained from NEN. Functional blocking antibodies and FITC-labeled antibodies to CD49d (the α4 chain of VLA-4) were obtained from Pharmingen.

Isolation and purification of T cells from lymph nodes: Female SJL/J mice were immunized s.c. with 400 μg bovine MBP and 60 μg *Mycobacterium tuberculosis* (H37RA; Difco Labs.) in IFA (Calbiochem). On 10 to 12 day of immunization, lymph nodes were collected from these mice and single cell suspension was prepared in RPMI 1640 medium containing 10% FBS, 2 mM L-glutamine, 50 μM β-ME, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells cultured at a concentration of 4-5 × 10^6 cells/ml in six-well plates were incubated with 50 μg/ml MBP for 4 days. The non adherent cells were collected and passed through the nylon wool column preincubated for a period of 30 min with RPMI 1640 supplemented with 10% FBS at 37 ^{0}C, 5% CO_2. The first 15-20 ml eluant was collected, centrifuged at 500g, and resuspended in RPMI 1640-FBS. Viability of the cells was checked by trypan blue exclusion.

Flow cytometry: The purity of lymph node cells, obtained after nylon wool column separation, was checked by flow cytometry using FITC-labeled anti-CD3 (Biosource International). Briefly, 1 X 10 ^{6} cells suspended in RPMI 1640-FBS were incubated in the dark with appropriately diluted FITC-labeled anti-CD3 at 4 ^{0}C for 30 min. Following incubation, cell suspension was centrifuged, washed thrice and resuspended in 500 μl of RPMI-FBS. The cells were then analyzed through FACS (Beckton Dickinson, USA). A minimum of 10,000 cells were accepted for FACS analysis. Cells were gated based on morphological characteristics. Apoptotic and necrotic cells were not accepted for FACS analysis. More than 98% cells were found as CD3 positive T cells. These T cell populations were used to stimulate microglial cells. Similarly, FITC-labeled antibodies to CD49d (Integrin α4 chain) (Pharmingen) was used to examine the surface expression of VLA-4 on T cells.

T cell proliferation assay: T cells isolated from MBP-immunized mice were cultured in 96 well U bottomed microtiter plate (Coaster) in 200 μl of RPMI 1640 containing 10% FBS, 2mM L-glutamine, 50 μM β-ME, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells (2 X 10^5 cells) in the wells were stimulated with different concentrations of MBP. Unstimulated cells were kept as controls. After 72 h of stimulation, cells were pulsed with [^{3}H]-thymidine (0.5 μCi/ well) for another 24 h. Cells were then harvested on GF/C glass fiber filter (Whatman) and the level of [^{3}H]-thymidine incorporation in cells was assessed by scintillation counter (Beckman).

Treatment of MBP-primed T cells with IFN-β before their co-culture with

microglial cells: T cells isolated from MBP-immunized mice were cultured in RPMI 1640 (supplemented with 10% FBS, 2mM L-glutamine, 50 μM β-ME, 100 U/ml penicillin, 100 μg/ml streptomycin) containing 50 μg/ml MBP in the presence or absence of different concentrations of IFN-β for 4 d before their co-culture with microglia. One day before this co-culture, another dose of IFN-β was administered. Cells were then centrifuged, washed, and used in co-culture.

Isolation of Mouse Primary Microglia: Microglial cells were isolated from mixed glial cultures according to the procedure of Guilian and Baker (18). Briefly, on day 7 to 9 the mixed glial cultures were washed 3 times with DMEM/F-12 and subjected to a shake at 240 rpm for 2 h at 37^0C on a rotary shaker. The floating cells were washed and seeded onto plastic tissue culture flasks and incubated at 37^0C for 2 h. The attached cells were removed by trypsinization and seeded on to new plates for further studies. Ninety to ninety-five percent of this preparation was found to be positive for Mac-1 surface antigen. For the induction of NO production, cells were stimulated with MBP-primed T cells in serum-free DMEM/F-12.

Mouse BV-2 microglial cells (kind gift from Virginia Bocchini of University of Perugia) were also maintained and induced with different stimuli as indicated above.

Assay for NO Synthesis: Synthesis of NO was determined by assay of culture supernatants for nitrite, a stable reaction product of NO with molecular oxygen. Briefly, supernatants were centrifuged to remove cells, and 400 μl of each supernatant was allowed to react with 200μl of Griess reagent (19-22) and incubated at room temperature for 15 min. The optical density of the assay samples was measured spectrophotometrically at 570 nm. Fresh culture media served as the blank. Nitrite concentrations were calculated from a standard curve derived from the reaction of $NaNO_2$ in the assay. Protein was measured by the procedure of Bradford (23).

Immunoblot Analysis for iNOS: Immunoblot analysis for iNOS was carried out as described earlier (20-22). Briefly, cells were detached by scraping, washed with Hank's buffer, and homogenized in 50mM Tris-HCl (pH 7.4) containing protease inhibitors (1 mM PMSF, 5 μg/ml aprotinin, 5 μg/ml pepstatin A, and 5 μg/ml leupeptin). After electrophoresis the proteins were transferred onto a nitrocellulose membrane, and the iNOS band was visualized by immunoblotting with antibodies against mouse macrophage iNOS and ^{125}I-labeled protein A (20-22).

RNA Isolation and Northern Blot Analysis: Cells were taken out of the culture

dishes directly by adding Ultraspec-II RNA reagent (Biotecx Laboratories, Inc.), and total RNA was isolated according to the manufacturer's protocol. For Northern blot analyses, 20 μg of total RNA was electrophoresed on 1.2% denaturing formaldehyde-agarose gels, electrotransferred to Hybond nylon membrane (Amersham Pharmacia Biotech), and hybridized at 68°C with ^{32}P-labeled cDNA probe using Express Hyb hybridization solution (CLONTECH) as described by the manufacturer. The cDNA probe was made by polymerase chain reaction amplification using two primers (forward primer: 5'-CTC CTT CAA AGA GGC AAA AAT A-3'; reverse primer: 5'-CAC TTC CTC CAG GAT GTT GT-3') (24-27). After hybridization, the filters were washed two or three times in solution I ($2 \times$ SSC, 0.05% SDS) for 1 h at room temperature followed by solution II (0.1 $\times$ SSC, 0.1% SDS) at 50 °C for another hour. The membranes were then dried and exposed to x-ray films (Kodak). The same amount of RNA was hybridized with probe for glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Results:

MBP-primed T cells induce the production of NO and the expression of iNOS in BV-2 microglial cells: T cells isolated from lymph nodes of MBP-primed mice proliferated in response to MBP, and maximum proliferation was observed at 50 or 100 μg/ml of MBP (Fig. 1). However, these cells did not proliferate in response to bovine serum albumin (BSA) (data not shown) suggesting that these cells were primed specifically against MBP. Therefore, in subsequent studies, these cells were treated with 50 μg/ml of MBP for priming. After priming, these T cells were purified by nylon wool column. As shown in the FACS analysis of Fig. 2, MBP-primed T cells were more than 98% pure after nylon wool column purification. Next we examined if these purified MBP-primed T cells can induce the production of NO in mouse BV-2 microglial cells. MBP-primed T cells were washed and added to BV-2 microglial cells in direct contact. There was no induction of NO production when MBP-primed T cells were added to microglial cells at a ratio of 0.1:1 (Fig. 3). However, the induction of NO production started from the ratio of 0.2:1 of T cell:microglia, reached to the maximum at the ratio of 0.7:1 and decreased at higher concentrations of T cells (Fig. 3). This decrease in NO production was due to the increase in microglial cell death in the presence of higher concentration of MBP-primed T cells (data not shown). In contrast to MBP-primed T cells, normal T cells were unable to induce the production of NO in BV-2 microglial cells (Table-1).

Next we examined if the contact between MBP-primed T cells and microglial cells is necessary to induce the production of NO. First, MBP-primed T cells were placed in a culture insert, where they were in close proximity to, but not contacting microglia. In contrast to marked induction of NO production by MBP-primed T cell:microglia contact (Fig. 3 and Table-1), very little induction of NO production was observed when MBP-primed T cells were placed within culture inserts (Fig. 3). Second, the conditioned supernatant of MBP-primed T cells were added to microglial cells. Fifty microliter of supernatant is equivalent to T cells sufficient for the ratio of 0.5:1 of T cell:microglia. The induction of NO production by 50 μl of that supernatant (7.7 ± 2.4 μg/24 h/mg protein) was very low as compared to that (256 ± 34 μg/24 h/mg protein) of 0.5:1 of T cell:microglia (Tables 1 and 2). These observations suggest that the induction of NO production by MBP-primed T cells primarily depend on the contact between T cells and microglia. However, higher volumes of supernatants of MBP-primed T cells did induce the production of NO in BV-2 glial cells by 5 to 9 fold (Table-2).

The inhibition of MBP-primed T cell-induced NO production by arginase, an enzyme that degrades the substrate (L-arginine) of NOS and L-NMA, a competitive inhibitor of NOS, but not by D-NMA, a negative control of L-NMA, suggests that MBP-primed T cells induce the production of NO in BV-2 microglial cells through NOS-dependent arginine metabolism (Table-1). To understand further the mechanism of NO production, we examined the effect of MBP-primed T cells on protein and mRNA levels of iNOS. Western blot analysis with antibodies against murine macrophage iNOS and Northern blot analysis for iNOS mRNA clearly showed that MBP-primed T cells alone are capable of inducing the expression of iNOS protein (Fig. 4A) and iNOS mRNA (Fig. 4B). Consistent to the induction of NO production, MBP-primed T cells did not induce the expression of iNOS protein and mRNA in BV-2 microglial cells when added at a ratio of 0.1:1. The induction of iNOS protein and mRNA started at the ratio of 0.2:1 of T cell:microglia, peaked at the ratio of 0.7:1 and decreased at higher concentration of T cells (Fig. 4).

Consistent to a role of NF-kB activation in the expression of iNOS (24-31), we have also found that MBP-primed T cell:microglia contact induced the activation of NF-kB in BV-2 microglial cells and that SN50, a specific inhibitor of NF-kB activation (32), but not that SN50M, a nonfunctional mutant of SN50, inhibited MBP-primed T cell-induced production of microglial NO (data not shown).

MBP-primed T cells induce the production of NO in mouse primary microglia: To

understand if MBP-primed T cells were able to induce the production of NO in primary cells, we examined the effect of MBP-primed T cells on the induction of NO production in mouse primary microglia (Table-3). Consistent to the induction of NO production in BV-2 microglial cells, MBP-primed T cells dose-dependently induced the production of NO in mouse primary microglia. Similar to in BV-2 cells, MBP-primed T cells were unable to induce the production of NO in primary microglia when added at a ratio of 0.1:1 of T cells and microglia (Table-3). However, the induction of NO production started from the ratio of 0.2:1 of T cell:microglia, reached to the maximum at the ratio of 0.5:1 and decreased at higher concentrations of T cells (Table-3).

Role of very late antigen-4 (VLA-4) in MBP-primed T cell-induced production of NO in BV-2 microglial cells: Integrins being present on cell surface are mainly involved in integrating cell-cell interaction (33). It has been shown that T cells activated by anti-CD3 express more VLA-4 integrin and that VLA-4 plays an important role in T cell contact-mediated activation of microglial cells (34). Therefore, we investigated if neuroantigen-primed T cells require VLA-4 to induce contact-mediated production of NO in microglial cells. At first, we analyzed the expression of VLA-4 on the surface of MBP-primed T cells by FACS analysis using FITC-labeled antibodies against the α4 chain of VLA-4 antigen. Figure 5A represents auto-fluorescence as this was observed in unconjugated normal T cells. As areas under M1 and M2 in figure 5 represent auto-fluorescence and fluorescence due to VLA-4 respectively, there was very little expression of VLA-4 on the surface of normal T cells (Fig. 5B) in contrast to marked expression of VLA-4 on the surface of MBP-primed T cells (Fig. 5C). Analysis of three separate experiments shows that MBP-priming induced the expression of VLA-4 by 16.85 ± 2.15 fold. These results suggest that neuroantigen priming induces the expression of VLA-4 on the surface of T cells.

Next to examine if this VLA-4 is involved in contact-mediated production of NO, we used functional blocking antibodies against CD49d (the α4 chain of VLA-4). It is apparent from Figure 6 that incubation of MBP-primed T cells with different concentration of antibodies against CD49d inhibited its ability to induce the production of NO in microglial cells. Although antibodies against CD49d at a dose of 10 or 20 μg/ml was not effective in blocking the production of NO (data not shown), marked inhibition was observed when antibodies were used at a concentration of 50 or 100 μg/ml. In contrast, control IgG did not block the ability of MBP-primed T cells to induce microglial production of NO (Fig. 6). These studies suggest that VLA-4 on the surface of MBP-primed T cells is involved in

contact-mediated induction of NO production in microglial cells.

Effect of IFN-β on MBP-primed T cell-induced production of NO in BV-2 microglial cells: Recent clinical trials have demonstrated that interferon-β (IFN-β) or betaserone decreases the number of relapses in relapsing-remitting MS (35,36). However the mode of action of IFN-β in MS remains unclear. Since MS is a T cell-mediated disease, and MBP-primed T cells induce iNOS in microglial cells, we investigated the effect of IFN-β on MBP-primed T cell-induced production of NO in BV-2 microglial cells. MBP-primed T-cells, with or without preconditioned with different doses of IFN-β, were co-cultured with BV-2 microglial cells for 24 h. It is evident from Fig. 7A that the production of NO in BV-2 glial cells was markedly inhibited by more than 90% when they were co-cultured with MBP-primed T cells preconditioned with IFN-β. On the other hand, addition of IFN-β to the co-culture of BV-2 glial cells and MBP-primed T cells, did not result in inhibition of NO production (Fig. 7B) suggesting that pretreatment of MBP-primed T cells with IFN-β is necessary to inhibit the ability of T cells to induce the production of NO. Surprisingly, IFN-β alone induced the production of NO in BV-2 microglial cells (Fig. 7C). These observations suggest that in spite of the ability of IFN-β itself to induce the production of NO in microglial cells, pretreatment of MBP-primed T cells with IFN-β inhibits the ability of MBP-primed T cells to induce contact-mediated production of NO in microglial cells.

Effect of IFN-β on the surface expression of VLA-4 in MBP-primed T cells: Since IFN-β inhibited the ability of MBP-primed T cells to induce contact-mediated production of NO in microglia, we investigated the effect of IFN-β on the expression of VLA-4 on the surface of MBP-primed T cells. MBP-primed T cells were treated with 50 U/ml of IFN-β during priming with 50 μg/ml of MBP for 4 d before the FACS analysis. One day before FACS analysis, another dose of IFN-β (50 U/ml) was administered. Cells were then centrifuged, washed, and used in co-culture. Consistent to the inhibitory effect of IFN-β on MBP-primed T cell-induced production of NO (Fig. 7A), pretreatment of MBP-primed T cells with IFN-β led to the inhibition of VLA-4 expression (compare Fig. 5D to Fig. 5C). Analysis and quantitation of FACS data obtained from four separate experiments showed that IFN-β treatment of MBP-primed T cells resulted in $50.8 \pm 12.1\%$ inhibition of VLA-4 expression. These results suggest that IFN-β inhibits the ability of MBP-primed T cells to induce microglial production of NO by inhibiting the expression of VLA-4 on the surface of MBP-primed T cells.

Discussion:

Activation of microglia, CNS-resident professional macrophages, has been implicated in the pathogenesis of a variety of neurodegenerative diseases, including multiple sclerosis (MS), Alzheimer's disease (AD), Creutzfeld-Jacob disease, and HIV-dementia (37). Upon activation, microglia produce and secrete potentially neuroinflammatory molecules including NO that plays a pivotal role in demyelination of MS patients (7,24,25,28). Bagasra et al (38) have shown that iNOS-expressing cells in MS brains were predominantly, but not exclusively, microglia/macrophages. Therefore, understanding the mechanism by which iNOS is induced in CNS microglia of MS patients can thus impact upon the rational treatment of the disease. However, the mechanism by which microglia are activated to induce the expression of iNOS for the production of NO in MS brain remains unclear. Although LPS is a potent inducer of iNOS in microglia, it has not been demonstrated to have a physiological relevance in MS (39). IFN-γ, alone or in combinations with other proinflammatory cytokines like TNF-α and IL-1β, can also induce iNOS in microglia (7,24,25,28), however, microglia isolated from adult human brain tend to be a poor source of iNOS-derived NO in response to these proinflammatory cytokines (40,41). These observations suggest that apart from proinflammatory cytokines, microglia can be activated by some other mechanism(s) for the induction of iNOS in the CNS of MS patients.

The infiltration of neuroantigen-specific T cells into the CNS is considered a key event in the pathogenesis of MS or EAE. However, the biological role of such T cells within the CNS is poorly understood. Several lines of evidence presented in this manuscript clearly support the conclusion that MBP-primed T cells potently induce the expression of iNOS in microglial cells through cell-cell contact. This conclusion is based on the following observations. First, MBP-primed T cells induce the production of NO and the expression of iNOS through the activation of NF-kB in mouse microglial cells. This effect was dose-dependent and it peaked at 0.7:1 or 0.5:1 of T cell:microglia. In contrast, normal T cells were unable to induce the production of NO and the expression of iNOS. Second, the placement of MBP-primed T cells in a culture insert, where they were in close proximity to, but not contacting microglia, was unable to induce the production of NO in microglial cells. Third, soluble factors of MBP-primed T cells equivalent to T cells of 0.5:1 or 0.7:1 of T cell:microglia were very poor inducers of NO.

Integrins and adhesion molecules are involved in cell-cell interaction. It has been shown that an increased number of lymphocytes express VLA-4 in active

MS lesions (42) and that blocking VLA-4 function with a monoclonal antibody prevents experimental allergic encephalomyelitis, an animal model of MS, in mice (43). Consistently, short-term treatment of MS patients with anti α4 integrin antibody significantly reduces BBB dysfunction as assessed by contrast-enhanced MRI (44). Earlier Yong and colleagues (34) have reported that this VLA-4-VCAM-1 interaction plays a crucial role when anti-CD3-stimulated T cells activate microglia through cell-cell contact prompting us to investigate the role of VLA-4 in neuroantigen-primed T cell-induced microglial production of NO. The observations presented in this manuscript that MBP-primed T cells expressed VLA-4 on their surface and that blocking of that VLA-4 by neutralizing antibodies against the α4 chain of VLA-4 inhibited the ability of MBP-primed T cells to induce the production of NO clearly suggest that VLA-4 integrin on the surface of neuroantigen-primed T cells plays an important role in contact-mediated induction of iNOS in microglia. NO being a major mediator in immune and autoimmune functions has been also shown to increase permeability of the BBB, allowing substances to enter into the brain passively leading to vasogenic edema and secondary brain damage (45). Although the precise molecular mechanisms for NO-induced breakdown of the BBB are not completely understood, in a cell culture model of the BBB, NO leads to a rapid breakdown in model barrier integrity and resulted in a reduction in cellular ATP content and GAPDH activity (46). If these cell culture studies have significance in vivo the CNS of EAE and MS, it is possible that neuroantigen-primed T cells may increase the permeability of BBB through contact-mediated induction of NO production in microglia and astroglia (Dasgupta and Pahan, unpublished observation) present near the BBB junction.

Sedgwick et al (47) have shown that microglia and T cells do interact in vivo. In the graft-versus-host disease model, activated microglia were found to cluster around T cell infiltrates, and to be associated with single or clustered microglia (47). Furthermore, microglia isolated from animals of graft-versus-host disease proliferated and exhibited functions of activated microglia, such as phagocytosis and motility (47). In light of these in vivo observations, and the proximity of activated T cells and microglia in the perivascular space or CNS parenchyma of MS lesions (48), the induction of iNOS by the contact between MBP-primed T cells and microglia described in this manuscript is relevant to MS pathogenesis. The in vitro model of our study used a 0.5:1 or 0.7:1 ratio of neuroantigen-primed T cells to microglia, and at present, it is unclear if this is a relevant ratio. Depending on the fact that microglia constitute only 2-5% of total

CNS cells in normal brain (37), the present study suggests that infiltration of very few neuroantigen-primed T cells would be sufficient to activate CNS microglia for contact-mediated induction of iNOS.

Several studies have shown that IFN-β decreases the number of relapses and MRI-detected lesions in relapsing/remitting MS patients (35,36,49). However, the precise mechanism(s) by which IFN-β is effective in the treatment of MS has remained unclear. It has been suggested that IFN-β attenuates the disease process of MS probably through mediating systemic immunity (50), decreasing T cell reactivity to produce IFN-γ (51), stimulating the production of IL-10, an anti-inflammatory cytokine (17), inhibiting antigen presentation to T cells (52), or modifying humoral immune response (53). Our current results have underlined another important mechanism by which IFN-β may be efficient in MS. In this regard, IFN-β blocks the ability of neuroantigen-primed T cells to induce contact-mediated production of the oligodendrocyte-toxic molecule, NO, in microglia by inhibiting the expression of VLA-4 on the surface of neuroantigen-primed T cells. It is not known if IFN-β can penetrate the blood-brain barrier. However, we have found that IFN-β alone can induce the production of NO and the activation of NF-kB (data not shown) in microglial cells suggesting that apart from inhibiting the functions of activated T cells if IFN-β itself can enter into the CNS it may augment the inflammatory response. Consistent to our observation, it has been found that IFN-β is beneficial in relapsing/remitting MS but not in acute MS attacks (54,55) when the integrity of the blood-brain barrier is questionable.

Consistent to the inhibitory effect of IFN-β on the surface expression of VLA-4 in neuroantigen-primed T cells (Fig. 5), after analyzing the cell surface expression of VLA-4 by flow cytometry in 10 MS patients before and during IFN-β treatment, Calabresi et al (56) have shown that the mean VLA-4 fluorescence of MS patients' lymphocytes decreases on treatment compared to untreated controls. However, this inhibitory effect of IFN-β on surface expression of VLA-4 was absent in in vitro studies with PBMC (56). By western blot analysis Chabot et al (34) have reported that the total cellular level of the 80-kDa CD49d is lower (about 40%) in T lymphocytes treated with IFN-β than in untreated cells. In contrast, under the same condition, the cell surface levels of α4 integrin as assessed by flow cytometry were not affected (34) leading to the speculation that IFN-β treatment might affect the affinity of integrin molecules without altering their expression on the cell surface. However, in our experiment, IFN-β treatment has been found to inhibit the mean VLA-4 fluorescence on the surface of neuroantigen (MBP)-primed T cells compared to untreated cells (Fig. 5). The only difference

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between these two studies is that activated T cells were incubated with IFN-β for 3 days in the former study by Chabot et al (34) but for 4 days in the present one (Fig. 5). Therefore, this discrepancy about the effect of IFN-β on surface expression of VLA-4 in T cells could be explained by the possible complexity involved in the turnover of cell surface integrins.

NO and peroxynitrite (reaction product of NO and O_2^-) are potentially toxic molecules to neurons and oligodendrocytes that may mediate toxicity through the formation of iron-NO complexes of iron containing enzyme systems, oxidation of protein sulfhydryl groups, nitration of proteins and nitrosylation of nucleic acids and DNA strand breaks (2). NO derived from microglia has also been implicated in the damage of myelin producing oligodendrocytes in demyelinating disorders like MS (7). Because neuroantigen-primed T cells infiltrate into the CNS of MS patients, the induction of iNOS expression by MBP-primed T cells in microglia suggests that neuroantigen-primed T cells may induce the neural injury in the inflamed CNS through the induction of NO production.

References:
1. Nathan, C. (1992) *FASEB J.* **6,** 3051-3064
2. Jaffrey, S. R., and Snyder, S. H. (1995) *Annu. Rev. Cell Dev. Biol*. **11,** 417-440
3. Galea, E., Feinstein, D. L., and Reis, D. J. (1992) *Proc. Natl. Acad. Sci. USA.* **89,** 10945-10949
4. Koprowski, H., Zheng, Y.M., Heber-Katz, E., Fraser, N., Rorke, L., Fu, Z. F., Hanlon, C., and Dietzshold, B. (1993) *Proc. Natl. Acad. Sci. USA.* **90,** 3024-3027
5. Mitrovic, B., Ignarro, L. J., Montestruque, S., Smoll, A., and Merril, J. E. (1994) *Neurosci.* **61,** 575-585
6. Murphy, G. M., Yang, L., and Cordell, B. (1998) *J. Biol. Chem*. **273,** 20967-20971
7. Merrill, J. E., Ignarro, L. J., Sherman, M. P., Melinek, J., and Lane, T. E. (1993) *J. Immunol.* **151,** 2132-2141
8. Kolb, H., and Kolb-Bachofen, V. (1992) *Immunol. Today* **13,** 157-160
9. McCatney-Francis, N., Allen, J. B., Mizel, D. E., Albina, J. E., Xie, Q-W,

Nathan, C. F., and Wahl, S. M. (1993) *J. Ex. Med.* **178,** 749-754

10. Ding, M., Zhang, M., Wong, J. L., Rogers, N. E., Ignarro, L. J., and Voskuhl, R. R. (1998) *J. Immunol.* **160,** 2560-2564

11. Johnson, A. W., Land, J. M., Thompson, E. J., Bolanos, J. P., Clark, J. B., and Heales, S. J. R. (1995) *J. Neurol. Neurosurg. Psychiatry* **58,** 107-115

12. Brenner, T., Brocke, S., Szafer, F., Sobel, R. A., Parkinson, J. F., Perez, D. H., and Steinman, L. (1997) *J. Immunol.* **158,** 2940-2946

13. Hooper, D. C., Scott, G. S., Zborek, A., Mikheeva, T., Kean, R. B., Koprowski, H., and Spitsin, S.V. (2000) *FASEB J.* **14,** 691-698

14. Brosan, C. F., Battistini, L., Raine, C. S., Dickson, D. W., Casadevall, A., and Lee, S. C. (1994) *Dev. Neurosci.* **16,** 152-161

15. Bo, L., Dawson, T. M., Wesselingh, S., Mork, S., Choi, S., Kong, P. A., Hanley, D., and Trapp, B. D. (1994) *Ann. Neurol.* **36,** 778-786

16. Utz, U., and McFarland, H. F. (1994) *J. Neuropathol. Exp. Neurol.* **53,** 351-358

17. Chabot, S., and Yong, V. W. (2000) *Neurology* **55,** 1497-1505

18. Giulian, D., and Baker, T. J. (1986) *J. Neurosci.* **6,** 2163-2178

19. Feinstein, D. L., Galea, E., Roberts, S., Berquist, H., Wang, H., and Reis, D. J. (1994) *J. Neurochem.* **62,** 315-321

20. Pahan, K., Namboodiri, A. M. S., Sheikh, F. G., Smith, B. T., and Singh, I. (1997) *J. Biol. Chem.* **272,** 7786-7791

21. Pahan, K., Sheikh, F. G., Khan, M., Namboodiri, A. M. S., and Singh, I. (1998) *J. Biol. Chem.* **273,** 2591-2600

22. Pahan, K., Raymond, J. R., and Singh, I. (1999) *J. Biol. Chem.* **274,** 7528-7536

23. Bradford, M. (1976) *Anal. Biochem.* **72,** 248-254

24. Jana, M., Liu, X., Koka, S., Ghosh, S., Petro, T. M., and Pahan, K. (2001) *J. Biol. Chem.* **276,** 44527-44533

25. Pahan, K., Sheikh, F. G., Liu, X., Hilger, S., McKinney, M., and Petro, T. M. (2001) *J. Biol. Chem.* **276,** 7899-7905

26. Pahan, K., Liu, X., McKinney, M. J., Wood, C., Sheikh, F. G., and Raymond, J. R. (2000) *J. Neurochem.* **74,** 2288-2295

27. Pahan, K., Liu, X., Wood, C., and Raymond, J.R. (2000) *FEBS Lett.* **472,** 203-207

28. Pahan, K., Sheikh, F. G., Namboodiri, A. M. S., and Singh, I. (1997) *J. Clin. Invest.* **100,** 2671-2679

29. Xie, Q., Kashiwabara, Y., and Nathan, C. (1994) *J. Biol. Chem.* **269,** 4705-

4708

30. Taylor, B. S., de Vera, M. E., Ganster, R. W., Wang, Q., Shapiro, R. A., Morris, S. M., Billiar, T. R., and Geller, D. A. (1998) *J. Biol. Chem.* **273,** 15148-15156

31. Pahan, K., Sheikh, F. G., Namboodiri, A. M. S., and Singh, I. (1998) *J. Biol. Chem.* **273,** 12219-12226

32. Lin, Y-Z., Yao, S. Y., Veach, R. A., Torgerson, T. R., and Hawiger, J. (1995) *J. Biol. Chem.* **270,** 14255-14258

33. Hynes, R. O. (1987) *Cell* **48,** 549-554

34. Chabot, S., Williams, G., and Yong, V. W. 1997. *J. Clin. Invest.* **100,** 604-612

35. Yong, V. W., Chabot, S., Stuve, O., and Williams, G. (1998) *Neurology* **51,** 682-689

36. Paty, D.W., Li, D.K.B., and the UBC MS/MRI Study Group, and the IFN Multiple Sclerosis Study Group. (1993) *Neurology* **43,** 662-667

37. Gonzalez-Scarano, F., and Baltuch, G. (1999) *Annu. Rev. Neurosci.* **22,** 219-240

38. Bagasra, O., Michaels, F. H., Zheng, Y. M., Bobroski, L. E., Spitsin, S. V., Fu, Z. F., Tawadros, R., and Koprowski, H. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 12041-12045

39. Martin, R., McFarland, H. F., and McFarlin, D. E. (1992) *Annu. Rev. Immunol.* **10,** 153-187

40. Zhao, M. L., Liu, J. S., He, D., Dickson, D. W., and Lee, S. C. (1998) *Brain Res.* **813,** 402-405

41. Peterson, P. K., Hu, S., Anderson, W. R., and Chao, C. C. (1994) *J. Infect Dis.* **170,** 457-460

42. Sedgwick, J. D., Ford, A. L., Foulcher, E., and Airriess, R. (1998) *J. Immunol.* **160,** 5320-5330

43. Traugott, U., Reinherz, E. L., and Raine, C. S. (1983) *Science* **218,** 308-310

44. Cannella, B., and Raine, C. S. (1995) *Ann. Neurol.* **37,** 424-435

45. Yednock, T., Cannon, C., Fritz, L., Sanchez-Madrid, R., Steinman, L., and Karin, N. (1992) *Nature* **356,** 63-66

46. Tubridy, N., Behan, P. O., Capildeo, R., Chaudhuri, A., Forbes, R., Hawkins, C. P., Hughes, R. A. C., Palace, J., Sharrack, B., Swingler, R., Young, C., Moseley, I. F., MacManus, D.G., Donoghue, S., and Miller, D. H., (1999) *Neurology* **53,** 466-472

47. Thiel, V. E., and Audus, K. L. (2001) *Antioxid. Redox Signal.* **3,** 273-278

48. Hurst, R. D., Azam, S., Hurst, A., and Clark, J. B. (2001) *Brain Res.* **894,** 181-

188

49. Rudick, R. A., Carpenter, C. S., Cookfair, D. L., Tuohy, V. K., and Ransohoff, R. M. (1996) *Neurology* **43,** 2080-2087.

50. IFN Multiple Sclerosis Study Group. (1993) *Neurology* **43,** 655-661

51. Noronha, A., Toscas, A., and Jensen, M. A. (1993) *J. Neuroimmunol.* **46,** 145-154

52. Noronha, A., Toscas, A., and Jensen, M. A. (1995) *Ann. Neurol.* **37,** 7-15

53. O'Gorman, M. R. G., Oger, J., and Kastrukoff, L. F. (1987) *Clin. Exp. Immunol.* **67,** 66-67

54. Whitaker, J. N. 1994. *Ann. Neurol.* **36,** S103-S107

55. Connelly, J. F. 1994. *Ann. Phamacother.* **28,** 610-616

56. Calabresi, P. A., Pelfrey, C. M., Tranquill, L. R., Maloni, H., McFarland, H. F. (1997) *Neurology* **49,** 1111-1116

Table-1. **Induction of NO production in mouse BV-2 microglial cells by MBP-primed T cells**

Treatments	Nitrite (μg/24 h/mg protein)
Control BV-2 cells	3.6 + 0.5
BV-2 + Normal T cells (1:0.5)	3.6 + 0.3
BV-2 + Normal T cells (1:1)	3.8 + 0.5
BV-2 + MBP-primed T cells (1:0.5)	256 + 34
BV-2 + MBP-primed T cells (1:0.5) + L-NMA	6.3 + 1.7
BV-2 + MBP-primed T cells (1:0.5) + D-NMA	255 + 43
BV-2 + MBP-primed T cells (1:0.5) + Arginase	4.6 + 1.5

BV-2 microglial cells received MBP-primed T cells at 1 to 0.5 ratio under serum-free condition in the presence or absence of L-NMA (0.1 mM), D-NMA (0.1 mM) and arginase (100 units/ml). After 24 h of incubation, concentrations of nitrite were measured in the supernatants as described under "Materials and Methods. Data are mean $\pm$ S.D. of three different experiments.

Table-2. **Induction of NO production in BV-2 microglial cells by supernatants of MBP-primed T cells**

Treatments	Nitrite (µg/24 h/mg protein)
Control BV-2 cells	3.6 ± 0.5
BV-2 + Normal T cell supernatant (200 µl)	3.6 ± 0.4
BV-2 + MBP-primed T cell supernatant (25 µl)	3.6 ± 0.4
BV-2 + MBP-primed T cell supernatant (50 µl)	7.7 ± 2.4
BV-2 + MBP-primed T cell supernatant (100 µl)	18.9 ± 2.9
BV-2 + MBP-primed T cell supernatant (200 µl)	39.6 ± 3.2

BV-2 microglial cells received different amount of supernatants of MBP-primed T cells under serum-free condition. The total volume of supernatant was adjusted to 1 ml. After 24 h of incubation, concentrations of nitrite were measured in the supernatants as described under Materials and Methods. Data are mean ± S.D. of three different experiments.

Table-3. **MBP-primed T cells induce the production of NO in mouse primary microglia**

Treatments	Nitrite (µg/24 h/mg protein)
Control microglia	3.4 ± 0.5

Microglia + Normal T cells (1:0.5)	3.45 $\pm$ 0.4
Microglia + Normal T cells (1:1)	3.6 $\pm$ 0.4
Microglia + MBP-primed T cells (1:0.1)	3.4 $\pm$ 0.4
Microglia + MBP-primed T cells (1:0.2)	156 $\pm$ 11
Microglia + MBP-primed T cells (1:0.5)	363 $\pm$ 46
Microglia + MBP-primed T cells (1:0.7)	349 $\pm$ 35
Microglia + MBP-primed T cells (1:1)	310 $\pm$ 23

Mouse primary microglia received MBP-primed T cells at different ratios under serum-free condition. After 24 h of incubation, concentrations of nitrite were measured in the supernatants as described under "Materials and Methods". Data are mean $\pm$ S.D. of three different experiments.

Legends to figures:

Fig. 1. **Proliferation of T lymphocytes with MBP.** T cells suspended in RPMI containing 10% FBS were treated with different concentrations of MBP. After 4 days of incubation, proliferation was assayed using [^{3}H]-thymidine as mentioned under "Materials and Methods." Data are mean $\pm$ S.D. of three different experiments.

Fig. 2. **FACS analysis of T lymphocytes with FITC-labeled anti-CD3.** Purified T cells were treated with 0.5 ml of appropriately diluted FITC-labeled anti-CD3 for 30 min followed by FACS analysis in a FACScan as mentioned under "Materials and Methods." A, FITC-unconjugated; B, FITC-conjugated.

Fig. 3. **MBP-primed T lymphocytes induce the production of NO in mouse BV-2 microglial cells.** BV-2 cells received different concentrations of MBP-primed T cells in direct contact or within insert under serum-free condition. After 24 h, supernatants were used for nitrite assay as mentioned under "Materials and Methods". Data are mean $\pm$ S.D. of three different experiments.

Fig. 4. **MBP-primed T lymphocytes induce the expression of iNOS in mouse BV-2 microglial cells.** BV-2 cells were stimulated with different concentrations of

MBP-primed T cells under serum-free condition. A) Cell homogenates were electrophoresed, transferred on nitrocellulose membrane and immunoblotted with antibodies against mouse macrophage iNOS as mentioned under "Materials and Methods". B) After 6 h of incubation, Northern blot analysis for iNOS mRNA was carried out as described under "Materials and Methods."

Fig. 5. **Expression of VLA4 on the surface of MBP-primed T cells.** Normal or MBP-primed T cells were treated with 0.5 ml of appropriately diluted FITC-labeled anti-VLA α4 for 30 min followed by FACS analysis. A, FITC-unconjugated normal T cells; B, FITC-conjugated normal T cells; C, FITC-conjugated MBP-primed T cells. D) Similarly MBP-primed T cells which were treated with 50 U/ml of IFN-β during priming with 50 μg/ml of MBP for 4 days were analyzed by FACS.

Fig. 6. **Functional blocking antibodies against the α4 chain of VLA4 inhibit the ability of MBP-primed T cells to induce the production of NO in BV-2 microglial cells.** MBP-primed T cells were mixed with either different concentrations of antibodies against the α4 chain of VLA4 or control IgG and rocked gently for 1 h at room temperature. Cells were centrifuged, washed twice and added to BV-2 microglial cells at a ratio of 0.7:1 T cell:microglia. After 24 h of stimulation, supernatants were used for nitrite assay. Data are mean $\pm$ S.D. of three different experiments.

Fig. 7. **Effect of IFN-β on MBP-primed T cell-induced production of NO in BV-2 microglial cells.** BV-2 cells were stimulated under serum-free condition with A) MBP-primed T cells which were pretreated with different concentrations of IFN-β, B) MBP-primed T cells followed by the treatment of different concentrations of IFN-β, and C) different concentrations of IFN-β. After 24 h of stimulation, supernatants were used for nitrite assay. Data are mean $\pm$ S.D. of three different experiments.
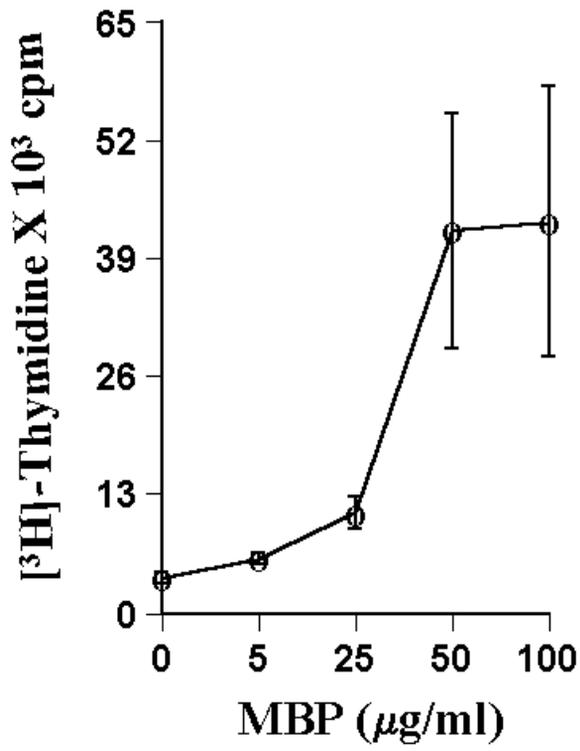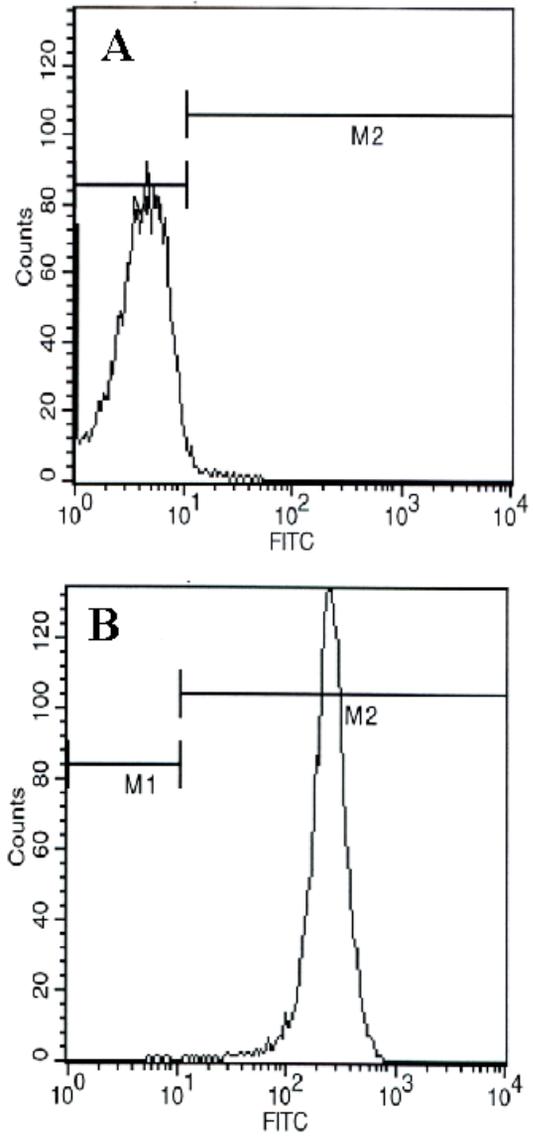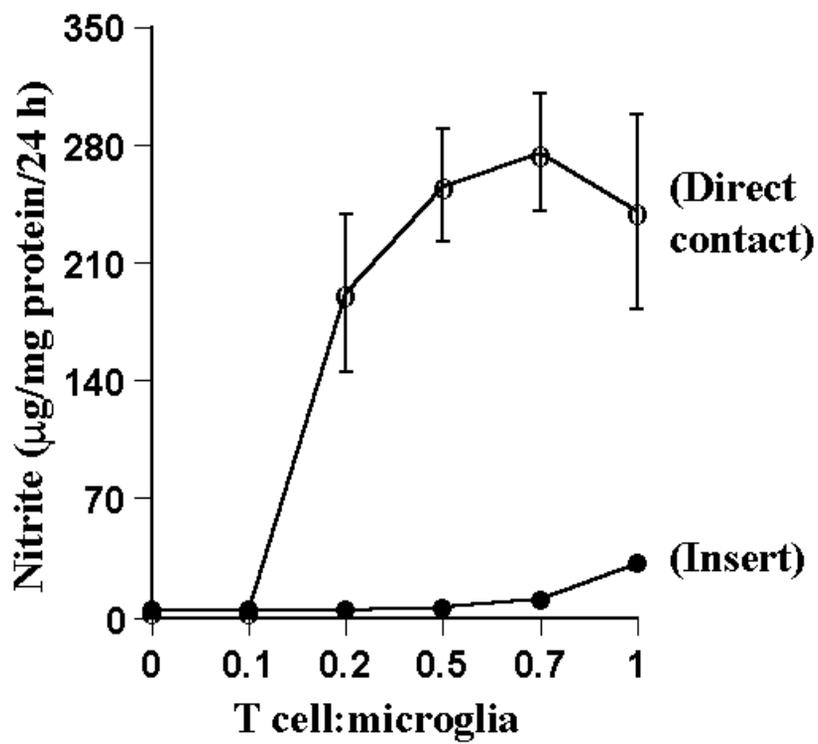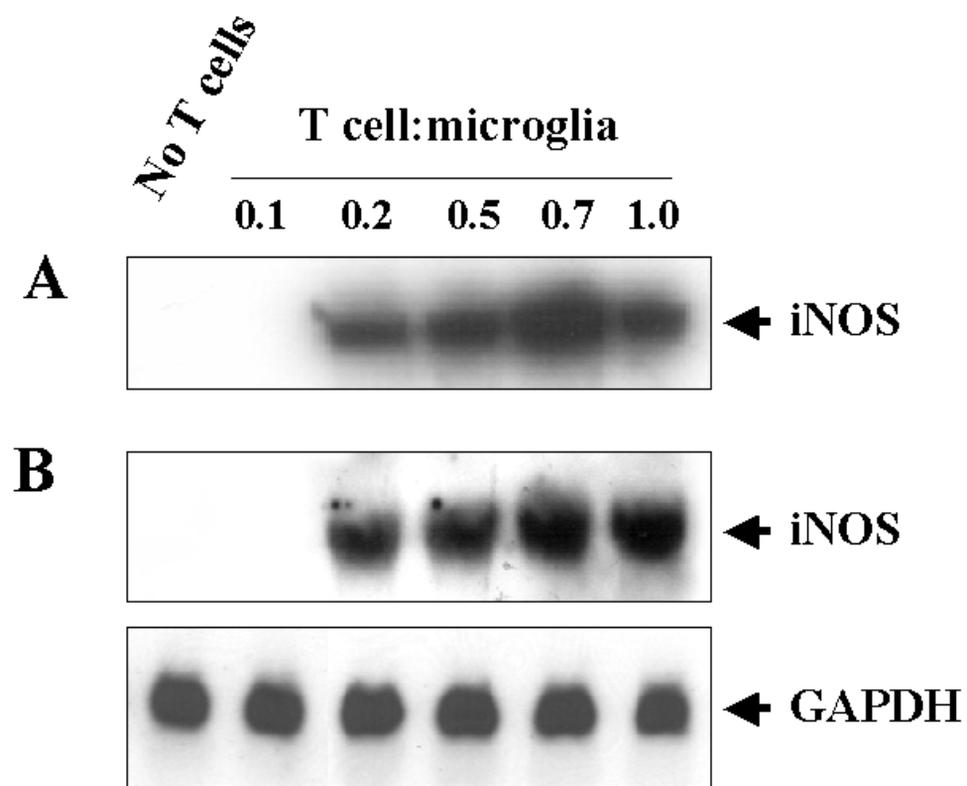
Fig. 1

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