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Mechanisms of Cytokine Production from Microglia-T Cell Interaction: Relevance to Multiple Sclerosis

by

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Abstract

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS). Even though the etiology of MS remains to be firmly established, the disease is nevertheless characterized by an immune-mediated tissue injury, which results in demyelination, axonal loss, and neurological impairments. Demyelinated MS lesions are characterized by the presence of activated T lymphocytes, the majority of which are thought to be antigen-non-specific, activated microglia/macrophages, and enhanced levels of inflammatory cytokines such as TNF-α. Given these characteristics, the present thesis tests the hypothesis that activated antigen-non-specific T lymphocytes interact with microglia to produce inflammatory cytokines, and that drugs used for the treatment of MS, namely interferon-β (IFNβ) and glatiramer acetate, modify this production of cytokines.

Results demonstrate that microglia-T cell interactions induce the production of cytokines, including TNF-α and IL-10, and that contact-dependent signaling, which occurs during microglia-T cell interaction, is critical for this production of cytokines. TNF-α production depends on 3 interactions: VLA-4/VCAM-1, CD40/CD40L and B7/CD28,CTLA-4; while IL-10 production results from CD40/CD40L, B7/CD28,CTLA-4 and CD23/ligands interactions. Intracellularly, TNF-α production induced by VCAM-1 signaling depends on NF-κB activation, since the phosphorylation and degradation of IκB-α, and the nuclear translocation of NF-κB occur following the ligation of VCAM-1 on PMA/IFNγ-treated U937 cells, used as a model of human adult microglia. Finally, IFNβ impacts upon cytokine production in microglia-T cell interaction by preferentially increasing the production of IL-10, and inhibiting that of TNF-α, IL-1β, IL-4, IL-12 and

IL-13. In contrast, glatiramer acetate inhibits the production of all inducible cytokines tested.

I conclude that the interaction of antigen-non-specific T cells with microglia may be an important pathogenic event of MS since it induces the production of inflammatory cytokines, and that IFNβ and glatiramer acetate modulate this production of cytokines to create a non-inflammatory milieu, a mechanism which helps account for their efficacy in MS.

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Dedication

I dedicate my thesis to everyone who has helped me throughout my Ph.D.

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Abbreviations and Symbols

Ag	Antigen
AIDS	Acquired immunodeficiency syndrome
ANOVA	Analysis of variance
AP-1	Activator protein-1
BBB	Blood brain barrier
bp	Base pairs
CD	Cluster of differentiation
CFA	Complete freund's adjuvant
CNS	Central nervous system
CSF	Cerebrospinal fluid
CTL	Cytotoxic T lymphocytes
CTLA-4	Cytotoxic-T-lymphocyte-associated-4
°C	Degree Celcius
DNA	Deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
EAE	Experimental autoimmune encephalomyelitis
EDTA	Ethylene diamine tetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
EMSA	Electrophoretic mobility shift assay
FADD	Fas-associated-death domain
FITC	Fluorescein isothiocyanate
FLICE	Fas-associated death domain interleukin1β-converting enzyme

GA	Glatiramer acetate
GD	Gadolinium
h	hour(s)
HCl	Hydrochloric acid
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HSP	Heat shock protein
IFN	Interferon
IgG	Immunoglobulin
IκB	Inhibitor kappa B
IL	Interleukin
ICAM-1	Intercellular cell adhesion molecule-1
kDa	Kilodalton
LFA-1	Leukocyte-function-antigen-1
LPS	Lipopolysaccharide
ng	Nanogram
NO	Nitric oxide
M	Molar
MAPK	Mitogen-activated protein kinase
MBP	Myelin basic protein
MHC	Major histocompatibility complex
µg	Microgram
µl	Microliter

μM	Micromolar
ml	milliliter
MMP	matrix metalloproteinase
MOG	myelin oligodendrocyte glycoprotein
mRNA	messenger ribonucleic acid
MS	Multiple Sclerosis
MRI	Magnetic resonance imaging
NF-AT	Nuclear factor of activated T cells
NF-κB	Nuclear factor kappa B
PAF	Platelet activating factor
PBMC	Peripheral blood mononuclear cells
PBS	phosphate buffered saline
PKC	Protein kinase C
pH	Log_{10} proton concentration
PLP	Proteolipid protein
PMA	phorbol-12-myristate-13-acetate
RAIDD	RIP-associated ICH-1/CED-3 homologous protein with a death domain
rpm	rotations per minute
RNA	Ribonucleic acid
RRMS	Relapsing-remitting multiple sclerosis
RT-PCR	Reverse-transcriptase polymerase chain reaction
SDS	Sodium dodecylsulfate

SPMS	Secondary progressive multiple sclerosis
STAT	Signal transducer and activator of transcription
TACE	TNF-alpha converting enzyme
TBE	Tris-borate/EDTA
TCR	T cell receptor
TGF	Transforming growth factor
TIMP	Tissue inhibitor of MMPs
Th	T helper cells
TNF-α	Tumor necrosis factor alpha
TNFR	TNF receptor
TRADD	TNFR-associated death domain
TRAF	TNFR-associated factors
TX-100	Triton X-100
UPA	Urokinase plasminogen activator
V	Volt
VCAM-1	Vascular cell adhesion molecule-1
VLA-4	Very late antigen-4

CHAPTER ONE

Introduction

1.1. Multiple Sclerosis

1.1.1. *What is Multiple Sclerosis?*

Multiple sclerosis (MS) is a common chronic inflammatory disease of the central nervous system (CNS) affecting primarily young adults, mostly women, worldwide (Williams et al, 1995). MS is characterized by episodic neurological symptoms that are often followed by fixed neurological deficits and increasing disability over a period of 30 to 40 years (Rudick et al, 1997). The onset is between 20 and 40 years of age in about two third of the cases (Kumar et al, 1992). The prevalence of MS is higher in temperate latitudes of both northern and southern hemispheres, and the incidence in populations of European descent is high when compared to that of Oriental, African and native peoples. The natural history of the disease is highly variable and often unpredictable. A standardized nomenclature to describe different forms of MS has been established (Lublin and Reingold, 1996). The most common pattern of MS is relapsing-remitting multiple sclerosis (RRMS), which affects 70% of patients who undergo symptomatic periods known as attacks or relapses followed by periods of remissions. Fifteen to 20 years after onset, most patients with RRMS develop a chronic or secondary progressive form of multiple sclerosis (SPMS). The acute or primary progressive MS, observed in only ten percent of patients, is characterized by a progression of the disease from onset with occasional stability and minor improvements. The progressive relapsing form of MS is a very rare form of MS that is characterized by acute relapses and continuing progression. Other forms of MS include the benign and malignant forms. The benign

form of MS is a disease in which patients remains functional for a long period of time (many years), while the malignant form affects patients with a rapid progressive course, leading to disability and even death shortly after onset (Lublin and Reingold, 1996). Common early manifestations are paresthesias, retrobulbar neuritis, mild sensory or motor symptoms in a limb, or cerebellar incoordination (Kumar et al, 1992). Recovery from early attacks is sometimes complete but, as the disease progresses, remissions become less complete. Although not all patients become totally disabled, the end stage is often marked by unsteadiness of gait, incontinence, and paralysis due to widespread cerebral and spinal cord demyelination (Kumar et al, 1992).

1.1.2. *Neuropathology of MS*

The condition of MS was first described about 160 years ago (cited in Hickey, 1999), and has since been the subject of numerous studies. The condition of MS is a result of CNS demyelination and axonal loss. Demyelinated regions are most commonly known as MS lesions. As demyelination progresses, lesions expand to form visible "plaques". MS plaques can be found scattered throughout the white matter of the cortex, brain stem, optic nerve and spinal cord, and their number and distribution are variable among MS patients. On cut section, MS plaques are described as irregularly-shaped, sharp-edged areas of demyelination varying in size from barely visible to many centimeters in diameter. They are initially slightly pink and swollen but as the disease progresses they become gray, sunken, and opalescent (Kumar et al, 1992).

Among the earliest and most persistent abnormality found in brains of MS patients is the breakdown of the blood brain barrier (BBB) (Birnbaum and Antel, 1998).

Breakdown of the BBB is believed to underlie the gadolinium (GD) enhancement detected by magnetic resonance imaging (MRI), which is used as a means to diagnose MS (McLean et al, 1993; Hickey, 1999). Although MRI is regarded as a more sensitive approach to monitor disease activity, a strict correlation with clinical relapse is not always found. However, there is evidence that the GD-lesion enhancement on MRI images correlates with the pathological localization of MS lesions which often determines the disability observed in the patients. For example, when GD-enhancing lesions are detected in the optic nerve, vision is often compromised (Arnason, 1999). While BBB breakdown may be an early and constant event in MS, its role in the pathophysiology of MS remains unclear since disease progression often occurs in the absence of GD-enhancement (Revesz et al, 1994).

Another prominent feature of the neuropathology of MS is the accumulation of infiltrated inflammatory mononuclear cells in perivenular spaces, where demyelination is subsequently observed (Matthews, 1999; Conlon et al, 1999). Most of these inflammatory cells infiltrating in the CNS are activated T lymphocytes (Woodroofe et al, 1986). Immunocytochemical analysis demonstrated that both subsets of T cells, namely CD4+ helper T cell (Th) and CD8+ cytotoxic T cells (CTL), are present in those infiltrates (Woodroofe et al, 1986; Traugott et al, 1983). In active chronic lesions, CD4+ T cells are mainly found into the adjacent normal-appearing white matter, while CD8+ T cells are commonly concentrated around lesion margin (Traugott et al, 1983). Based on studies performed with EAE, and animal model of MS, the majority of infiltrated T cells have no specific or unknown antigen reactivity, while only few (about 1.5%) are reactive to myelin antigens (Sedgwick et al, 1987; Cross et al, 1993a, 1993b). It is not clear how T

cells aggravate MS, but it is believed that antigen-specific T cells play a role in the initiation of the disease, and that antigen-non-specific T cells are involved in its progression. Other cell type present in perivascular inflammatory cuffs are lipid-containing monocytes/macrophages (Nyland et al, 1982). Some B lymphocytes or plasma cells and γδ T cells have also been detected in MS lesions (Traugott et al, 1983; Nyland et al, 1982; Wucherpfennig et al, 1992; Hvas et al, 1993). There is also evidence that neutrophils and mast cells are present in MS lesions, and that they play a role in the pathogenesis of MS (Toms et al, 1990; Tchorzewski et al, 1976; Prosiegel et al, 1987).

Figure 1. The MS lesion. Multiple Sclerosis. Perivascular infiltration of mononuclear cells. A myelin stain reveals demyelination adjacent to a small vessel in the white matter. (Adapted from Morris. Basic Pathology edited by Kumar et al, 1992)

MS lesions are also characterized by microglia reactivity. Microglia are the resident immune cells of the CNS, which perform functions of macrophages, such as phagocytosis and antigen presentation. In MS lesions, microglia are often rounded in morphology, which is a characteristic of their activated state (Bo et al, 1994). Upon activation, microglia express MHC class II (Mattiace et al, 1990; Panek and Benveniste, 1995). Chronic active MS lesions are characterized by an enrichment of MHC class-II

positive, lipid-lapen microglia at the border of lesions, and perivascularly (Trapp et al, 1999; Bo et al, 1994; Hayes et al, 1987). Microglia activation in MS is thought to contribute to the aberrant immune reactivity of MS lesions by serving as antigen-presenting cells, and by producing pathogenic molecules, such as pro-inflammatory cytokines, matrix metalloproteinases, and free radicals (Benveniste, 1997; Sriram amd Rodriguez, 1997; Cuzner, 1997). For more details, see section 1.2.4.

Although MS plaques are primarily inflammatory and demyelinating, oligodendrocyte death, axonal loss, and reactive astrogliosis are other neuropathological features of MS lesions (reviewed in Trapp et al, 1999; reviewed in Noseworthy, 1999). It is not clear whether oligodendrocyte death is a result of indirect damage to myelin or whether it is directly induced; several intermediate forms of these extremes are also possible. There is evidence that oligodendrocytes undergo both apoptotic and necrotic death (Gu et al, 1999; Dowling et al, 1999; Akassoglou et al, 1998; Yoon et al, 1998; Ladiwala et al, 1998; 1999; Bonetti and Raine, 1997; D'Souza et al, 1995; 1996). Mechanisms underlying axonal injury are unclear, but it is believed that it is associated with neurological disability in MS (reviewed in Trapp et al, 1999; Silber and Sharief, 1999; Perry and Anthony, 1999; Lee et al, 2000).

1.1.3. *Mechanisms underlying the pathology of MS*

Despite much effort devoted to the search of the cause of MS, the etiology of MS remains unknown. Given the variability of MS, it is widely believed that multiple factors are involved in the pathogenecity of MS. Indeed, various lines of research favor the hypothesis that the manifestation of MS involves an immunological process, with

possible autoimmune properties, as well as the interplay between environmental and genetic factors.

Studies of populations migrating to and from geographic locations where MS incidence is high suggest that an environmental factor contributes to the development of MS (Kurtzke et al, 1980). Furthermore, the observation that infectious diseases can precipitate relapses in 25% of cases led to the concept that infectious agents may be involved in the etiology of MS (Sibley et al, 1985). Although there has been much effort in isolating the virus triggering MS, results obtained from viral-isolation studies have been unsatisfactory since viral sequences isolated from MS patients have been found also in control brain and non-neural tissue (reviewed in Noseworthy, 1999). Molecular mimicry is a mechanism by which viruses or other infectious agents may participate in the development of MS. It was shown that viral or bacterial proteins express antigenic determinants whose structures mimic determinants on self myelin antigens leading to the activation of autoreactive T cells, and the induction of an inappropriate response against myelin antigens (Jahnke et al, 1985; Miller and Heath, 1993). Indeed, experimental data indicates that MBP-specific T cell clones often have degenerate peptide recognition (Hausmann and Wucherpfenning, 1997). Several viral- and bacterial-derived peptides were found to stimulate the proliferation of human MBP-specific T cells (Wucherpfenning and Strominger, 1995). The development of autoimmune demyelination induced by viral antigens that have sequence homology with immmunodominant epitope has been demonstrated (Fujinami and Olstone, 1985). In addition, it is possible that infectious agents are directly implicated in demyelination. This is emphasized by the observation that intracerebral infection of Theiler's murine

encephalomyelitis virus (TMEV) into susceptible strains of mice (SJL and PLJ) leads to virus persistence and demyelination similar to that found in MS (Tsunoda et al, 1996). The possibility that immune responses to proteins of infectious agents expressed also in humans because of their phylogenic conservation, has also been explored. For example, heat shock proteins (HSPs) are expressed by a variety of infectious agents for which they serve as immunodominant proteins. In humans, HSPs are expressed in response to stressful stimuli, and they have been localized in oligodendrocytes within MS lesions (Selmaj et al, 1991), suggesting that immune responses to HSPs induced during the course of an infection can result in the immune-attack of oligodendrocytes, and in subsequent demyelination.

A role for a genetic predisposition in MS was suggested by results obtained from epidemiological studies. Indeed, it was recognized that there is a slightly higher incidence of disease among siblings of MS patients (Sadovnick and Ebers, 1995). The most convincing evidence of genetic predisposition in MS is derived from twin studies. It indicates that disease concordance in monozygotic (identical) twins is significantly greater than in dizygotic (non-identical) twins, and that the smaller risk for dizygotic twins was still increased approximately by 20-40 fold compared to that of the general population (Sadovnick et al, 1997; Cooper et al, 1999). There has been an extensive effort provided to identify the susceptibility genes of MS. Although the results were of modest significance, genetic studies, using techniques of microsatellite mapping and two-stage multianalytical genomic screening to establish linkage disequilibrium, revealed that MS is associated with major histocompatibility complex (MHC) class II locus (reviewed in Ebers and Dyment, 1998; Ebers et al, 1996). Genomic typing analyses demonstrated

that the incidence of the HLA-Dw2, -DRw15, -DQw6, -DRB*1501, -DQA1*0102, and -DQB1*0602 haplotypes is much higher in MS patients of northern Europeans origins than the control population (Ebers et al, 1996). It is believed that MHC genes contribute no more than 10% of the genetic susceptibility of MS (Risch, 1987), which most likely involves the interaction of multiple MS susceptibility genes. There is also data showing that genes encoding the TCR beta chain are associated with MS (Seboun et al, 1989; Beall et al, 1993), but those results have never been confirmed.

Based on studies performed with an animal model of MS, experimental autoimmune encephalomyelitis (EAE), many investigators believe that MS is an autoimmune disease. EAE is a demyelinating disease mediated by autoreactive Th1-type CD4+ T cells that recognize myelin protein encephalitogenic epitopes. Demyelinating lesions of EAE resemble those of a delayed-type hypersensitivity (DTH) reaction, which are characterized by the presence of macrophages and infiltrating CD4+ T lymphocytes. Mice injected with myelin antigens or spinal cord homogenate and complete Freund's adjuvant (CFA) develop EAE. Many antigens can be used to induce disease in mice including myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG), proteolipid protein (PLP), and myelin associated glycoprotein (MAG). PLP EAE mice develop a chronic progressive form of the disease, whereas MBP EAE mice develop a relapsing-remitting form of the disease; MOG EAE may demonstrate a chronic progression or a relapsing course depending on the strain of the mice used. The concept that EAE is a T-cell-mediated disease is based on results obtained from studies of encephalitogenic T cells clones and of T cell subset depletion, which demonstrated that CD4+ cells are the predominant cells type responsible for the transfer of EAE to normal

mice (Ben-Nun et al, 1981; Pettinelli and McFarlin, 1981). Nonetheless, EAE can be induced in mice lacking TCR cells, albeit at reduced frequency and variable severity (Koh et al, 1994). While T cells play a role in the initiation of EAE, macrophages/microglia are thought to be the main effector cells responsible for tissues damage caused in EAE. In Lewis rats depleted of macrophages using mannosylated lipososomes containing dichloromethylene diphosphate, the severity of the disease was shown to be suppressed (Huitinga et al, 1990). Autoimmune diseases, such as EAE, often develop when mechanisms responsible for central and peripheral tolerance to self-antigens are dysregulated. In the case of EAE, there is evidence suggesting that myelin antigens are expressed in the thymus, and that their expression can influence the generation of potential autoimmune T-cell repertoires. How autoreactive T cells escape tolerance in the thymus is unclear. However, one mechanism has been revealed through the use of a transgenic mouse expressing a T cell receptor specific for a myelin antigen. T cells specific for the N-terminal epitope of MBP escape tolerance through low avidity interaction. The affinity of antigen binding to MHC also proves to be important for induction of peripheral tolerance (King and Sarvetnick, 1997).

Despite the pathological resemblance of MS with EAE, it has been difficult to show with confidence that MS is an autoimmune disease. First, unlike EAE, it is not clear whether MS is a T-cell-mediated disease, since MS lesions may develop in the absence of T cell infiltrates, which suggest that initiating factors other than T cells are implicated (Cuzner, 1997). Moreover, it has been proposed that disease activity could be the result of non-antigen specific factors, such as cytokines, released from the activation of the peripheral immune system during infections (Birnbaum and Antel, 1998). Cytokines may

then act on the blood brain barrier to upregulate adhesion molecules, which would facilitate the entry of activated cells into the CNS. Consequently, the nonspecific recruitment of cells to areas of demyelination may be an important pathogenic event of MS. Second, the nature of the myelin antigen(s) responsible for the pathogenic immune responses is unknown. Recent data suggest that multiple antigens may be involved in the continuation of autoimmune destructions through a process recently defined as *epitope spreading*. This phenomenon occurs when a multitude of epitopes distinct from, and non-cross-reactive with, an inducing epitope become major targets of an ongoing immune response. In MS, it is no thought that the progression of disease is accompanied by the decline of primary T cell autoreactivity associated with disease onset and by the concurrent emergence of autoreactivity directed against other epitopes of myelin protein or determinant (reviewed in Tuohy et al, 1998). Therefore, this suggests that epitope spreading is a pathogenic event of MS. Third, autoreactive T cells to myelin antigens are not only found in MS pateints, but can be isolated also from healthy individuals (Pette et al, 1990; Hafler et all, 1987; Olsson et al, 1992).

Despite its unknown etiology, MS is generally referred to as an inflammatory-demyelinating disease. As mentioned in section 1.1.2, profound immune reactivity is observed in MS lesions, which are characterized by perivascular-lymphocytic cuffing, consisting mainly of activated antigen-non-specific T cells and few myelin-reactive T cells, and microglia reactivity. The role of T cells in MS is not clear. A prevailing view supposes that myelin-reactive T cells are involved in the initiation of the disease, while activated antigen-non-specific T cells play a role in its progression (Sedgwick et al, 1987; Cross et al, 1993). Similarly, the role of reactive or activated microglia in MS is not

entirely understood, but there is evidence that they are directly involved in demyelination due to their ability to phagocytose myelin (Li et al, 1996; deJong et al, 1997; Smith, 1993; 1999). It is believed that activated microglia contribute to CNS inflammation by serving as antigen presenting cells and by producing molecules that regulate pathogenic events of MS, such as oligodendrocyte death, axonal loss and reactive astrogliosis. These molecules include inflammatory cytokines, such as IFNγ, TNF-α, interleukin-1 (IL-1), IL-6 and IL-12, chemokines, matrix metalloproteinases, and free radicals (Benveniste, 1997; Sriram amd Rodriguez, 1997; Cuzner, 1997). Coincidently, increased levels of these molecules can be located in MS lesions (McDonnell et al, 1998; Ferrante et al, 1998; Ford et al, 1996; Brosnan et al, 1995; Cannella and Raine, 1995). Functions of cytokines and their role in MS are described in details in section 1.4. Adhesion molecules are important in regulating cell-cell interactions involved in inflammatory responses, including leukocyte migration, antigen presentation and cellular activation (Madri et al, 1996). Enhanced levels of VCAM-1, VLA-4, intercellular cell adhesion molecule-1 (ICAM-1) and leukocyte function antigen-1 (LFA-1) have been detected in MS lesions (Canella and Raine, 1995). Characteristics of adhesion molecules and their role in MS are described in more details in section 1.5.

1.1.4. *Therapies of MS*

Various drugs have been used in the treatment of MS to impact upon immune-mediated attacks resulting in demyelination and axonal loss. For example, treatment of acute relapses or exacerbations is dominated by corticosteroids, such as methylprenisolone and corticotropin, known to have potent anti-inflammatory and

immunosuppressive effects. Corticosteroid therapy shortens the duration of the relapse and accelerates recovery (Milligan et al, 1987). Treatments for chronic progressive MS usually consists of nonspecific immunosuppression using drugs such as methotrexate, a dihydrofolate reductase inhibitor, the alkylating agent cyclophosphamide, and cyclosporine. Several drugs are used in the treatment of RRMS including corticosteroids, the purine analog azathioprine, intravenous immunoglobulin, interferon beta, and glatiramer acetate (Arnason, 1999).

On the basis of results obtained from large multicenter clinical trials, the two forms of recombinant interferonβ, namely IFNβ-1a (Avonex ® and Rebif®) and IFNβ-1b (Betaseron®), and glatiramer acetate (Copaxone®) were approved by the U.S. Food and Drug Administration (FDA) to be used for the treatment of RRMS. Patients with RRMS were shown to have the best response to treatment, whereas those with the progressive form of the disease were less responsive (reviewed in Rudick et al, 1997). The response to these experimental therapies was determined by clinical relapse rate, the development of new lesions detected by magnetic resonance imaging (MRI), and in the case of IFNβ-1a, by the disability progression.

IFNβ-1a is a recombinant glycosylated protein produced by Chinese-hamster ovary cells with an amino acid sequence identical to that of natural interferon beta, whereas interferonβ-1b is a recombinant non-glycosylated protein produced by *Escherichia coli* in which serine is substituted for cysteine at position 17. Multicenter clinical trial has demonstrated that IFNβ-1b reduces the number of relapses in relapsing-remitting MS, as well as the frequency of lesion formation detected by magnetic-resonance imaging (Paty et al, 1993; The IFNβ Multiple Sclerosis Study Group, 1993,

1995; Lublin et al, 1996). The precise mechanisms of action underlying the efficacy of IFNβ-1b in MS are still unknown. IFNβ-1b is a type I interferon with an antiviral action (Sen et al, 1993), and was initially tested as a potential drug in MS given this property and because of a suspected viral etiology of the disease (Jacobs et al, 1996). Presently, IFNβ is thought by some to be a general immunosuppressive drug (reviewed in Yong et al, 1998a). It is well established that IFNβ inhibits T cell proliferation and activation (Noronha et al. 1993; Rudick et al, 1993; Zdravkovic et al, 1994; Rudick et al, 1996a). Some reports have also indicated that IFNβ can improve the suppressor function of T cells (Noronha et al., 1992; Arnason, 1995). Moreover, IFNβ can modulate antigen presentation due to its ability to downregulate the IFNγ-enhanced expression of MHC class II molecule on the surface of antigen presenting cells, such as on monocytes or macrophages (Ling et al, 1985; Panith et al, 1989; Soilu-Hanninen et al, 1995). However, contradicting results were reported by another group (Rudick et al, 1989). IFNβ treatment also affects the cell surface expression of adhesion molecules involved in leukocyte migration. For example, Calabresi et al (1997) reported that T cells from MS patients treated with IFNβ-1b exhibit a significantly lower level of VLA-4, an integrin required for T cell to migrate in the CNS (Baron et al, 1993). Another mechanisms by which IFNβ may affect leukocyte migration is by inhibiting the production of MMP-9 required for the degradation of extracellular matrix molecules, such as fibronectin, during T cell extravasation (Stuve et al, 1996). Finally, IFNβ was shown to affect the production of inflammatory cytokines by T lymphocytes and cells of the monocytic lineage. Several groups have demonstrated that IFNβ-1b inhibits the production of IFNγ by activated T lymphocytes (Panith et al, 1987; and Noronha et al, 1993). In addition, treatment of

monocytes with IFNβ results in a significant decrease in the production of TNF-α (Dhanami et al, 1994). In contrast, IFNβ-1a treatment induced the production of the anti-inflammatory cytokine IL-10 by cultured PBMC (Porrini et al, 1995; Rudick et al, 1996b), and in MS patients, increased serum levels of IL-10 was found 24 hours after the injection with IFNβ-1a (Rudick et al, 1998). In another study, anti-IL-10 antibody significantly blocked the effects of IFNβ-1b on ConA-induced suppressor cell functions and IFNγ production (Porrini et al, 1998). Overall, it appears that IFNβ induces an anti-inflammatory response by inducing the production of anti-inflammatory cytokines while inhibiting the production of pro-inflammatory cytokines (Yong et al, 1998a).

Glatiramer Acetate (GA, Capaxone®) is a mixture of random synthetic polypeptides composed of L-alanine, L-glutamic acid, L-lysine, and L-tyrosine in defined ratios initially designed to mimic MBP and induce EAE. However, it was not encephalitogenic, but instead, it suppressed MBP-induced EAE (Teitelbaum et al, 1971). Clinical trials have demonstrated the efficacy of GA in the treatment of RRMS (Bornstein et al, 1987; Johnson et al, 1995; 1998). The mechanism by which GA exerts its beneficial effect in MS remains unclear, but there is evidence to suggest it has an immunomodulatory function. It was shown that GA is cross-reactive to myelin basic protein (MBP), and inhibits the proliferation of MBP-specific T cells by blocking the secretion of IL-2 and the production of IL-2 receptors (Teitelbaum et al, 1992). Moreover, GA inhibits T cell activation by competing with MBP for binding to the MHC or by displacing MBP from the MHC (Teitelbaum et al, 1996; Racke et al, 1992; Ben-Nun et al, 1996; Arnon et al, 1996). Another possible mechanism of action includes the induction of suppressor function by T cells (Aharoni et al, 1993). Furthermore, it was

shown that GA suppresses EAE by various routes of administration including subcutaneous, intramuscularly, and even orally. Its effectiveness was shown in different species and was found to be independent of the inducing encephalitogen and type of disease. GA does not affect humoral immune responses to EAE non-related antigens since antibody against particulate antigen (bacteriophage T4), proteins (BSA), and haptens (DNP, poly-D-Ala) are not produced. GA does not interfere with vaccination against various infections caused by bacteria (shigella), viruses (influenza) and parasites (schistosoma mansoni). Moreover, GA treatment does not affect other experimental autoimmune diseases, such as experimental myasthenia gravis, experimental thyroiditis, experimental systemic lupus erythematosus (SLE), and experimental diabetes suggesting that GA is not a general immunosuppressive agent. In EAE, adoptive transfer of specific T cells treated with GA led to their unresponsiveness in vivo.

Since glatiramer acetate does not reach the CNS, how do cells suppress the disease in the CNS? It is believed that GA induces suppressor T cell function, which results in the secretion of anti-inflammatory (or Th2) cytokines. A mechanism by which GA is effective in MS has been proposed. First, GA binds to MHC class II expressed on antigen presenting cells in the periphery, which induces the development of suppressor Th2/Th3 cells. Those suppressor T cells then enter the CNS as they circulate, and undergo reactivation within the CNS resulting in the local secretion of anti-inflammatory cytokines and in the attenuation of inflammation. Thus, the outcome of GA treatment results in the diminution of immune-mediated demyelination leading to the amelioration of the disease. Recently, oral GA was shown to induce oral tolerance and disease suppression of both rat and mouse models of EAE (Teitelbaum et al, 1999). The

relevance of these results are of significance since it raises the possibility of a growing use of GA for the treatment of MS, and the abolishment of other drugs that are injected.

1.2. Microglia: the CNS macrophages

1.2.1. *Phenotypic properties of microglia*

Microglia are the principal immune effector cells of the central nervous system (CNS), originally described by Rio-Hargeta (1919-1932). They constitute 5-15% of the total glial cell population present in the adult CNS, and it is estimated that there are as many microglia cells as neurons emphasizing their importance in the CNS (Kreutzberg, 1987). Microglia cells are distributed throughout the CNS although their density varies between CNS regions. For example, in the mouse brain, the highest microglia density is encountered in the hippocampus, the olfaltory telecephalon, portions of the basal ganglia, and the substantia nigra (Lawson et al, 1990).

The functional plasticity of microglia is shown by their capacity to adopt various morphologies depending on their activation state. Resting microglia appear as branched ramified glial cells, while activated microglia have an ameoboid shape and closely resemble macrophages with a large rounded cell body with retracted or shortened processes (Streit, 1995). Ramified-like microglia represent a relatively permanent population with little turnover in the adult nervous system, and are uniformely dispersed throughout the adult CNS, more common in gray than white matter (Perry and Gordon, 1988). In contrast, ameoboid-like microglia are abundant in the developing brain, and are believed to phagocytose debris from naturally occuring cell death in late embryonic and postnatal stages of development (Ferrer et al, 1990; Hume et al, 1983). Microglia can also

be found as hyper-ramified state, which represents an intermediate stage between ramified and amoeboid forms (Streit et al, 1999). It is believed that the hyper-ramification of microglia is due to hypertrophy, an early stage of microglia activation.

In the normal adult CNS, two distinct populations of microglial cells have been described, namely, parenchymal and perivascular microglia. Parenchymal microglia are located in the CNS parenchyma, and are in a resting state as shown by their ramified morphology. On the other hand, perivascular microglia are found in the vicinity of blood vessels enclosed within the basal lamina, and are distinguished from parenchymal microglia by their amoeboid shape suggesting that they are activated cells. Perivascular cells express high level of MHC class II in contrast to parenchymal microglia (Graeber and Streit, 1990). Recent experiments demonstrated that the intracerebral injection of the neuronal tracer substance, Fluoro-Gold (FG), results in the labelling of perivascular microglial cells, but not in the labelling of parenchymal microglia (Pennell and Streit, 1998). Therefore, due to this capacity to take up foreign particles, it is believed that perivascular microglia represents the only population of constitutive phagocytes in the CNS (Gehmann et al, 1995; Hickey et al, 1988).

Immunocytochemically, microglia can be detected using antibodies that recognize numerous molecules. Those include MHC class I and II, CD4, CD11a (LFA-1), CD11b (CR3 complement receptor/Mac-1), CD11c (CR4, p150.95), CD14 (LPS receptor), CD45, CD64 (FcγRI), CD68, and F4/80 (Streit, 1995; Rezaie and Male, 1999). The expression level of these molecules varies depending on the state of activation of microglia. For example, MHC class II is highly expressed on activated microglia under pathological conditions, while low or undetectable levels are expressed on resting

microglia (Mattiace et al, 1990; McGeer et al, 1987). It is difficult to distinguish the intrinsic population of microglia from invading monocytes/macrophages since both cell population can express those molecules. One criterion that has been proposed relates to multiple surface markers. Sedgwick and coworkers (1991) demonstrated by flow cytometry that murine microglia express constitutively low levels of CD45 and negligible CD11b, designated $CD11b^{-}/CD45^{low}$ while monocytes/macrophages express high levels of CD11b and CD45 ($CD11b^{+}/CD45^{high}$).

1.2.2. *Origin of microglia*

The origin of microglia is still a controversial issue, but a substantial body of evidence supports the theory that microglia are of mesodermal origin, and that they are related to cells of the monocyte/macrophage lineage which derive from bone marrow cells. Another theory argues that microglia arise from neuroectodermal-derived glioblast progenitors (Hao et al, 1991; Kurz and Christ, 1998; Richardson et al, 1993).

The hypothesis that microglia are myelomonocytic cells of mesodermal origin was first proposed by del Rio-Hortega (1932) who postulated that microglia arise from blood-borne cells of leukocytic morphology which invade the brain from the meninges (cited in Rezaie and Male, 1999). Many studies have now reconfirmed this hypothesis, and further demonstrate that parenchymal microglia settle the CNS antenatally, being derived from bone marrow precursor cells that colonize the CNS early during embryogenesis. It was shown that the colonisation of the CNS by microglia coincides with vascularisation, formation of radial glia, neuronal migration and myelination primarily in the 4^{th}- 5^{th} months of gestation in the humans and beyond (Rezaie and Male, 1999). Once in the CNS, microglia influx generally conforms to a route following white

matter tracts to gray areas (Cuadros and Navascues, 1998; Rezaie and Male, 1999). As microglia migrate through the CNS parenchyma, they have an amoeboid shape. After reaching their definitive location, it appears that amoedoid microglia differentiate into ramified microglia, which are distributed more or less homogeneously throughout the entire CNS parenchyma. Moreover, studies of the developing retina provided insights about the origin of microglia which supports the monocytic origin (Ashwell et al. 1989; Boya et al, 1987; Hume et al, 1983).

After embryogenesis, perivascular microglia, but not parenchymal microglia, derive from circulating monocytes, as shown using bone marrow chimeras (Alliot et al, 1991). In these studies, rats of Strain A are lethally irradiated and then reconstituted with bone marrow cells taken from Strain A/Strain B hybrids, and antibodies against MHC class I are then used to detect the donor origin. Strain B-specific MHC class I positive cells belong to the pool of transplanted cells which have invaded the CNS following bone marrow transplantation. These studies have shown that, under normal conditions, perivascular microglia are regularly replaced from the bone marrow in adult animals (Hickey et al, 1992;De Groot et al, 1992; Unger et all, 1993; Krall et al, 1994), whereas parenchymal microglia undergo no or very little turnover with bone marrow-derived cells. (De Groot et al, 1992; Hickey et al, 1992; Matsumoto et al, 1987; Eglitis and Mezey, 1997).

The view that at least some microglial cells are of neuroectodermal origin is supported by several studies. Autoradiographic analyses of the genesis of microglia within mouse hippocampus showed the presence of glioblast, which differentiate into both microglia and astrocytes (Kitamura et al, 1984). Fedoroff and colleagues provided evidence that microglial cells and astrocytes may even derive from the same progenitor

cell (Hao et al, 1991; Fedoroff and Hao, 1991). They showed that clones derived from a single newborn mouse brain cell contained both astrocytes and microglial cells. These results raise also the possibility that astrocytes, like microglial cells, can be derived from hematopoietic cells that have entered into the CNS. This agrees with the finding by Eglitis and Mezey (1997) that astrocytes of donor origin appear in mice subjected to bone marrow grafting.

1.2.3. *Functions of microglia*

Functions of resting microglia are still largely unknown, as are the factors that contribute to the maintenance of the quiescent state of microglia. All known functions of microglia are performed by activated microglia, which include migration, phagocytosis, processing/presentation of antigens, and the production and release of several inflammatory mediators including cytokines, chemokines, reactive oxygen intermediates, nitric oxide, complement proteins, coagulation proteins and proteases (reviewed in Cuzner, 1997).

The phagocytic function of microglia has long been known. In 1928, Penfield noted that parenchymal microglia migrate to the lesion site induced in newborn animals where they phagocytose debris as shown by their foamy appearance and the intracellular formation of lipoid and iron granules bodies. Like other professional phagocytes, activated microglia possess a respiratory burst system that can generate large quantities of free radicals from oxygen molecules, such as hydroxyl radicals, singlet oxygen species, and hydrogen peroxide (McGeer and McGeer, 1999), and they possess many phagocytic-related enzymes, such as nucleoside diphosphatase and acid phosphatase (Boya et al,

1979; Ling et al, 1982; Murabe and Sano, 1982, Fujimoto et al, 1989; Castellano et al, 1991). All groups of Fc receptors, FcRI, FcRII, and FcRIII, which mediate antibody-dependent phagocytosis by binding to the Fc portion of immunoglobulins, have been shown on the surface of both reactive and perivascular microglia (Ulvestad et al, 1994b). *In vitro*, microglial phagocytic activity is modified by the presence of astrocytes and cytokines. For example, coculturing microglia with astrocytes was shown to suppress phagocytosis (De Witt et al, 1998). Preincubation of cultured microglia with granulocyte/macrophage colony stimulating factor (GM-CSF), IFN-γ, and TNF-α enhanced their phagocytic activity, while transforming growth factor-β-1 and IL-4 inhibited this function (Von Zahn et al, 1997; Rezaie and Male, 1999). Adult human derived microglia phagocytose antibody-coated targets, as shown by increased NADPH oxidase activity, due to their ability to express Fcγ receptors (Ulvestad et al, 1994b).

A role for microglia as antigen-presenting cells has been proposed due to their ability to express MHC class II. In situ, human resting microglia of ramified morphology express low levels of MHC class II molecules. Under normal conditions, perivascular microglia, expressing high levels of MHC class II, function as effective antigen presenting cells (Ford et al, 1995), while parenchymal microglia fail to perform this function (Carson et al, 1998; Havenith et al, 1998). However, under pathological conditions, the expression of MHC class II is widely upregulated on parenchymal microglia, as they become activated. Mechanisms responsible for the upregulation of MHC class II on activated microglia are not entirely understood, but in culture, it is inducible by cytokines, such as IFN-γ (Cuzner et al, 1997; Dickson et la, 1993). Microglia can process antigen in vitro (Aloisi et al, 1999; 1998). Sedgwick and colleagues (1993) demonstrated that immediately

ex vivo microglia, isolated from the CNS of healthy Brown Norway rat, do not express MHC class II, and that they fail to activate MBP-reactive CD4+ T cells. However, under inflammatory conditions, MHC class II is expressed on the surface of microglia isolated from the spinal cord of EAE animals (Ford et al, 1995). The antigen-presenting capacity of microglia was also demonstrated in the murine and human systems. Human adult microglia derived from brain biopsy specimen from patients who underwent surgical resection as a means to treat intractable epilepsy or brain tumors express MHC class II and activate T cells in vitro (Becher and Antel, 1996; Ulvestad et al, 1994a). In culture, IFN-γ is known to induce MHC class II expression on human adult microglia (Becher and Antel, 1996). Microglia also express the important costimulatory molecules required for adequate antigen presentation. In vitro and in situ expression of B7-1 (CD80) and B7-2 (CD86) by activated microglia has been shown (Williams et al, 1994; Dangond et al, 1997; de Simone et al, 1995; Satoh et al, 1995), and blocking B7 interaction results in the reduced antigen presentation capacity of microglia (Williams et al, 1994). Recent evidence indicates that the activation state of murine microglia determines whether microglia induce MBP-specific T cell anergy or T cell activation. Microglia become professional APC only after a mutlistep activation process involving both stimulation through cytokines, such as GM-CSF and IFN-γ, and cognate interactions, such as B7/CD28 and CD40/CD40L (Matyszak et al, 1999).

Activated microglia are important sources of molecules that are involved in CNS inflammation. For example, in vitro experiments demonstrated that activated microglia can produce pro-inflammatory cytokines, which include tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), IL-6, IL-12, IL-15, and interferon-γ (IFN-γ) (reviewed in Perry et

al, 1995). Cytokines have multiple effects in the CNS, which can be both beneficial and detrimental depending on their concentrations. Cytokine functions in the CNS include the control of neuronal and glial differentiation, proliferation, and survival. Given those functions, cytokines have the potential to influence neuronal and glial plasticity, degeneration as well as development and regeneration of the nervous system (reviewed in Munoz-Fernandez and Fresno, 1998). Other products of activated microglia are secreted proteases, such as elastase, urokinase plasminogen activator (uPA), matrix metalloproteinase-2 (MMP-2), and MMP-9. For example, Cuzner and co-workers (1996) demonstrated by in situ hybridization that MMP-2 and MMP-9 mRNAs are predominantly expressed in microglia throughout normal white matter. MMPs have the potential to degrade basement membrane and other matrix components, and to catalyse the release of membrane-bound inflammatory cytokines such as TNF-α (Black et al, 1997; Gearing et al, 1994). Due to their ability to produce neurotoxins, it is believed that microglia may have neurotoxic functions. In vitro, microglia produce a large amount of glutamate and aspartate (Piani et al, 1991), and sustained high extracellular levels of these amino acids was shown to cause NMDA receptor-mediated neuronal injury in vivo (reviewed in Nicoletti et al, 1999). Complement proteins, which provides signals for scavenger activation, opsonization, and membrane-attack-complexes (MAC)-mediated direct lysis of cells, are also produced by activated microglia (reviewed in El Khoury et al, 1998). Platelet activating factor (PAF) is a lipid molecule shown to be involved in inflammatory processes and in cell-cell communication. In the CNS, there is growing evidence that PAF is a mediator of neuro-injury caused by stroke and trauma by neuronal cells. PAF production by human fetal microglia was shown to be induced by both TNF-α and LPS in

a concentration-dependent manner (Gremo et al, 1997). Finally, microglia produce and secrete chemokines, which are small proteins (8-10 kD) that induce chemotaxis, tissue extravasation and functional modulation of a wide variety of leukocytes during inflammation. Chemokines are also thought to be pivotal regulatory molecules implicated in cellular communication, and mediate their biological activities through G-protein-coupled-cell-surface-receptors. The binding of chemokines to their receptors results in the activation of MAPK-associated and CREB-associated signal transduction pathways (Asencio and Campbell, 1999). Evidence suggest that chemokines and their receptors are important in CNS development and regeneration (Rezaie ad Male, 1999). Fractalkine (neurotactin), a CXXXC chemokine, and its receptor are highly expressed by microglia in rat brains (Nishiyori et al, 1998).

1.2.4. *Microglia in MS*

Microglia respond to virtually any, even minor, pathological events in the CNS. In most pathological conditions, microglia are aided by infiltrating hematogenous macrophages. Microglia activation appears to play a central role in the pathogenesis of MS and other CNS pathologies, such as cerebral ischemia, brain abscesses, traumatic brain injury, Alzheimer's disease, experimental globoid cell dystrophy, AIDS dementia complex and cerebral malaria.

In MS, a large number of microglia accumulate in and around lesions of demyelinating areas in brains of MS patients (Raine, 1994). Immunocytochemically, microglia activation markers can be used to characterize the distribution of these cells in MS plaques including MHC class II, Mac-1 and LFA-1 (Mattiace et al, 1990; Raine,

1994; Trapp et al, 1999). MHC class II positive cells are present in active and chronic demyelinated MS lesions. At the edge and at the center of the demyelinating region of MS brains, neuropathological analyses demonstrate that the morphology of microglia appears to be elongated and ameoboid respectively, characteristic of their activated state (Bo et al. 1994); resting microglia in non-inflammed brains have a ramified shape. In MS brains, it was recently shown that microglia proliferate (Schonrock e al, 1998). Microglia are the first CNS cell type to respond to several types of CNS injury. Upon activation, microglia express cellular adhesion molecules, such as ICAM-1, and VCAM-1, involved in the regulation of immune responses. It has been well documented that enhanced levels of ICAM and VCAM-1 are present in active MS lesions (Canella and Raine, 1995). In MS brains, it was recently shown that microglia proliferate (Schonrock e al, 1998).

An active role of microglia in demyelination has been proposed. In MS, the presence of phagocytic cells with a foamy appearance are found primarily in new lesions where active myelin phagocytosis is proceeding, and are seen frequently in active MS plaques (Smith, 1999; 1998; 1993). *In vitro*, cultured microglia have the ability to phagocytose myelin (Smith, 1993). For example, it was shown that the short incubation of ^{14}C-lipid-labeled myelin with microglia results in the rapid intracellular metabolic conversion of myelin into cholesterol ester and triglyceride (Trotter et al, 1986). Mechanisms of myelin phagocytosis by microglia *in vitro* appear to involve MAC-2, a galactoside-specific lectin (Williams et al, 1994; Reichert et al, 1994). There is also *in vivo* evidence that microglia phagocytose myelin. By the use of bone marrow chimeras in Lewis rats with EAE, Rinner et al (1995) demonstrated that resident microglia contained myelin degradation products (Li et al, 1993). Prineas and colleagues reported (1981,

1984) that myelin is attached at sites where receptors are involved in phagocytosis, suggesting that myelin is ingested by receptor-mediated phagocytosis. Receptors implicated in phagocytosis include Fc receptors (FcRI, FcRII, and FcRIII), complement receptors (CR3) and scavenger receptors. FcR and CR3 can act as a ligand for myelin (Bruck and Friede, 1990), and are thus thought to be important mechanisms of myelin destruction *in vivo*. Enhanced levels of Fc receptor and CR3 expression on microglia in MS lesions have been reported (Compston et al, 1989; Nyland et al, 1980; Ulvestad et al, 1994).

Activated microglia play a central role in regulating inflammatory responses of MS lesions. For example, it is believed that activated microglia plays a role in the initation of the immune response by presenting myelin antigens to infiltrating myelin-reactive T lymphocytes. As a result, myelin-reactive T cells become activated, differentiate and secrete cytokines that are pathogenic in MS, such as TNF-α (see section 1.4.2.)(Aloisi et al, 1998). By producing a large number of inflammatory molecules, such as cytokines, chemokines and proteases, possibly through their contact with antigen non-specific T cells, activated microglia augment CNS local immune responses, which contribute to the progression of MS (Cammer et al, 1978; Gijbels, et al, 1993; Proost et al, 1993). Finally, under certain conditions, it was shown that activated microglia also have the capacity to downregulate T cell responses (Sedgwick et al, 1998a). Therefore, it appears that activated microglia regulate the initiation, progression and termination of CNS inflammation of MS by dictating the fate of the immune response.

1.2.5. *Mechanisms of microglia activation*

Microglial activation is a multi-step process characterized by changes in cellular morphology, cell size, cell number, and in cell surface molecule expression. Mechanisms of microglial activation are not completely understood, but few have been described. Clearly, microglial activation is accompanied by an alteration in gene expression, which also results in the synthesis of inflammatory mediators, such as reactive oxygen and nitrogen intermediates, proteolytic enzymes, arachidonic acid metabolites and proinflammatory cytokines.

Microglia undergo morphological transformations from a ramified to an amoeboid form, through mechanisms that remain unclear. However, the ramification of amoeboid microglia at least *in vitro* is dependent on a Ca^{2+}-ATPase, as shown using the specific inhibitor thapsigargin (Yagi et al, 1999). A commonly described cellular morphological change of activated microglia is hypertrophy. Hypertrophic microglia develop enlarged cell processes, which give the cells a bushy appearance. Hypertrophy is usually apparent by 24 hours after CNS injury, which correlates with an upregulation of the complement receptor CR3 (Graeber et al, 1988). It is thought that hypertrophy of microglia is a hallmark of their proliferation. Microglial proliferation has been shown in vivo in various models of CNS injuries (Streit et al, 1988; Lawson et al, 1992; Amat et al, 1996). Following 2-3 days of CNS injury, it was shown that microglia begin proliferating, and their numbers reach maximal levels after 4-7 days (Streit et al, 1999). In vitro, the proliferation of microglia cells can be regulated by soluble factors, including colony stimulating factors, such as M-CSF and GM-CSF, primarily produced by astrocytes (Suzumura et al, 1990) and neurotrophins (Elkabes et al, 1996). Accordingly, it is possible that microglia proliferation may be regulated indirectly via neuronal-

astroglial signaling. Moreover, evidence suggests that molecules produced by hematogeneous macrophages are also involved. After transection of a CNS fiber tract, microglia are insufficiently activated, and this correlates with a lack of hematogenous macrophages infiltration into the degenerating nerve stump (Stroll and Jander, 1999). Moreover, CNS injuries where there is the breakdown of the BBB, such as cerebral ischemia, brain abscesses and stab wounds elicit prompt microglia activation associated with macrophage recruitment (Stroll and Jander, 1999).

Activators of microglia include the bacterial lipopolysaccharide (LPS) and IFNγ. In vitro, treatment with a combination of LPS and IFNγ induces the production of reactive nitrogen oxides (NO, $ONOO^-$), reactive oxygen intermediates (O_2^-, H_2O_2), and enzymes, such as lysozyme, cathepsin B/L, and acid hydrolases (reviewed in Rezaie and Male, 1999). This has also been shown in *in vivo* experiments. For instance, after injecting a mixture of LPS and IFN-γ in the rat hippocampus, microglia are activated as shown by morphological changes and by an increase in IL-1β and iNOS immunostaining (Hartlage-Rubsamen et al, 1999).

Direct contact between T lymphocytes and microglia is another mechanism involved in microglia activation. We have demonstrated that microglia-T cell interactions results in the significant production of cytokines, such as TNF-α and IL-10 (Chapter 3 and 4 / Chabot et al, 1997; 1999). Some selectivity of ligand-receptor pairs in microglia-T cell interactions was revealed in this study. While the CD40/CD40L and CD28-CTLA-4/B7 pathways regulate both IL-10 and TNF-α, the VLA-4/VCAM-1 interaction was specific for TNF-α; in contrast, the CD23 system affected IL-10 but not TNF-α (Chapter 3 and 4 / Chabot et al, 1997; 1999). Similarly, the ligation of microglial CD40 by CD40L

was shown to induce the production of TNF-α (Tan et al, 1999a). Cross-linking of VCAM-1 on microglia was shown to induce TNF-α transcription (Chapter 3 / Chabot et al, 1997) through a mechanism which likely involves the activation of NF-κB (Chapter 6 / Chabot et al, submitted).

Microglia cultured from rat, mouse and human fetal nervous system express an inward rectifying K+ channel, but no outward currents, which makes them more sensitive to changes in extracellular potassium than any other cell type in the brain (Kettenmann, et al, 1993; McLarnon et al, 1997; Rezaie and Male, 1999). In the cortical spreading depression, microglia respond to neuronal depolarizations, which are associated with increased potassium fluxes across membranes in the absence of neuronal damage, suggesting that inward rectifying K+ channel may play a role in macrophage activation (Gehrmann et al, 1993). Other ion channels have been implicated in microglia activation, including Cl^- channels. Indeed, it was shown that chloride channel blockers, and not blockers of Na+ or K channels, inhibit microglia activation by preventing their ramification from an amoeboid form (Eder et al, 1998), and by blocking the production of NO (Brown et al, 1998).

Results obtained from in vitro studies suggest that the cotransmitter adenosine triphosphate (ATP), released from neurons during CNS injury, is involved in microglia activation. Microglia express P2-purinoreceptors, which bind to ATP. The extracellular application of ATP to cultured microglia from mouse brains induces complex membrane currents resulting in the depolarization of microglia (Kettenmann et al, 1993; Walz et al, 1993; Haas et al, 1996; Illes et al, 1996). It is not clear whether ATP is a direct activator of microglia, or whether it acts more as a modulator of microglia activation. Ferrari et al

(1997) demonstrated that LPS-induced released of IL-1β is modulated in microglia cells lines via purinergic receptors.

Other activators of microglia include complement fragments, gangliosides, PAF and the stress-inducible small heat shock protein, alpha crystallin. Complement fragments, such as C5a and C3a, which are generated from complement activation, activate microglia by inducing Ca^{2+} signaling (Moller et al, 1997). In the case of PAF, it was shown that it induces an increase of $Ca(^{2+})$ influx in microglia by causing the activation of store-operated-$Ca(^{2+})$ channels (Wang et al, 1999). Gangliosides, such as GM1, GD1a, GD1b, GT1b, and GQ1b, are glycosphingolipid containing sialic acid residues that are located in mammalian cell membranes. It was shown that GT1b can induce the production of NO, TNF-α and COX-2 or rat microglia, while GM1 and GD1a induce the production of NO only (Pyo et al, 1999). Finally, alpha-crystallin induces the activation of microglia *in vitro* by inducing the production of NO and inducible NO synthase (iNOS) (Bhat and Sharma, 1999). Alpha crystallin also stimulates the synthesis of the pro-inflammatory cytokine TNF-α (Bhat and Sharma, 1999).

Deactivators of microglia have also been identified. The best described deactivators of microglia are the anti-inflammatory cytokines IL-10 and TGF-β. For example, it was shown that IL-10 inhibits the LPS-induced microglial production of TNF-α, IL-1β, lysosomal enzyme activity, superoxide anion, and the chemokine RANTES (Sawada et al, 1999; Hu et al, 1999). Ligation of CD40 on microglia induces the production of TNF-α (Tan et al, 1999b). However, upon treatment with IL-10 and TGF-β, CD40-induced production of TNF-α is inhibited (Tan et al, 1999b). Finally, the expression of MHC class II and the class II transactivator (CIITA) transcription factor

induced by IFN-γ was shown to be inhibited by IL-10 and TGF-β in primary murine microglia (O'Keefe et al, 1999). Another deactivator of microglia is prostaglandin. For example, Petrova and co-workers (1999) demonstrated that prostaglandin-E2 (PGE2) selectively inhibits the production of TNF-α (by 95%) and IL-6 in LPS-stimulated microglia. Cyclooxyenase-2 (COX-2), pro-IL-1β, or inducible NO synthase production was not affected by such a treatment.

1.3. T lymphocytes

1.3.1. *T Lymphocyte functions*

T Lymphocytes are best known for the role they play in cell-mediated immunity due to their ability to recognize and respond to foreign antigens. T lymphocytes precursors arise from the bone marrow and then migrate to the thymus where they differentiate into functionally distinct populations, the best defined of which are the CD4+ T helper (Th) and CD8+ cytotoxic T cells (CTL). As its name suggests, Th cells' major function is to help other cell types to perform their effector functions, whereas CTL play an important role in the lysis of virally-infected cells, tumor cells, or allografts. Both populations of T cells recognize antigens associated with major histocompatibility complex (MHC) molecules via the T cell receptor (TCR). Th cells recognize foreign peptides presented in association with the MHC class II molecule while CTL recognize virally-derived or self peptides presented on the MHC class I molecule. In the CNS, cells expressing MHC class II molecule are the microglia; astrocytes are also suspected of having antigen-presenting capacity. As a result of MHC-restricted antigenic recognition,

naïve T cells become activated. In the case of CD4+ Th cells, antigenic stimulation leads to the differentiation and development of 2 Th subsets, namely Th1, and Th2, which produce distinct patterns of cytokines. Th1 cells secrete IL-2 and IFN-γ, and Th2 cells produce IL-4, IL-10 and IL-13. When Th1 cells are preferentially induced, the main immune response is cell-mediated, macrophages being the effector cells, a Th2 response results in humoral immunity. The factors determining whether CD4+ T cells will differentiate into a Th1 or a Th2 cell are not fully understood. The nature of the MHC:peptide ligand interaction and the co-stimulators used to drive the response are thought to be involved (Janeway et al, 1999). Other Th subpopulations have also been described. TGFβ-producing Th cells are now known as Th3 cells (MacDonald, 1999), and Th cells that produce IL-10 following B7-H1 co-stimulation are thought to comprise another Th subset (Dong et al, 1999).

T cell activation is a process consisting in a series of molecular events resulting in the proliferation or clonal expansion of antigen-specific T cells and in the acquisition of their effector functions. T cell proliferation is mediated primarily by an autocrine growth pathway. T cells that are being activated secrete the growth-promoting cytokine, IL-2, and upregulate their expression of cell surface receptors specific for IL-2. Membrane events necessary for T cell activation to occur involves the interaction between MHC: peptide and the TCR complex, which include the TCR αβ or γδ heterodimer, the γ, δ, and ε CD3 chains, and ζ and η proteins. The MHC:peptide/TCR interaction results in the differentiation, proliferation and effector functions of T cells by inducing signal transduction pathways (reviewed in Janeway et al, 1999). Upon MHC: peptide recognition by TCR, antigen recognition activation motifs (ITAMs) present in the

cytoplasmic tail of CD3 proteins are phosphorylated by activated tyrosine kinases, which include Fyn and Lck. The phosphorylation of ITAMs allows the binding of the protein tyrosine kinase ZAP-70, which in turns leads to the activation of various signaling pathways including the phospholipase C-γ (PLC-γ), protein kinase C, and MAP kinase pathways. As a result, gene transcription is initiated by transcription factors such as NF-kB, NF-AT, and AP-1. Other membrane accessory molecules involved in signal transduction include the Igα and Igβ proteins, CD4 or CD8, CD45, LFA-1, CD2, and CD28 or CTLA-4 (Abbas et al, 1997). To simulate the MHC/TCR interaction, the cross-linking of TCR/CD3 complex with monoclonal antibodies to CD3, such as OKT3, is often employed as a mean to activate T cells *in vitro*. OKT3 treatment of T cells results in IL-2 production, in the expression of CD25, the alpha chain of the IL-2 receptor, and in the proliferation of T cells as shown by H^3-thymidine uptake assays (Van Wauwe et al, 1984). This mitogenic activity induced by OKT3 was shown to depend on the presence of monocytes (Ceuppens and Van Vaeck, 1989), which express co-stimulatory molecules required for the full activation of T cells. Co-stimulatory molecules include B7 molecules (B7-1, B7-2) expressed on the surface of antigen-presenting cells and their ligands CD28 or CTLA-4 (Hathcock et al, 1994; Thompson and Allison, 1997). Reagents used to block co-stimulation, such as CTLA-4-Ig, a fusion protein of CTLA-4 and human IgG1, strongly inhibits T cell responses (Janeway and Bottomly, 1994).

1.3.2. *Migration of T lymhocytes into the CNS*

The recruitment of inflammatory cells to sites of inflammation is a complex multi-step process, which involves the tethering, rolling, and firm adhesion of leukocytes

to the vascular wall. Each step is regulated by various interactions between leukocytes and endothelial cells. These interactions involve the cell adhesion molecules (CAMs). Members of the the selectin family, such as P-, E-, and L-selectin, are responsible for the low affinity interactions occuring during the tethering and rolling phase of leukocyte recruitment. On the other hand, the β2-integrins (CD11/CD18), which include LFA-1, are involved in the adhesion step, by mediating high affinity binding to their receptors ICAM-1 and ICAM-2, members of the immunoglobulin family (Springer, 1990). An alternative pathway has also been identified by several groups in which the α4-integrins (α4β1 and α4β7) mediate all three steps of leukocyte recruitment (Jones et al, 1994; Alon et al, 1995; Johnston et al, 1996). The α4β1, also called VLA-4, and α4β7 both bind to the vascular adhesion cell molecule (VCAM-1), which is also a member of the immunoglobulin family. Integrin-dependent interactions trigger the extravasation of leukocytes. For example, the cross-linking of β1-integrins mediate a signalling cascade which leads to the upregulation of the expression of matrix metalloproteinases (MMPs) necessary for the degradation of the basement membrane that forms a post-endothelial barrier (Huhtala et al, 1995).

The CNS has long been considered to be an immunoprivilege site because of the observation that allografts survive longer in the CNS than in any other organs (Head et al, 1985). The immunological privilege of the CNS was thought to be due to four factors: (1) low expression levels of MHC class II in the parenchyma, (2) lack of professional antigen presenting cells, such as dendritic cells, (3) the lack of a lymphatic drainage system, and (4) the presence of a tight and highly specialized vascular wall, the blood brain barrier (BBB). There may also be a parenchymal resistance to immune responses. However,

despite this "privilege", inflammatory cells still migrate to the CNS (Traugott et al, 1983; Renno et al, 1995). It should be noted that BBB damage occurs during these chronic inflammatory diseases, causing leakage in the perivascular space. Nevertheless, it appears that there is no passive recruitment of leukocytes into the inflamed CNS, suggested by the absence of erythrocytes in inflammatory infiltrate indicating that leukocytes migrate across the BBB and that it is an active process (Engelhardt et al, 1995; 1998). There is also evidence indicating that α4-integrin is important for the recruitment of inflammatory cells during chronic inflammatory diseases (Johnston et al, 1996). For instance, in EAE, Baron et al (1993) showed that α4-integrin expression on MBP-specific T cells directly correlated with their entry into the brain parenchyma, and with their ability to transfer EAE. Moreover, the administration of blocking antibodies against α4-integrin 2 days following adoptive transfer of EAE was shown to prevent the accumulation of leukocytes in the CNS of the rat and the mouse (Yednock et al, 1992; Baron et al, 1993). In EAE, the α4β7 integrin is expressed at very low levels on the surface of infiltrating T cells, suggesting that only α4β1 integrin is responsible for migration of T cells into the CNS (Engelhardt et al, 1995; Male et al, 1994). The role of α4-integrins in the recruitment of inflammatory cells during EAE remains to be confirmed in other models of chronic inflammatory diseases of the CNS. Moreover, it is not clear whether the α4-integrin pathway applies to a specific stage of the inflammatory response.

1.3.3 *Phenotypes of T lymphocytes in MS*

It is well-known that naïve T cells cannot readily penetrate the blood-brain barrier (BBB), and that only activated T cells easily enter the CNS (Fabry et al, 1994). The presence of activated T cells expressing the receptor for IL-2 has been reported in the

brains of patients with MS (Hickey et al, 1991). Moreover, the activated phenotype of T cells in MS is further demonstrated by the observation that the majority of infiltrated T cells exhibit the memory cell markers. Most cells (both CD4+ and CD8+) isolated from the CSF of MS patients express CD45RO+, a marker of memory cells (Vrethem et al, 1998). Although Ag-specific T cells reactive to MBP exist in the periphery of most individuals, the frequency of T cells responsive to MBP, PLP, MAG and MOG is increased in MS patients compared to healthy controls (Olsson et al, 1992; reviewed in Birnbaum and Antel, 1998). Despite this increased number of antigen-specific T cells, the majority of T cells (over 99%) present in MS plaques are Ag-non-specific T cells, which are thought to be involved in the exacerbation of the disease (reviewed in Martin et al, 1992; Fabry et al, 1994; Hickey et al, 1991).

It has long been known that CD4+ T cells are present in MS lesions (Traugott et al, 1983a). Similarly, CD4+ T cells are found in the CSF of MS patients, and comprise at least 75% of all cells isolated (Fleischer et al, 1984). On the basis of their classes of T-cell receptor (TCR), the majority of those CD4+ T cells around areas of MS plaques were found to be of the α/β phenotype (Traugott et al, 1983a). A preferential accumulation of T cells expressing the Vβ5.2 chain of the TCR in the brains of MS patients has been described, which is similar to that of MBP-specific T cell clones used in EAE (Oksenberg et al, 1990). Moreover, a small number of γ/δ T cells, which are thought to have autoreactive potential, has also been observed around oligodendrocytes at the edges of plaques (Selmaj et al, 1991a). However, results obtained from a sequence analysis study demonstrated no MS specific expansion of one or more clones of particular type of TCR γ/δ-bearing T cells (Hvas et al, 1993). The presence of CD8+ T cells in MS lesions is also

well known (Traugott et al, 1983a), and in the CSF, they constitute about 25% of cells. However, the role of CD8+ T cells in MS is not very well understood. *In vitro* studies demonstrated that CD8+ T cells may be involved in the destruction of oligodendrocytes. Jurewicz et al (1998) showed that oligodendrocytes express MHC class I, and that they are susceptible to cytotoxic killing by MBP-specific CD8+ T lymphocytes.

1.4. Cytokines

1.4.1. *What are cytokines?*

Cytokines are defined as regulatory proteins that are generally secreted by immune cells and by a variety of other cells, which tend to have multiple target cells as well as multiple actions in modulating inflammatory and immune responses. Most cytokines are simple polypeptides or glycoproteins with a molecular weight of 30 kDa or less produced by more than one type of cell. Cytokine production is regulated at the transcriptional level and is usually inducible and transient. In contrast to the endocrine mode of action of hormones, the radius of action of cytokines is usually short, and defined as autocrine or paracrine. Cytokines produce their actions by binding to specific high-affinity cell surface receptors which results in the alteration of gene expression and phenotypical changes, such as modulation of cell proliferation, cell differentiation, and a change in the expression of various differentiated functions. The range of actions displayed by an individual cytokine can be broad and diverse, and the same stimulatory or inhibitory action can be performed by more than one cytokine creating redundancy. Cytokines are classified into families based on their structural features, which include the family of IL-1, IL-2/IL-4, IL-6/IL-12, IFNs, TNFs, transforming growth factors (TGFs),

and chemokines. The production of cytokines in many cell types can be regulated by several signaling molecules including cell membrane receptors, protein kinases (e.g. src, Jak, Tyk, PKA, PKC, MAP kinases), protein phosphatases (e.g. PP1, PP2A, PP2B, PTP), G-protein related proteins (e.g. Ras), and transcription factors, such as NF-κB and the STAT proteins (reviewed in Thompson et al, 1998).

The spectrum of cytokines produced during disease conditions appears to have a decisive influence on the outcome of MS. Pro-inflammatory cytokines are involved in the amplification of inflammatory responses, and thus believed to be pathogenic in MS. Those include IFNγ, TNFβ, TNFα and IL-12 (Brosnan et al, 1995; Canella and Raine, 1995; Yednock et al, 1992; Ferrante et al, 1998; Ford et al, 1996). On the other hand, anti-inflammatory cytokines, such as IL-4, IL-5, IL-10, and IL-13, are thought to have beneficial actions in MS because they participate in the downregulation of local inflammatory responses (Samoilova et al, 1998; van Boxel-Dezaire et al, 1999; Huang et al, 1999).

1.4.2. *TNF-α*

Tumor necrosis factor-alpha (TNF-α) is a potent pleiotropic pro-inflammatory cytokine which plays an important role in the regulation of inflammatory and immune responses. TNF-α is produced by many cells types, including cells of the monocytic/macrophage lineage, epithelial cells, osteoblasts, smooth mucle cells, fibroblasts, and keratinocytes (reviewed in Thompson, 1998). In the CNS, the primary source of TNF-α is the microglia, but it can also be produced by astrocytes and neurons (Giora et al, 1994). There are two forms of TNF-α which arise from the same gene: the

26-kDa transmembrane TNF-α and the 17-kDa secreted TNF-α. Transmembrane TNF-α is displayed on the plasma membrane and is then proteolytically cleaved between alanine and valine in the extracellular domain leading to the release of secreted TNF-α (Kriegler et al, 1988). Both forms of TNF-α are biologically active, although they appear to have different functions. Secreted TNF-α is thought to be the form mediating most biological effects known of TNF-α, whereas the function of transmembrane TNF-α appears to be restricted to the cytotoxic effect of TNF through cell-cell contact (Kriegler et al, 1988). The biosynthesis of TNF-α is tightly regulated at many different levels to ensure the silence of the TNF gene under normal circumstances. At the transcriptional level, a number of regulatory sequences are found in the promoter region of TNF-α gene including three nuclear factor (NF)-κB sites κ1, κ2 and κ3, three nuclear factor of activated T cells (NFAT), binding sites for NFATp, NFAT-149, NFAT-117 and NFAT-76, one Y box, one cAMP-responsive element (CRE) for activation transcription factor-2 (ATF-2)/Jun, two SV40 promoter-1 (SP-1) sites, one activating protein-1 (AP-1) and one AP-2 binding site for Fos/Jun, and an early growth responsive-1 (Egr-1) binding site (reviewed in Thompson, 1998). At the translational level, the presence of an AU-rich sequence has been shown to decrease mRNA stability (Wilson and Treisman, 1988), and at the post-translational level, the processing of pro-TNF-α (transmembrane TNF-α) to yield mature secreted form of TNF-α is achieved by the TNF-alpha converting enzyme (TACE), a member of the adamalysin family of proteases (Black et al, 1997; Gearing et al, 1994, 1995).

TNF-α acts as a trimeric molecule, and this structure was shown to be important for receptor interaction (Zhang et al, 1992). There are two receptors for TNF-α, namely

TNF receptor type I (p60 or p55 or TNFR1), and type II (p75 or TNFR2), which are present on virtually all cell types except for red blood cells (Hohmann et al, 1989). Both TNF receptors are type I membrane glycoproteins with the N-terminus located at the exterior of the cell. Like other members of the TNFR superfamily, TNFR1 and TNFR2 contain a highly conserved cysteine-rich motif in their extracellular ligand-binding domain. The signaling events induced by TNFR1 following TNF-α binding are more complex, and can have contrasting effects on apoptosis. For this reason, TNFR1 is thought to play a dual role in apoptosis. Like most members of the TNFR superfamily, TNFR1 contain a death domain in its cytoplasmic portion, which interacts with the death domain of another adaptor molecule, the TNFR-associated death domain (TRADD). TRADD binds simultaneously with other adaptor proteins. Indeed, a study demonstrated that TRADD can interact with FADD, TRAF2, and RIP (Tartaglia et al, 1993; Hsu et al, 1995, Hsu *et al*, 1996). RIP, in turn, was shown to associate with another adaptor molecule, RAIDD (RIP-associated ICH-1/CED-3-homologous protein with a death domain) (Duan and Dixit, 1997). The interaction of TRADD with different adaptor molecules results in the activation of different pathways. For example, the TRADD-FADD interaction probably induces the activation of FLICE leading to the activation of caspases and apoptosis, whereas the TRADD-TRAF2 and TRADD-RIP interactions to induce the activation of the transcription factor NF-κB (Hsu et al, 1995; 1996), associated with the suppression of apoptosis. During resting conditions NF-κB is bound to its inhibitor IκB. Upon stimulation, IκB becomes phosphorylated and degraded, allowing the translocation of NF-κB to the nucleus. The fact that NF-κB can prevent apoptosis is based on three lines of evidence. First, inhibition of NF-κB translocation enhanced apoptotic

killing induced by ionizing radiation or daunorubicin (a cancer therapeutic compound) (Wang et al, 1996). Second, cells expressing a dominant-negative IκB (IκBM) have enhanced sensitivity to TNF-α-induced apoptosis, and the TNF-α treatment of fibroblasts and macrophages isolated from RelA-deficient mice results in a decrease in cells survival (Van Antwerp et al, 1996; Beg and Baltimore, 1996). It is not understood how NF-κB can have this suppressing effect on apoptosis, and whether different members of the NF-κB family play different roles in the regulation of apoptosis remains to be determined.

Biologic effects of TNF-α are diverse as shown in Table 1. In the CNS particularly, TNF-α has multiple effects, which can be both beneficial and detrimental, depending on its concentration. These include the control of neuronal and glial differentiation, proliferation, differentiation and survival, thus influencing neuronal and glial plasticity, degeneration as well as development and regeneration of the nervous system (reviewed in Munoz-Fernandez and Fresno, 1998). For example, at low concentrations, TNF-α increases neuronal survival and protects hippocampal, septal, and cortical neurons against glucose-deprivation-induced injury, excitatory amino acid toxicity and amyloid beta-epetide toxicity expression (Carlson et al, 1999; Kaltschmidt et al, 1999; Barger et al, 1995; Cheng et al, 1994), possibly through a mechanism involving the induction of the NF-kB-dependent induction of bcl-2 and bcl-x (Tamatani et al, 1999). At high concentrations, TNF-α is toxic to other CNS cell types including oligodendrocytes. Indeed, it has been shown that TNF-α causes the apoptotic death of oligodendrocytes through mechanisms involving the activation of jun kinase (JNK) and the induction of p53 (Ladiwala et al, 1998; 1999; Selmaj et al, 1988; Louis et al, 1993; D'Souza et al, 1995).

Table 1. Some of the biologic effects of TNF.

Immune cells	Non-immune cells	*In vivo*
Monocytes-macrophages Activation and autoinduction of TNF Induction of IL-1, 6, 8, GM-CSF, MCSF, INF-γ, NGF, TGF-β, PDGF and PGE_2 Transmigration and chemotaxis Stimulation of metabolism Inhibition of differentiation Suppression of proliferation Internalization of complement-coated virus	*Vascular endothelial cells* Modulation of angiogenesis Increasing permeability Enhanced expression of MHC I Procoagulant and antifibrinolytic Increasing permeability of albumin and water Suppression of proliferation Rearrangement of cytoskeleton Induction of NO synthase, IL-1, IL-3 receptor, G-CSF, GM-CSF, ICAM-1, VCAM-1, P- and E-selectin, surface antigen, platelet-activating factor, prostacyclin Inhibition of integrin B30, thromomodulin, glutathione, protein S	*Central nervous system* Fever Anorexia Altered pituitary hormone secretion *Cardiovascular* Shock ARDS Capillary leakage syndrome *Gastrointestinal* Ischemia Colitis Hepatic necrosis Inhibition of albumin expression Decreased catalase activity in liver Suppression of HBV gene expression
Polymorphonuclear leukocytes Priming of integrin response Release of granule components Increasing phagocytic capacity Enhanced production of superoxide Increased adherence to extracellular matrix Suppression of chemotaxis to *N*-formyl-1-leucyl-1-phenylalanine Suppression of cell surface expression of sialophlorin CD43	*Fibroblasts* Induction of proliferation Induction of IL-1, 6, INF-β_2, leukemia inhibitory factor, metalloproteinases (MMTs) Suppression of respiratory activity Inhibition of collagen synthesis, MMT inhibitor *Adipocytes* Enhanced release of free fatty acids and glycerol Suppression of lipoprotein lipase (LPL)	*Metabolic* LPL suppression Net protein catabolism Net lipid catabolism Stress hormone release Insulin resistance *Inflammatory* Activation of cell cytotoxicity Enhanced NK cell function Mediation of IL-2 tumor toxicity Protective role in cutaneous leishmaniasis
Lymphocytes Induction of T-cell colony formation Induction of superoxide in B cells Activation of cytotoxic T-cell invasiveness Induction of apoptosis in mature T cells	*Endocrine system* Stimulation of adrenocorticotrophic hormone and prolactin Inhibition of thyroid stimulating hormone, follicle stimulating hormone and growth hormone Enhancing IL-1 inhibition of steroidogenesis	

Adapted from Zhang and Tracey. The Cytokine Handbook. Ed. bY Angus W. Thompson.

It is thought that TNF-α plays a major role in increasing leukocyte trafficking to the CNS through an alteration of the blood-brain barrier (BBB) integrity. For instance, TNF-α induces the activation of endothelial cells by causing the upregulation of cell adhesion molecules, such as ICAM-1 and VCAM-1, by brain endothelial cells (Wong et al, 1999). TNF-α can also induce the production of other inflammatory mediators, such

as IL-1, IL-6, IL-8, IL-12, prostaglandins, and chemokines, by microglia and astrocytes, contributing to the amplification of CNS inflammation (reviewed in Giora et al, 1994). Finally, a role for TNF-α in fever and anorexia has been proposed because of its effect on hypothalamic centers that regulate body temperature and appetite (Plata-Salaman, et al, 1991; Krueger et al, 1998; Hayley, et al, 1999).

There is evidence to suggest that TNF-α is involved in the pathogenesis of CNS diseases including meningococcal meningitis, human immunodeficiency virus (HIV) infections, Alzheimer's disease, brain ischemia, and MS. For example, in the case of MS, the level of TNFα is elevated in the serum, cerebrospinal fluid as well as in the brain lesions of MS patients, and this correlates with disease activity (Canella et al, 1995; Hofman et al, 1989; Selmaj et al, 1991; Rieckmann et al, 1995). Moreover, peripheral blood mononuclear (PBMC) isolated from peripheral blood of MS patients at the time of a relapse produce significantly increased levels of TNF-α when compared to PBMC from controls (Glabinski et al, 1995). In mice, studies using transgenic animals overexpressing TNFα in the CNS demonstrate a role for TNF-α in CNS demyelinating disease. Indeed, Probert et al. (1995) demonstrated that these mice develop a spontaneous inflammatory demyelinating disease similar to MS; these results were also demonstrated by Taupin et al. (1997). In EAE, the administration of soluble TNFα receptors or TNFα antibodies as a mean to attenuate TNF-α biologic effects was shown to prevent the transfer of EAE and to abrogate autoimmune demyelination (Ruddle et al, 1990; Selmaj et al, 1991b, 1995a, 1995b). It is believed that the pathogenic role of TNFα in MS/EAE is associated with its ability to damage the myelin/oligodendrocyte complex, and to enhance leukocyte migration through the direct upregulation of adhesion molecules. In vitro studies

demonstrated that TNFα causes apoptotic death of oligodendrocytes (Selmaj et al, 1988; Louis et al, 1993; D'Souza et al, 1995), and demyelination of mouse optic nerve axons results from the intravitreal injection of TNFα in vivo (Butt et al, 1994). In EAE sensitized-animals treated with TNF binding protein (TNFbp), a polyethylene glycol-linked form of TNFR1, a reduction in VCAM-1 and VLA-4 staining was observed which corresponded to inhibition of CNS inflammation and the prevention of clinical signs of EAE (Selmaj et al, 1998).

1.4.3. *IL-10*

IL-10 is an important anti-inflammatory cytokine produced by a variety of cells including monocytes/macrophages, B cells, T cells, and epithelial cells. Mechanisms underlying the production of IL-10 are not well understood. However, it was recently shown that the ligation of B7-H1, a third member of the B7 family, co-stimulates T cells responses to polyclonal stimuli, and induces the secretion of IL-10 (Dong et al, 1999). Human IL-10 is a 18kDa protein which is homologous to viral IL-10 (vIL-10) derived from Epstein-Barr virus (EBV) since their amino acid sequence is 84% identical (de Waal Malefyt et al, 1991). IL-10 binds with high affinity to the IL-10 receptor (IL-10R), a member of the interferon receptor family (Ho et al, 1993). Like IFNs, IL-10 signals through the JAK-STAT system. IL-10R ligation induces tyrosine phosphorylation of tyrosine kinases JAK1 and TYK2 (Ho et al, 1995). Tyrosine phosphorylated DNA binding STAT1, STAT3, and in some cases STAT5 were detected in cells stimulated with IL-10 (Wehinberg et al, 1996).

IL-10 was first described as a cytokine synthesis inhibitory factor (CSIF) produced by mouse Th2 clones, which inhibited the production of cytokines by Th1

clones (Fiorentino et al, 1989). Based on these results, IL-10 is thought to be involved in the development and maintenance of a Th2 response as well as for the suppression of a Th1 response (Mosmann and Moore, 1991). In addition to this ability to affect Th cells functions, the most predominant effect of IL-10 is on cells of the monocytic lineage as it is considered to be a mediator of macrophage deactivation (Bogdan et al, 1991). For instance, IL-10 is a potent inhibitor of the synthesis of pro-inflammatory cytokine, including TNFα, IL-1α, IL-6 and IL-12, through a mechanism which may involve the inhibition of NF-kB activation (de Waal Malefyt et al, 1991; Oswald et al, 1992; Bogdan et al, 1991; Wang et al, 1995). IL-10 also blocks the ability of macrophages to present antigens because it downregulates the IFN-γ-induced expression of MHC class II and B7 costimulatory molecules (Frei et al, 1994; Ding et al, 1993; Iglesias et al, 1997). The anti-inflammatory role of IL-10 is further supported by the phenotype of IL-10 knockout mice. These mice develop an inflammation of the gut, namely chronic enterocolitis, which is associated with an aberrant MHC class II expression and an increase in proinflammatory cytokine production and CD4+ Th1-like responses (Kuhn et al, 1993; Berg et al, 1996).

In the CNS, IL-10 is produced by microglia and astrocytes (Williams et al, 1996; Mizuno et al, 1994). IL-10 production by microglia can be induced by LPS, and this is enhanced by the lipid mediator prostaglandin E2 (PGE2) (Aloisi et al, 1999c). Effects of IL-10 on microglia includes the reduction of IL-1 and TNF-α production, as well as the expression of cytokine receptors for IL-2, IL-6, but not for IL-4 (Sawada et al, 1999), downregulation of MHC class II, B7 molecules and CD40 (Wei et al, 1999). In astrocytes, IL-10 suppresses the IL-1β-induced production of IL-6 (Pousset et al, 1999).

There is also evidence that IL-10 is neuroprotective. IL-10 treatment of post-ischemic hypothermia priovided long-lasting neuroprotection of CA1 hippocampus following transient global ischemia in rats (Dietrich et al, 1999). Finally, a role for IL-10 in HPA-axis activation has been proposed. It was shown that treatment of IL-10 results in an increase in the production of corticotrophin releasing factor from rat hypothalamic median eminence (Stefano et al, 1998).

There is evidence to suggest that IL-10 plays a role in the remission phase of MS by inhibiting ongoing immune responses. MS patients have decreased levels of IL-10 mRNA compared to controls and a significant increase to normal levels were noted when active lesions became apparent by MRI monitoring (van Boxel-Dezaire et al, 1999). In another study, IL-10-secreting cells were isolated from the blood of MS patients mainly at the time of remission (Correale et al, 1995). Similarly, the appearance of IL-10 mRNA expression in the CNS of EAE mice correlates with recovery (Kennedy et al, 1992) The administration of IL-10 by a recombinant vaccinia construct was found to inhibit EAE in mice (Willenborg et al. 1995). The development of EAE in these IL-10-deficient mice was also shown to be accelerated suggesting that IL-10 plays a role in disease progression and recovery (Samoilova et al, 1998). Indeed, it is most likely that IL-10 does not play a role in the induction of EAE, since the administration of recombinant IL-10 or anti-IL-10 monoclonal antibody before onset of signs had no effect when given early post-sensitization (Canella et al, 1996).

1.5. Molecules involved in cell-cell interactions

1.5.1 *Cell adhesion molecules and other interacting molecules*

Cellular adhesion molecules are known to be of fundamental importance in leukocyte migration and in immune responses by mediating cell-cell and cell-extracellular matrix (ECM) interactions. Families of cell adhesion molecules have been defined on the basis of structural homologies and include selectins, cadherins, immunoglobulin (Ig) superfamily, and integrins. Cell-cell interactions are also mediated by members of the tumor necrosis factor receptor (TNFR) superfamily.

In particular, the Ig superfamily consists of cell-surface proteins containing a variable number of Ig-like domains with conserved cysteine sequences that form disulfide bonds to stabilize β-sheets or the tertiary structures (Williams and Barclay, 1988). Members of the Ig superfamily likely derive by divergent evolution from a common precursor gene encoding the Ig domain. Many members of the Ig superfamily play a central role in the regulation of immune responses. Those include the immunoglubulins, T cell receptor (TCR), MHC class I and II, CD2, CD3γ, δ and ε, CD4, CD8, CD28, and the costimulatory molecules B7-1 and B7-2 (Abbas et al, 1997). A subset of the Ig superfamily proteins is often expressed on endothelial cells and is specialized for binding to integrins, and are often referred as cell adhesion molecules (CAMs). Members of this subset are the intercellular adhesion molecule-1 (ICAM-1: CD54), ICAM-2 (CD102), ICAM-3, ICAM-4, ICAM-5, vascular cell adhesion molecule-1 (VCAM-1: CD106), platelet-endothelial cell ahdesion molecule-1 (PECAM-1:CD31), and the mucosal addressin (MadCAM-1) (reviewed in Wang and Springer, 1998; Carlos and Harlan, 1994). The expression of ICAM-1 and VCAM-1, in particular, is inducible and can be greatly increased by cytokines produced at inflammatory sites. ICAM-2 and MadCAM, on the other hand, are constitutively expressed on endothelial cells (Wang

amd Springer, 1998). The ICAMs bind to integrins that contain I domain, whereas VCAM-1 and MadCAM-1 bind integrins that lack I domains (Wang and Springer, 1998).

Integrins are transmembrane cell surface proteins composed of noncovalently linked heterodimeric α and β chains. To date, there are 8 known β chains subunits and 15 α subunits. Integrins have been arranged in subfamilies according to the β subunits, which associate with one to more different α subunits. As many as 21 different integrin combinations have been reported. Integrin α chains have characteristic features. First, there are 7 tandem repeats of 60 amino acids that serve as divalent cation-binding domain. Second, a region of 180 amino acids, termed I, is inserted between tandem repeats and is important in ligand binding. Third, the cytoplasmic domain of integrins is short, and contains a conserved sequence, which is thought to be critical for the modulation of integrin affinity (O'Toole et al, 1994). Integrin β chains also have common structural characteristics including conserved tandem repeats of four cysteine-rich regions that are essential for tertiary structure, and a conserved unit located close from the NH2-terminus that is critical for the maintenace of the αβ heterodimer. A conserved region in the cytoplasmic domain of integrin β chains is thought to play a role in affinity modulation, and in integrin signaling. Integrins bind to ligands in the extracellular matrix (ECM) or on the surface of other cell types to mediate either cell-substratum or cell-cell adhesion. Within cells, these interactions induce changes in cytoskeletal organization, protein phosphorylation and gene expression (Schoenwaelder, and Burridge, 1999). Integrin activation involves conformational changes that are often mediated by intracellular signals (or affinity modulation) and receptor clustering (or avidity modulation) (reviewed in Hughes and Pfaff, 1999).

The TNF receptor (TNFR) superfamily is a group of type I membrane proteins which are best known for the role they play in the regulation of cell survival versus apoptosis. All members of this family are characterized by several cysteine rich domains (CRDs) in their amino terminal region, and some members also possess a cytoplasmic sequence called death domain (Smith et ak 1994). Members of the TNFR family include the two receptors of TNF-α, TNFR1 and TNFR2, CD95 (or Fas), nerve-growth factor (NGF) receptor, CD27, CD30, CD40, Ox40, 4-1BB, SVT2, DR3 (or Wsl-1/Apo-3/TNF receptor-related apoptosis mediating protein (TRAMP)/lymphocyte associted receptor of death (LARD)), DR4, DR5 (or TNF-related apoptosis inducing ligand (TRAIL-R2)/ TRAIL receptor inducer of cell killing 2 (TRICK2), TRAIL receptor without an intracellular domain (TRID)/DcR1, and glucocorticoid-induced tumor necrosis factor receptor family-related gene (GITR) (Wang and Lenardo, 1997). Upon binding of ligands of TNFR, trimerization of the receptor is initiated which permits the immediate recruitment of signaling proteins to the cytoplasmic moiety of the receptor. Many signaling events are induced from TNFR, including the activation of caspases, protein kinase C, NF-κB, and mitogen-activated protein kinase (MAPK) (Wang and Lenardo, 1997).

1.5.2. *VCAM-1/VLA-4*

VCAM-1, a member of the Ig superfamily, is expressed on many cell types, including endothelial cells, follicular dendritic cells (Huang et al, 1995), macrophages (Walton et al, 1994), macrophage-like cells such as microglia (Chabot et al, 1997), astrocytes (Rosenman et al, 1995; Lee and Benveniste, 1999), epithelial cells (Shu et al,

1993), and myoblasts (Iademarco et al, 1993). The initial molecular cloning of VCAM-1 reported six extracellular Ig-like domains (VCAM-1-6dD) (Osborn et al, 1989). However, it is now known that VCAM-1-6D lacks domain 4 which is produced by alternative splicing of a form of VCAM-1 that contains 7 Ig domains (VCAM-1-7D). *In vivo*, the VCAM-7D, expressed predominantly on activated endothelium, is the most abundant form (Huang et al, 1995). The pair of Ig domains 1 and 2, 4 and 3, 5 and 6 show the greatest homology suggesting that the unit 1, 2, 3 may arose from gene duplication (Hession et al, 1991). Several forms of VCAM-1 exist. The most common form of VCAM-1 has a transmembrane domain, but a glycophosphatidylinositol-anchored isoform and a non-anchored isoform of VCAM-1 lacking the transmembrane domain has also reported (Kinashi et al, 1995). Moreover, a soluble form of VCAM-1 (sVCAM-1) was found. A 100kDa form of sVCAM-1 can be cleaved from T lymphocytes upon phorbol ester stimulation through a mechanism involving matrix metalloproteinases (Leca et al, 1995). The function of sVCAM-1 is not completely understood but evidence suggests that it plays a role in chemotaxis by inducing migration and recruitment of T cells through the activation of kinases (Kitani et al, 1998).

VCAM-1 expression on endothelial cells is induced by cytokines, such as IFN-γ, TNF-α, and IL-1β, and this induced VCAM-1 expressed is inhibited by nitric oxide (Spiecker et al, 1997; 1998), and PPARα activators (Marx et al, 1999). In endothelial cells, VCAM-1 transcription is regulated by the transcription factor NF-κB (Shu et al, 1993), which binds to NF-κB sites located in the promoter region of VCAM-1 gene (Iademarco et al, 1992).

To date, four ligands of VCAM-1 have been identified, namely $\alpha_4\beta_1$ (also called very-late-antigen-4 (VLA-4) or CD49d/CD29), $\alpha_4\beta_7$, $\alpha_9\beta_1$ (or VLA-9) and $\alpha_D\beta_2$. Mutagenesis studies have shown that residues in Ig domain 2 of VCAM-1 as well as those in domain 1 may be involved in integrin binding (Osborn et al, 1994). It is thought that an aspartic acid residue, which resides near the bottom of Ig domain 1, plays a key role in integrin binding of VCAM-1 since it is complementary to a more-pocket-like structure in the alpha 4 integrins to which they bind (reviewed in Wang and Springer, 1998). The constitutive expression of α4 integrins ($\alpha_4\beta_1$ and $\alpha_4\beta_7$) is present on most leukocytes, including lymphocytes, monocytes, basophils, eosinophils, NK cells, and neutrophils (reviewed in Foster, 1996; Reinhartd et al, 1997). The binding of $\alpha_4\beta_1$ is not specific to VCAM-1, since it is also the counterreceptor of MadCAM, fibronectin, thrombospondin (TSP-1), and the bacterial outer membrane protein invasin (Carlos and Harlan, 1994). Interactions of α4 integrins to endothelial VCAM-1 mediates the binding of leukocytes to endothelial cells under flow, as shown using blocking antibodies, which results in leukocyte arrest and extravasation. There is evidence that α4-integrin-dependent migration of leukocytes to the CNS, in particular, is regulated by the $\alpha_4\beta_1$ (VLA-4) only, since $\alpha_4\beta_7$ appears to play a minor role (Baron et al, 1993; Engelhardt et al, 1995). The engagement of α4 integrins to VCAM-1 expressed on antigen-presenting cells plays a role in costimulation by initiating T cell activation and proliferation (Damle and Aruffo, 1991; Damle and Aruffo, 1992). In contrast to the broad cellular expression of α4 integrin, $\alpha_9\beta_1$ is expressed on neutrophils only, and it is upregulated after neutrophil activation (Taooka et al, 1999). Based on monoclonal antibody studies, it is believed that $\alpha_9\beta_1$ plays a critical role in neutrophil migration (Shang et al, 1999). Many

cell types express $\alpha_D\beta_2$, including mast cells, monocytes, macrophages, NK cells, a subpopulation of T and B cells, basophils, eosinophils and neutrophils (Grayson et al, 1997; 1998, Van der Vieren et al, 1999). Using VCAM-1 mutants, Van der Vieren and coworkers (1999) demonstrated that the binding site of VCAM-1 for $\alpha_D\beta_2$ overlaps with that of $\alpha4$ integrins. ICAM-3 is another receptor of $\alpha_D\beta_2$ integrin (Van der Vieren et al, 1995).

Accumulating evidence demonstrates that VCAM-1/VLA-4 interaction is involved in the pathogenesis of MS as regulators of leukocyte transendothelial migration through the blood brain barrier. Elevated expression of VCAM-1 and VLA-4 was found to be significantly increased in chronic active MS lesions when compared to normal brain tissue, which does not express detectable VCAM-1 (Canella and Raine, 1995; Brosnan et al, 1995). The enhanced expression of VCAM-1 was confined to endothelial cells and microglia, and was detected in MS lesions of the active-chronic form while acute MS lesions exhibit low level of VCAM-1 expression (Cannella and Raine, 1995). In the EAE model, in situ immunohistochemical analysis of mouse brain demonstrated an upregulation of VCAM-1 expression by day 14 which preceded EAE clinical symptoms, suggesting a causal role of VCAM-1 in the initiation of CNS inflammation (Dopp et al, 1994). Increased levels of sVCAM-1 were found in the serum and CSF of progressive MS patients (McDonnell et al, 1999; 1998; Frigerio et al, 1998a, 1998b), and this enhanced level was shown to correlate with MRI activity (Riechmann et all, 1997). VLA-4 appears to be necessary for T cell migration to the CNS since VLA-4-deficient antigen-specific T cells fail to cross the blood brain barrier (Baron et al, 1993; Kuchroo et al, 1993). Monoclonal antibodies against VLA-4 can successfully prevent, suppress or reverse,

the development of two different models of EAE by diminishing the infiltration of VLA-4+ cells into the brain (Soilu-Hanninen et al, 1997; Yednock et al, 1992). In addition, the expression of VLA-4 on myelin-specific T cell clones is associated with encephalitogenecity in EAE (Baron et al, 1993). Brain sections of EAE mice demonstrated that as T cells migrated further into the tissue that VLA-4 expression was lost suggesting that the role of VLA-4 in EAE is at the level of cell extravasation into perivascular tissue (Romanic et al, 1997; Madri et al, 1996). Furthermore, VLA-4 is necessary for the adhesion of lymphocytes to brain capillary endothelial cells in vitro as shown using specific monoclonal antibodies (Male et al, 1994; de Vries et al, 1994). From all published evidence, it is thought that VLA-4 is the most important integrin involved in transendothelial migration of T cells in EAE. Finally, the role of VLA-4 in MS has recently been firmly established by results obtained from a randomized, double-blind, placebo-controlled clinical trial performed in 72 patients with active-relapsing-remitting and secondary progressive MS which aimed to test the efficacy of antegren, a monoclonal antibody against α4 integrin, on MS clinical course. Short term treatment with antegren resulted in a significant reduction of new lesion formation detected by MRI (Tubridy et al, 1999).

1.6. Microglia-T cell interaction

Due to the ability of microglia to serve as antigen-presenting cells, microglia-T cells interactions have been described in the context of antigen presentation. It is still controversial whether microglia-T cells interaction results in the promotion or termination of an immune response. Several groups demonstrated that murine microglia can stimulate the proliferation and differentiation of allogeneic naïve T cells into Th1

effector cells (Carson et al, 1999; Aloisi et al, 1998). However, others reported that freshly isolated microglia stimulated apoptosis rather than proliferation of an MBP-specific T cell line (Sedgwick et al, 1998; Ford et al, 1996).

Do microglia and T cells interact in vivo? In the graft-versus-host disease (GvHD) model, activated microglia were shown to cluster around T cell infiltrate, and to be associated with single or clustered microglia expressing MHC class II (Sedwick et al, 1998). Microglia isolated from GvHD animals were also shown to proliferate, and to exhibit functions of activated microglia, such as phagocytosis and motility (Sedgwick et al, 1998). These results suggest that microglia activation occurs as a consequence of their interaction with infiltrated T cells. Direct contact between microglia and T cells was further demonstrated by Raivich and co-workers (1998), who showed that CD3+ T cells infiltrate the site of injury 3 days following facial nerve resection, and form direct contact with microglia. Interestingly, the influx of T cells correlated with an increase in the production of cytokines, such as IL-1β and TNF-α, as detected by RT-PCR.

1.7. General hypothesis and specific objectives

A hallmark of MS pathology is the demyelinating lesion in the CNS. Evidence suggest that MS lesions are characterized by the presence of non-antigen specific T lymphocytes, activated microglia and augmented levels of inflammatory cytokines. On the basis of these observations, several questions arise:

1) What is the role of non-antigen specific T cells in MS?

2) How do microglia become activated in MS lesions?

3) How are inflammatory cytokines produced in MS lesions?

4) How do drugs effective in the treatment of MS affect CNS inflammation?

Answers to these questions remain unclear. Therefore, **in this thesis, I test the general hypothesis that non-antigen specific T cells interact with microglia, which results in microglial activation and the production of cytokines, such as TNF-α and IL-10 (Figure 2).** I further address the mechanisms involved in cytokine production following microglia-T cell interactions. In order to test the proposed hypothesis, an *in vitro* system of microglia-T cell interactions was developed. In this system, human adult microglia derived from brain biopsy specimens from patients who underwent surgical resection as a means to treat intractable epilepsy or brain tumors are co-cultured with T lymphocytes isolated from peripheral blood of healthy individuals and activated with anti-CD3 (OKT3) for a period of 72 hours.

Five objectives address the general hypothesis of this thesis. These are:

Objective 1*:* To determine whether, and how, the production of the oligodendrocyte-toxic cytokine, TNFα, is generated from microglia-T cells interactions (***Chapter 3***).

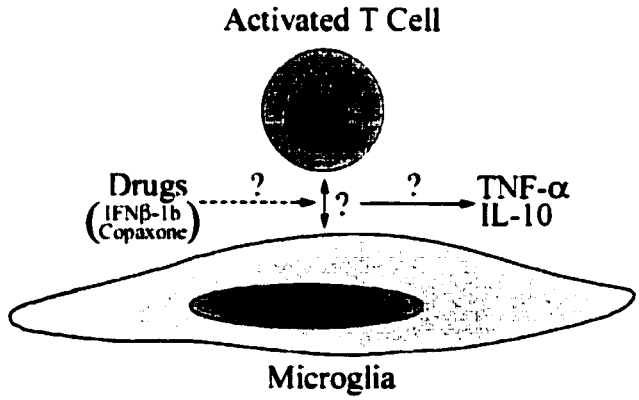
Objective 2*:* To determine whether, and how, the production of the anti-inflammatory cytokine IL-10 is generated from microglia-T cell interactions (***Chapter 4***).

Objective 3: To determine whether differentiated U937 cells, a promocytoid cell line that differentiates into macrophages, can serve as a model for human adult microglia (***Chapter 5***).

Objective 4: To determine whether VCAM-1 acts as signaling receptor to induce the production of TNF-α (***Chapter 6***).

Objective 5: To determine whether drugs used in the treatment of MS, such as IFNβ and glatiramer acetate, affects the production of cytokine generated from microglia-T cell interactions (***Chapter 7***).

MICROGLIA - T CELL INTERACTIONS



HYPOTHESIS: Do interactions between microglia and T cells result in cytokine production?

Figure 2. Schematic representation of the proposed general hypothesis.

CHAPTER TWO

Materials and Methods

2.1. Cell cultures and treatment

2.1.1. *Microglia culture*

Human adult microglia were isolated from resected brain specimens from patients undergoing surgery to treat intractable epilepsy or brain tumors as previously described (Williams et al, 1992, Yong and Antel, 1997; Sheng et al, 1995). Fetal microglia were isolated from fetal human brain obtained at legal and therapeutic abortions as described by Lee et al (1992). Specimens ranged in gestational age from 14 to 20 weeks. The use of adult or fetal materials has been approved by our local institutional ethics committee. After washing brain tissues with phosphate-buffer-saline (1X PBS), trypsinization of the tissue was achieved using 0.25% trypsin (GIBCO/BRL) and 50μg/ml DNAse (Sigma). The cell suspension was then passed through a Buchnel funnel nylon mesh, and remaining fragments were mechanically disrupted with a 10-ml pipet. Cell suspensions were centrifuged at 1200 rpm for 10 min. After resuspension of the cell pellets with phosphate-buffered saline (PBS), cells were added to Percoll (Pharmacia) to generate a final Percoll concentration of 30%. Cell debris and the myelin layer were aspirated off, after which the viable cell layer was collected. Cell suspensions were washed with PBS, and primary cultures were left overnight in T25 culture flasks (Nunc, Becton Dickinson, Missisauga, ON) at 37^0C, 5% CO_2. Microglia are adherent cells, while oligodendrocytes remain floating. Thus to obtain microglia-enriched cultures from the primary culture of mixed glial cells, floating cells (oligodendrocytes) were removed. Standardization of microglia

cultures was performed on the basis of morphological properties. Adherent cells were then trypsinized (0.25% trypsin) and microglia (purity over 95%) were plated in 96 well plate at a density of 2.5 X 10^4 cells per well. Microglia culture medium was minimum essential medium supplemented with 5% fetal calf serum (FCS), 0.1% dextrose and 20 μg/ml gentamicin (all obtained from GIBCO/BRL, Burlington, Ontario, Canada). Where indicated, microglia were treated with culture medium containing various concentrations (see results) of anti-CD40 (R&D System, Minneapolis, MN), anti-CD23 (Dako, Denmark) or CTLA-4-Fc (R& System, Minneapolis, MN) for a period of 30 minutes at room temperature before their co-culture with T cells. Also, in some experiments, microglia were treated with lipopolysaccharide (LPS) derived from E.Coli (Sigma, Oakville, Canada).

2.1.2. *Activated T cell culture*

Mononuclear cells were obtained from peripheral blood of healthy individuals or from epileptic patients, and were activated with an anti-CD3 antibody, OKT3 (1 ng/ml), generously provided by Dr. Jack P. Antel (McGill University, Montreal Neurological Institute, Montreal) for 72 hours; the purity of T cells at the end of this activation period was over 90% consisting roughly of equal quantities of CD4+ and CD8+ T cells (data not shown). Mononuclear cells (MNC) were isolated from the blood of healthy individuals using Ficoll Paque (Pharmacia Biotech). After two washes, cells were grown in the serum free medium, AIM V (GIBCO/BRL), and were activated with 1 ng/ml of an anti-CD3 antibody (OKT3) for a period of 72 hours. Flow cytometry analysis of the MNC population after the activation period indicated that CD3+ cells constituted about 90 %

of the total cell population with approximately 60 % CD4+ and 30 % CD8+. B lymphocytes (CD19+) and NK cells (CD56+) consisted of 5-6 % of the total MNC population, and no monocytes (CD14+) were detected. Henceforth, given that the majority of cells in the MNC population are T cells, it will be referred as T lymphocytes. When indicated, anti-CD3-activated T cells were treated with various concentrations of IFNβ-1b (provided by Berlex Laboratories, Richmond, CA) or glatiramer acetate (GA, obtained from TEVA pharmaceuticals, Israel) 3 hours after initiating the OKT3 treatment, for 72 hours. IFNβ- or GA-treated T cells received an additional treatment 3 hours prior to their co-culture with microglia or PMA/IFNγ-treated U937 cells. T cells were counted and 5.0 X 10^4 cells per well were added to the microglia.

In the experiment involving purified CD4+ or CD8+ T cells, the same number of purified cells (5.0 X 10^4) was added for co-culture with microglia. To obtain purified populations of CD4 or CD8, anti-CD3 activated cells or IFNβ-treated T cells were incubated with magnetic beads coated with a monoclonal antibody against CD4 or CD8 (Dynal, NY) for a period of 30 minutes at 4^0C under constant agitation. Rosetted CD4+ or CD8+ T cells were isolated using a magnet. In order to detach beads from purified cells, cells were incubated for 16-20 hours at 37^0C in a CO_2 incubator, and detached beads were then removed by placing the tube on a magnet. In some cases, activated T cells (5 x 10^4) were also pretreated with mouse anti-human CD49d (Clone HP2/1, Serotec, Toronto, Ontario), anti-LFA-1a (CD11a) (Becton Dickinson, Mississauga, Ontario), or purified mouse IgG1 (isotype control) antibody (Chemicon, Temecula, CA), for 1 hour, before they were co-cultured with microglia. In the VLA-4 function-blocking

experiment, T cells were pretreated with 25 μg/ml of anti-VLA-4 or IgG1 isotype control for a period of 30 minutes at 4^0C under constant agitation. Cells were then centrifuged for 2 minutes at 3000 rpm before resuspending them for their co-culture with microglia. T cells were counted and 5.0 X 10^4 cells per well were added to PMA/IFNγ-treated U937 cells. Where indicated, conditioned medium collected from activated T cells or IFNβ-treated T cells (SUP) was added to microglia or PMA/IFNγ-treated U937 cells. Finally, for some experiments, activated T cells or IFNβ-treated T cells were placed in tissue culture inserts (Becton Dickinson), where they were in close proximity, but not contacting the microglia or PMA/IFNγ-treated U937 cells.

2.1.3. *U937 culture*

The U937 human macrophage cell line, obtained from the American Type Culture Collection (Rockville, MD), is a human pro-monocytoid cell line that differentiates into macrophages upon treatment with phorbol-12-myristate-13-acetate (PMA), a potent activator of protein kinase C (PKC). U937 cells were cultured in RPMI 1640 (GIBCO BRL) containing 10% FCS and 10 μM β-mercaptoethanol. To differentiate the U937 line into macrophages, floating cells were treated with PMA (30 ng/ml; Sigma, St-Louis, MO), and were seeded into 96 well plates (Sumilon, Japan) at a density of 2.5 X 10^4 cells per well for experiments in Objective 3, and in 6 well plates (Sumilon, Japan) at a density of 3 X 10^6 cells per well for experiments presented in Objective 4. After 2 days of culture with PMA, which resulted in adherence, cells were treated with 100 IU/ml of recombinant IFN-γ (Genzyme, Cambridge, MA) for 24 hours. Where indicated, PMA/IFNγ-treated U937 cells were treated with culture medium

containing 5 μg/ml of anti-CD40 (R&D Systems, Mississauga, Ontario, Canada), anti-CD23 (Dako, Denmark), CTLA-4-Fc (R&D Systems, Mississauga, Ontario, Canada) or IgG_1 isotype control (Pharmingen, Mississauga, Ontario, Canada), for a period of 30 minutes at room temperature, before their co-culture with activated T cells.

2.1.4. *Microglia-T cell co-cultures*

Human adult microglia, human fetal microglia or PMA/IFNγ-treated U937 cells (2.5×10^4) were plated per well of a 96-well tissue-culture plate; anti-CD3 activated T cells, IFNβ-treated T or GA-treated T cells (5.0×10^4) were then added to the microglia culture for 24 hours unless otherwise indicated.

2.1.5. *VCAM cross-linking*

Cross-linking of VCAM-1 was performed by exposing IFNγ-treated human adult microglia or PMA/IFNγ-treated U937 cells to an antibody mixture containing mouse IgG_1-anti-human-VCAM-1 (1μg/ml; Chemicon, Temecula, CA) or mouse IgG_1 isotype control (Pharmingen, Mississauga, Ontario, Canada), and F(ab)2 fragment (0.5 μg/ml; Jackson Laboratories, Mississauga, Ontario, Canada), used as a cross-linker. To serve as a positive control of NF-κB activation, 10 μg/ml of lipopolysaccharide (LPS) derived from E.Coli (Sigma, St-Louis, MO) was used (Collart et al, 1990).

2.2. Cytokine measurements

Protein levels of cytokines (TNF-α, IL-1β, IL-4, IL-6, IL-10, IL-12 and IL-13) found in the conditioned medium of co-cultures of T cells (untreated, IFNβ- or GA-

treated) and human adult microglia, human fetal microglia or PMA/IFNγ-treated U937 cells were measured using the appropriate ELISA kit (Medicorp, Montreal, Quebec, Canada). Unless otherwise stated, all conditioned media were collected after 24h of microglia-T cell co-cultures. The assay was performed following detailed instructions from the manufacturer.

2.3. Flow cytometry

2.3.1. *Surface Staining*

T cells were incubated with one of 4 antibodies (PS/2 and HP2/1 described above, anti-VLAα4-FITC from Serotec or anti-VLAα4-PE from Becton Dickinson) for 30 minutes and incubated at 4 ^{0}C as previously described (27). In another experiment, untreated and IFNβ-treated activated T cells were collected for flow cytometry analyses, and staining was performed in a single-step process by incubating cells for a period of 30 min at 4°C with the following conjugated antibodies: anti-CD40L IgG_1-PE, anti-CD28 IgG_1-PE, anti-CTLA-4 IgG_{2a}-PE, all obtained from Becton Dickinson, or anti CD11b-IgG_1-PE obtained from Immunotech (France). Appropriate IgG isotype controls (purchased from Becton Dickinson) were also used to serve as negative control. Stained cells were analysed with an argon laser FACScan equipped with Consort 30 and Lysys II software (Becton Dickinson); data was collected on 15000 cells per condition.

Adherent PMA/IFNγ-treated U937 were trypsinized using 0.05% Trypsin and 0.02% EDTA. After several washes with PBS containing 0.05% NaN_3 and 2% fetal calf serum, cells were incubated on ice with mouse IgG_1-anti-human-VCAM (Chemicon

International Inc., Temecula, CA), mouse IgG1-anti-human-CD23-PE (Immunotech, France), IgG1-anti-human-CD40-PE (Immunotech, France), IgG1-PE isotype control (Immunotech, France) or with IgG_1 isotype control (Pharmingen, Mississauga, Ontario, Canada) for a period of 45 minutes. In the case of VCAM-1 staining and its approprirate IgG control, this was followed by a 30 minutes incubation with goat-anti-mouse-$F(ab)_2$-FITC (Immunotech, France). Staining of PMA/IFNγ-treated U937 cells (15000 events) was analyzed with a COULTER EPICS XL-MCL flow cytometer using SYSTEM II™ Software Version 1.0. For the verification of Flow cytometer's optical alignment.

2.3.2. *Intracellular Staining*

For intracellular staining of IL-10, lone cultures of anti-CD3 activated T lymphocytes, IFNβ-treated T cells, human adult microglia, and PMA/IFNγ-treated U937 cells or co-cultures of T cells with human adult microglia or PMA/IFNγ-treated U937 cells were treated for 4-6 hours with with Golgi Stop™ (Pharmingen Canada, Mississauga, ON), a solution containing a mixture of ethanol and the protein transport inhibitor, monensin. At the end of their culture period, cells were collected for flow cytometry analyses. Cells were stained with primary antibodies for a period of 30 minutes at 4°C. These were anti-CD3 IgG_1-FITC (Becton Dickinson, Mississauga, Ontario, Canada) in the case of T cells, anti-CD64 IgG_1-FITC (Becton Dickinson) for the staining of microglia, or an appropriate IgG_1 isotype control. Cells were then washed 2 times with PBS containing 3% FCS. To allow intracellular staining to occur, fixation of cells was achieved using 100 µl of cytofix/cytoperm solution containing ethanol (Pharmingen, Canada) for 20 min at 4°C. Cell permeability was maintained by

washing cells with Perm /Wash solution (Pharmingen, Ontario). Cells were then incubated with anti-IL-10-IgG2a-PE or with an appropriate isotype control (both antibodies purchased at Pharmingen, Canada) for 30 minutes at 4°C. Staining was analysed by flow cytometry using an argon laser FACS® equipped with consort 30 and lysys II software (Becton Dickinson); data was collected on 15000 cells per condition. No antibody cross-reactivity was detected since all IgG isotype control stains were negative.

2.4. Reverse-transcriptase Polymerase chain reaction (RT-PCR)

Total RNA was extracted using TRIZOL reagent (GIBCO BRL, Burlington, Ontario). RNA was precipitated using isopropanol, and RNA pellets were washed using 70% ethanol. Total RNA was quantified by spectrophotometry. The levels of transcripts encoding human TNF-α, IL-10 and β-actin were determined using semi-quantitative RT-PCR. The following sequence of primers were used in the RT-PCR experiments:

TNF-α 5'-GAGTGACAAGCCTGTAGCCCATGTTGTAGCA-3'(sense)

TNF-α 5'-GCAATGATCCCAAAGTAGACCTGCCCAGACT-3'(antisense)

IL-10 5'-ATGCCCCAAGCTGAGAACCAAGACCCA-3'(sense)

IL-10 5'-TCTCAAGGGGCTGGGTCAGCTATCCCA-3'(anti-sense)

β-actin 5'-GCCCTGGACACCAACTATTGC-3'(sense)

β-actin 5'-GCTGCACTT GCAGGAGCGCAC-3'(antisense)

In objective 1 and 2, RNA (0.5 μg) was reverse-transcribed and amplified in a single-step process as previously described (Chabot et al, 1997). Thirty five cycles of amplification were used for TNF-α or IL-10, and 25 cycles for β-actin; these were in

the linear range of amplification. cDNA products were run on a 1.5% agarose gel containing ethidium bromide, and visualized under UV light. TNF-α, IL-10 and β-actin transcripts were 444 bp, 464 bp and 351 bp, respectively.

In experiments performed in Objective 4, RNA was reverse transcribed (RT) and amplified by PCR in a two-steps reaction using the Gene Amp RNA PCR kit (Perkin Elmer, Mississauga, Ontario, Canada). Reverse transcription was achieved by combining 0.5 μg of total RNA, 5 mM Mg_2Cl, 1X PCR buffer II, 1 mM dGTP, 1 mM dATP, 1 mM dTTP, 1 mM dCTP, 1U/μl of RNase inhibitor, 2.5 mM of Oligo $d(T)_{16}$ primers, and 2.5 U/μl of MuLV Reverse Transcriptase. Reactions tubes were incubated at 42^0C for 15 min, followed by a incubation at 99^0C for 5 minutes. For PCR reactions, a mixture containing 2mM Mg_2Cl, 1X PCR Buffer II, 2.5 U/μl, and 0.5 μM of specific primers was added to each sample. Twenty eight PCR cycles were used for the amplification of TNF-α and β-actin transcripts, All reactions were performed using a GeneAmp PCR System 9600 (Perkin Elmer). The identity of PCR products was confirmed by purifying and sequencing the products; sequence analysis was performed by BLAST search.

2.5. Immunocytochemistry

In objectives 1 and 2, live human adult microglia cells were seeded in 16-wells Lab-Tek (Gibco-BRL) chambers, and were incubated with mouse anti-human VCAM-1 (1:100 dilution, Chemicon, Temecula, CA), mouse anti-human CD40, mouse anti-human CD80 or mouse anti-human CD23 (5 μg/ml each) for a period of 1 hour at room temperature, or with the diluting medium of the antibody, as a control. Cells were then

washed in PBS followed by an incubation of 1 hour with goat anti-mouse-FITC or -rhodamine (10 μg/ml). Cells were then fixed for 10 minutes with 4% paraformaldehyde, and were viewed using an immunofluorescence microscope. Note that the pre-absorption of VCAM-1 antibody, using recombinant human VCAM-1 (R&D System), attenuated the VCAM-1 signal obtained without this treatment, indicating that the VCAM-1 antibody used for this technique is specific for a VCAM-1 epitope.

In objective 4, PMA/IFNγ-treated U937 cells (1 X 10^6 cells/ml) were seeded into 4 wells culture chambers (Nunc, Burlington, Ontario, Canada). Following treatments, cells were fixed with cold methanol (10 minutes at room temperature), and fixed cells were incubated for 1 hour with 1 μg/ml of the following primary antibody: mouse anti-NF-κB p65, rabbit-anti-IκB-α (C-21), and rabbit-anti-IκB-β (N-20) (Santa Cruz Laboratories). To ensure the specificity of these antibodies for their epitopes, pre-absorbtion of the antibodies with an excess amount of the corresponding blocking peptide (Santa Cruz Laboratories) was performed. After several washes with PBS, biotinylated goat-anti-rabbit or mouse antibody (Chemicon International, Temecula, CA) was then added to cells, followed by an incubation with steptavidin-FITC (Chemicon International, Temecula, CA). Mounting was performed using glycergyl (Dako, Carpinteria, CA), and staining was visualized using a scanning confocal microscope. Note that a pre-absorption treatment with blocking peptides attenuated the signal obtained without this treatment, indicating that the antibodies are specific for their respective epitopes.

2.6. Western Blotting

Total cell lysates were obtained using a lysis buffer containing 10 mM Tris-HCl, 0.15 M NaCl, 2 mM EDTA, 2 mM EGTA, 1 mM DTT, 1% Triton-X and freshly added 1mM PMSF, 1 μg/ml leupeptin, 1 μg/ml antipain, 1 μg/ml pepstatin A, and 1 μg/ml pepstatin. To obtain nuclear extracts, cells were first trypsinized using 0.25% trypsin-0.02% EDTA and after which they were collected by centrifugation (1200 rpm, 5 min). Pellets were resuspended into nuclear extract Buffer containing 15 mM KCl, 3.75 mM NaCl, 0.25 mM EDTA, 0.05 mM EGTA, 3.75 mM Tris-HCl (pH. 7.4), 0.5 mM spermidine and 0.15 mM spermine. After centrifugation, cells were lysed in ddH_2O, and were gently resuspended until 95% lysis could be achieved, as verified by microscopy. Nuclear extracts were lysed using the lysis buffer described above.

Twenty μg of total cell lysates or 5 μg of nuclear extracts were used for SDS-PAGE (10% acrylamide-SDS gel). Wet transfer of proteins was performed using PVDF membranes (Bio Rad, Mississauga, Ontario, Canada), and non-specific binding sites were blocked by immersing the membrane into 15 ml of blocking solution (5% skim milk in PBS-buffered saline containing 0.1% Tween-20 (PBS-T)). After several washes with PBS-T, membranes were incubated with 1 μg/ml of primary antibodies diluted into PBS-T for a period of 1 hour. The following primary antibodies were used: anti-human CD49d (PS/2) purified from cultured medium conditioned by the hybridoma cell line, CRL-1911 (ATCC), rabbit anti-human IκB-α (C–21) (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-human IκB-β (N-20) (Santa Cruz Biotechnology), rabbit anti-human IkB-ε (Santa Cruz Biotechnology), anti-phospho-IkB-α (Ser 32) (New England Biolabs Inc, Beverly, MA) or rabbit-anti-human (p65) NF-κB (Upstate Biotechnology, Lake Placid, NY). This was followed by an incubation of 45 minutes with anti-rabbit

IgG linked with horseradish peroxidase (HRP) (Amersham Pharmacia Biotech, Baie d'Urfe, Quebec, Canada). Finally, ECL-HRP substrate prepared from the ECL kit (Amersham Pharmacia Biotech) was added to the membrane for 1 minute, and immunoblotting was visualized by exposing the membrane to an XJB-1 X-ray film (Kodak, Rochester, NY). To detect CD49d protein levels (Objective 1), the signal was amplified using the Vistra Fluorescence Western blotting kit (Amersham, Sunnyvale, CA), and protein expression levels were quantified using a Fluorimager (Molecular Dynamics).

2.7. Electrophoretic mobility Shift Assay (EMSA)

The consensus oligonucleotide sequence for NF-κB was: 5'-AGTTGAGGGGACTTTCCCAGGC-3'. Oligonucleotides were synthesized and chemically 5'-end-labelled with Dioxigenin (Dig) by Oligo Express (Amersham Pharmacia Biotech). An NF-κB-mutant 5'-AGTTGAGGCGACTTTCCCAGGC-3', with a "G"→"C" substitution (as highlighted) in the NF-κB/*Rel* DNA binding motif or a 100X excess of unlabelled NF-κB oligonucleotides were used as negative controls. Annealing of oligonucleotides was achieved by mixing single-stranded oligonucleotides in a molar ratio of 1:1 in TEN buffer (10mM Tris-HCl, 1mM EDTA, 0.1 M NaCl, pH 8.0). The oligonucleotide mixture was heated at 95°C for 10 minutes, and then incubated at room temperature for 30 minutes. In order to allow binding of NF-κB to double-stranded (ds) oligonucleotides, 1 pmol of ds-labeled-oligonucleotides, 1 μg of polydeoxyinosinic-deoxycytidylic acid (poly-dI-dC), 20 μg of nuclear extract (see section 2.6), and 20 μl of TEN buffer were incubated at 30°C for 30 minutes. To determine the specificity of binding, an excess (100X) of unlabelled ds-NF-κB

oligonucleotides was added 10 minutes before the addition of the labeled oligonucleotide. Following the binding reaction, samples were loaded on a pre-run 8% acrylamide gel in 0.25X TBE buffer. Transfer of oligonucleotides onto a nylon membrane (Amersham Pharmacia Biotech) was then achieved by electroblotting, followed by the UV cross-linking of oligonucleotides using a transilluminator. To block nonspecific binding, the nylon membrane was immersed for 30 minutes in 30 ml of blocking buffer containing 10% blocking reagent (Boeringher Mannheim, Laval, Quebec, Canada). In order to detect Dig-labelled oligonucleotide, the nylon membrane was incubated with 75 U/ml of anti-Dig-POD antibody (Boeringher Mannheim) for 1 hour, followed by an immersion into ECL substrate (Amersham Pharmacia Biotech) for 1 minute. Immunoblotting was visualized by exposing the nylon membrane to an X-ray film (XJB-1 from Kodak).

2.8. Statistical analyses

Since every set of experiment involved multiple groups, statistical analyses (compared to controls) were conducted using one way ANOVA with Bonferroni's post ANOVA comparisons. Significance was set at $p \leq 0.05$.

CHAPTER THREE

Mechanisms of TNF-α Production from Microglia-T cell Interactions

(Chabot S., Williams G., and V.W. Yong. 1997. Microglia production of TNF-α is induced by activated T lymphocytes: Involvement of VLA-4 and inhibition by interferonβ-1b: J. Clin. Invest. 100: 604)

3.1. Introduction

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS). The infiltration of immune cells such as T lymphocytes and monocytes/macrophages into the CNS triggers a cascade of inflammatory reactions, regulated by cytokines, which leads to demyelination, axonal loss and neurological impairments (Raine, 1994; Traugott et al, 1983). An important regulator of these events is the potent pro-inflammatory cytokine tumor necrosis factor alpha (TNF-α). The level of TNF-α is found to be elevated in the serum, cerebrospinal fluid and brain lesions of MS patients, and is correlated with the disease activity (Canella and Raine, 1995; Hofman et al, 1989; Selmaj et al, 1991; Reickman et al, 1995). TNF-α is also implicated in the pathogenecity of an inflammatory disease of the CNS in mice, experimental allergic encephalomyelitis (EAE), often used as a model for MS. The administration of antibodies to TNF-α or soluble TNF-α receptors prevents the transfer of EAE and abrogates autoimmune demyelination (Ruddle et al, 1990; Selmaj et al, 1995; Selmaj and Raine, 1995). Moreover, during TNF-α receptor-mediated inhibition of EAE, lymphocyte trafficking into the CNS becomes impaired (Korner et al, 1995). Finally, transgenic mice

overexpressing TNF-α in the CNS develop CNS inflammation and Wallerian degeneration (Probert et al, 1995).

Biological functions of TNF-α in the CNS are multiple. In particular, TNF-α regulates CNS inflammation by inducing the production of inflammatory mediators, such as IL-1, IL-6, IL-8, IL-12, prostaglandins and chemokines (reviewed in Giora et al, 1994). Importantly, TNF-α can also influence lymphocyte trafficking across endothelium by upregulating the expression of various adhesion molecules, such as ICAM-1 and VCAM-1, involved in this process (Wong et al, 1999; Springer, 1990). With relevance to MS, there is evidence that TNF-α plays a direct role in the process of demyelination. Indeed, TNF-α directly induces *in vitro* the apoptotic death of the myelin-producing cells in the brain, the oligodendrocytes through mechanisms involving the activation of JNK and the induction of p53 (Selmaj et al, 1988; Louis et al, 1993; D'Souza et al, 1995; Ladiwala et al, 1998). Moreover, intravitreal injection of TNF-α *in vivo* causes demyelination of mouse optic nerve axons (Butt et al, 1994).

Another important regulator of leukocyte migration into the CNS is the α4β1-integrin (or very-late antigen-4, VLA-4). For instance, it was shown that VLA-4 is necessary for the adhesion of lymphocytes to brain capillary endothelial cells (Male et al, 1994; de Vries et al, 1994), and that VLA-4 is the most important integrin involved in transendothelial migration of T cells in EAE (Baron et al, 1993). Furthermore, monoclonal antibodies against α4 can successfully prevent, suppress or reverse, the development of EAE by blocking the migration of VLA-4-expressing cells into the brain (Soilu-Hanninen et al, 19987; Yednock et al, 1992).

The mechanism by which TNF-α is generated in the MS brain is unclear, but it is known that the resident CNS macrophage, the microglia, is an important source (Giora et al, 1994; Hofman et al, 1989; Renno et al, 1995). In this chapter, I investigated whether, and how, the interaction of activated T-lymphocytes with microglia *in vitro* can generate TNF-α through a mechanism that involves VLA-4.

3.2. Results

3.2.1. *Morphology of human adult microglia in culture*

Adult human microglial cells in culture can assume various morphologies (Williams et al, 1992), but the majority tend to be bipolar (elongated) (Fig. 3). In co-culture with activated T cells, microglia becomes amoeboid (rounded) in appearance, a morphological transformation that is suggestive of an increased activation state (del Rio-Hortega and Penfield, 1927). The change in morphology of microglia following co-culture with activated T cells is apparent by 4h, and is most marked at 24h. Another feature of T cell:microglia co-culture is that activated T cells tend to clump and aggregate around microglial cells (results not shown).

Figure 3. Microglia in culture. Phase contrast micrograph shows morphological appearance of microglia used in this study. Original magnification: x500.

3.2.2. *TNF-α production in microglia-T cell co-cultures*

When activated T cells were co-incubated with microglia for 24h, the resultant conditioned medium, when assayed for TNF-α protein levels, contained significant amounts of TNF-α when compared to microglia or T cells by themselves (Fig. 4A). To ascertain whether the TNF-α production was mediated by soluble factors or direct microglia-T cell surface interactions, the conditioned medium from activated T cells was added to microglia cultures; very little TNF–α production resulted, indicating that soluble factors were unlikely to be involved. This was supported by cell culture insert experiments where microglia cells were incubated in close proximity to, but not contacting, the T cells; minimal TNF-α was generated under this condition (Fig. 4A). These results suggest that T cell-microglia contact was necessary for the generation of TNF-α.

The cell surface molecules encoded by the major histocompatibility complex (MHC) gene clusters are crucial for antigen presentation to T cells and their activation, and are the principal determinants of graft rejection. To determine whether the interaction between activated T cells and microglia is MHC-restricted, i.e. that it involves MHC molecules, we incubated syngeneic (T cells and microglia were from the same donor) or allogeneic (T cells and microglia were from different donors) activated T cells with microglia. Both T cell types elicited the production of TNF-α (Fig. 4B), indicating that the generation of TNF-α was not MHC restricted. Stout and colleagues have also reported that activated T cells can provide antigen-nonspecific, MHC-non-restricted cognate signals that induce TNF-α production by IFN-γ-primed peripheral macrophages (Stout and Suttles, 1993; Suttles et al, 1994).

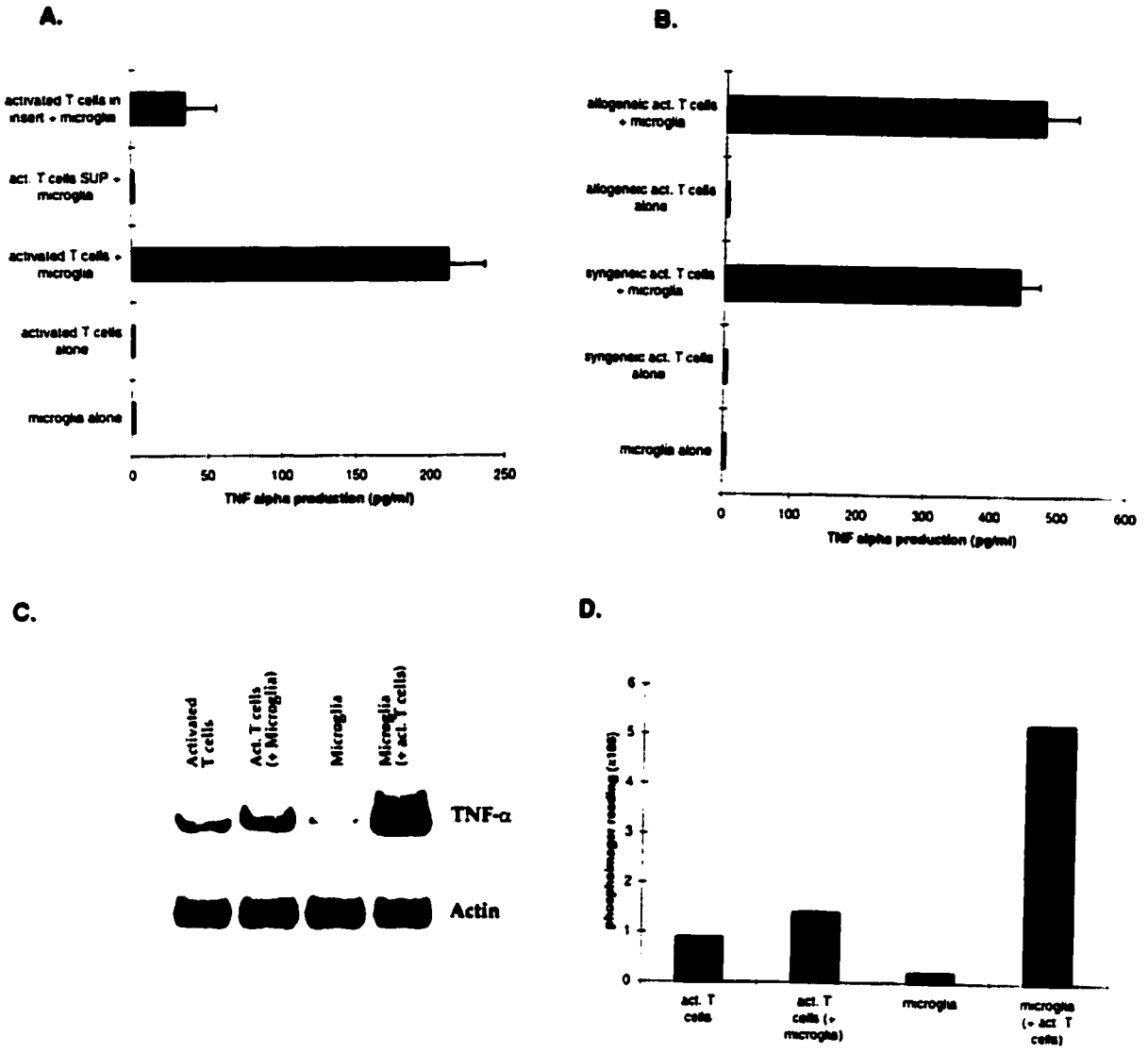


Figure 4: TNF-α production in microglia-T cell co-cultures. A: While microglia or activated T lymphocytes in isolation secrete negligible amounts of TNF-α into the culture medium, their co-culture for 24h resulted in substantial TNF-α production. This result (mean ± SEM of triplicates), shown by a representative series of experiments in panel A. The TNF-α produced by T cell-microglia co-culture cannot be reproduced if activated T cell supernatant (sup), rather than cells, were exposed to microglia, or if T cells were contained within cell culture inserts in close proximity to, but not contacting, microglia. **B:** Both allogeneic or syngeneic activated T cells induce the production of TNF-α by microglia, and to approximately the same extent. This result was also repeated when another series of allogeneic or syngeneic T cell: microglia co-cultures was used. **C:** The RNA isolated from activated T cells (lane 1) or microglia (lane 3) contains detectable amounts of TNF-α transcripts. The level of TNF-α mRNA in microglia increases significantly following their co-culture with activated T cells (lane 4); TNF-α mRNA elevates only modestly in T cells following their incubation with microglia (lane 2). The level of actin transcripts shows equal loading for each sample. The relative level of the TNF-α mRNA is quantitated using a phosphoimager and plotted in **D**.

T cells and microglia are both potential sources of TNF-α (Fig. 4C). To determine which cell type, or both, is the principal source of TNF-α in the T cell-microglia co-cultures, we took advantage of the fact that activated T cells, unlike microglia, are loosely adherent during their initial period of co-culture with microglia. Six hours following the addition of activated T cells to microglia, the loosely adherent T cells were removed by several washes of culture medium and collected. Microscopy confirmed the removal of T cells from the adherent microglia. When the RNA of both cell populations was analyzed, the level of transcript for TNF-α was dramatically increased in the microglia fraction, but not in the T cell fraction (Fig. 4C and D). We conclude that microglia were the major source of TNF-α in T cell-microglia co-cultures.

3.2.3 *VLA-4 is required for TNF-α to be produced in microglia-T cell co-cultures*

In order to elucidate the identity of the cell surface molecules involved in the interactions between microglia and activated T lymphocytes in producing TNF-α, we studied the contribution of an integrin found on T lymphocytes, the very late antigen-4 (VLA-4 or α4β1 integrin). VLA-4 and its ligand, vascular cell adhesion molecule-1 (VCAM- 1), found on microglia (Fig. 5B), are expressed at a higher level than normal in lesions of MS, as are other adhesion molecules including ICAM-1 (Cannella and Raine, 1995). Monoclonal antibodies against VLA-4 can successfully prevent, suppress or reverse, the development of EAE in rats (Yednock et al) or guinea pigs (Kent et al, 1995). In addition, the expression of VLA-4 on myelin-specific T cell clones is associated with encephalitogenecity in EAE (Kuchroo et al, 1993; Tanaka et al, 1993). We therefore treated activated T lymphocytes with a neutralizing antibody against CD49d, the α chain of VLA-4,

and found that it attenuated the subsequent secretion of TNF-α in T cell-microglia co-cultures in a dose-dependent fashion (Fig. 5A). In contrast, an antibody to LFA-1 (leukocyte function antigen-1), another integrin found on T cells, did not affect TNF-α production. We should note, however, that high concentrations of the VLA-4 antibody (25 or 50 μg/ml) were required to reduce TNF-α production. Nonetheless, we should note too that 50 μg/ml of the LFA-1 antibody, or the isotype control, did not affect TNF-α levels at all (Fig. 5A), suggesting the specificity of the reduction of TNF-α production when VLA-4 is blocked.

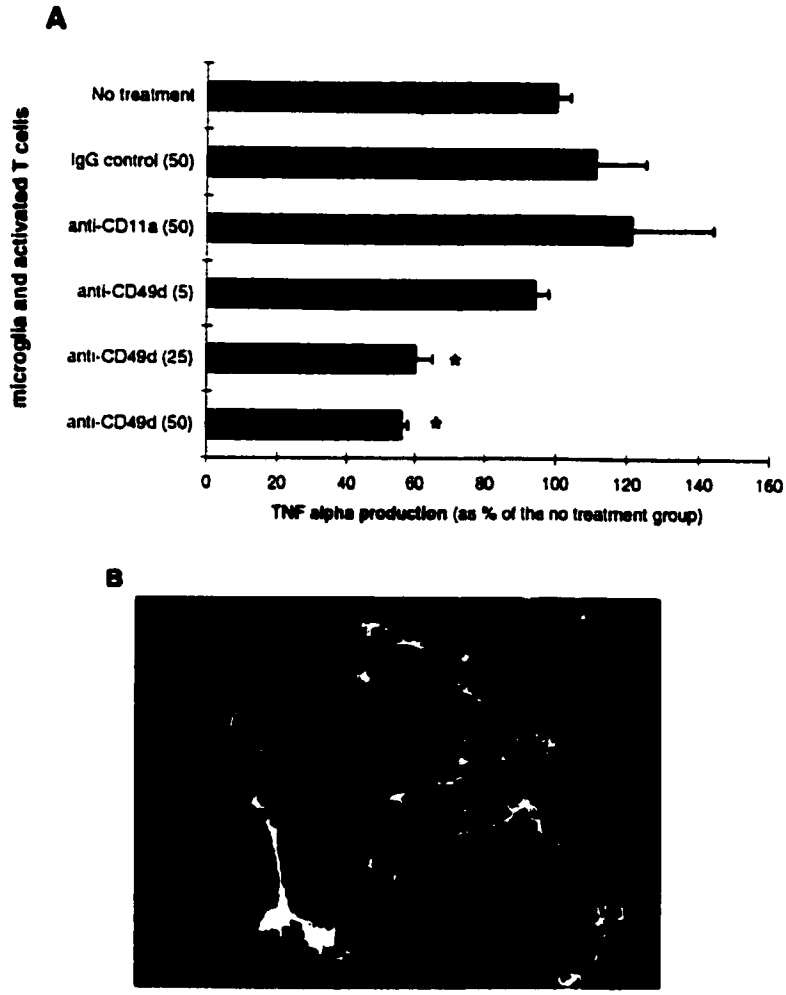


Figure 5: TNF-α production from microglia-T cell interactions depends on VLA-4 (CD49d or α4). A: Levels of TNF-α in the cell culture supernatant when activated T cells are treated with different concentrations of anti-CD49d (concentrations in parentheses in μg/ml) before being exposed to microglia. Values are mean of triplicate analyses ± SEM, and are normalized to the no treatment sample. Note that 50 μg/ml of an IgG isotype control, or an anti-LFA antibody (anti-CD11a), did not affect TNF-α levels, in contrast to the anti-CD49d treatment. *$p<0.05$ compared to IgG control (one way ANOVA with Duncan's multiple comparisons). **B:** Immunoreactivity of VCAM-1 of microglia, to confirm that this ligand for T cell integrin is present on the surface of microglia.

3.2.4. *VCAM-1 cross-linking induces TNF-α production*

What is the interacting partner of VLA-4 on microglia? VCAM-1 is an obvious candidate since microglia cells express this adhesion molecule (Fig. 5B). To test the involvement of VCAM-1, we first tested whether a blocking antibody against could inhibit TNF-α production in microglia-T cell interaction. Results demonstrated however that the anti-VCAM-1 blocking antibody had an inducing effect on TNF-α production suggesting that the antibody was crosslinking-VCAM-1 and inducing signal transduction pathways. Thus, on the basis of these observations, I addressed whether the cross-linking of VCAM-1 on the surface of microglia was sufficient to generate TNF-α. Figure 6 demonstrates that the addition of $F(ab)_2$ goat-anti-mouse Ig fragment alone enhanced TNF-α production by microglia, likely because of non-specific interaction with microglia or because of the presence of LPS in the antibody solution. To prevent this effect, polymyxin B could have been used to inactivate LPS activity. An isotype control for the VCAM-1 antibody also elicited TNF-α transcription to similar level as that for the $F(ab)_2$ group alone, and this is likely due to the $F(ab)_2$ Ig fragment. However, in the presence of the VCAM-1 antibody and $F(ab)_2$ Ig fragment, TNF-α mRNA level was clearly elevated over all control groups. Thus, the cross-linking of VCAM-1 is sufficient to induce signaling for the TNF-α production. Previously, the cross-linking of a related adhesion molecule, ICAM-1, on a rheumatoid synovial cell line, was reported to induce the transcription of IL-1β (Koyama et al, 1996). Hence, adhesion molecules can function not only as adhesive substrates, but also as transducer of signals for cytokine production. On balance, the findings suggest that the production of TNF-α by microglia may involve the VLA-4, and its receptor, VCAM-1, on microglia.

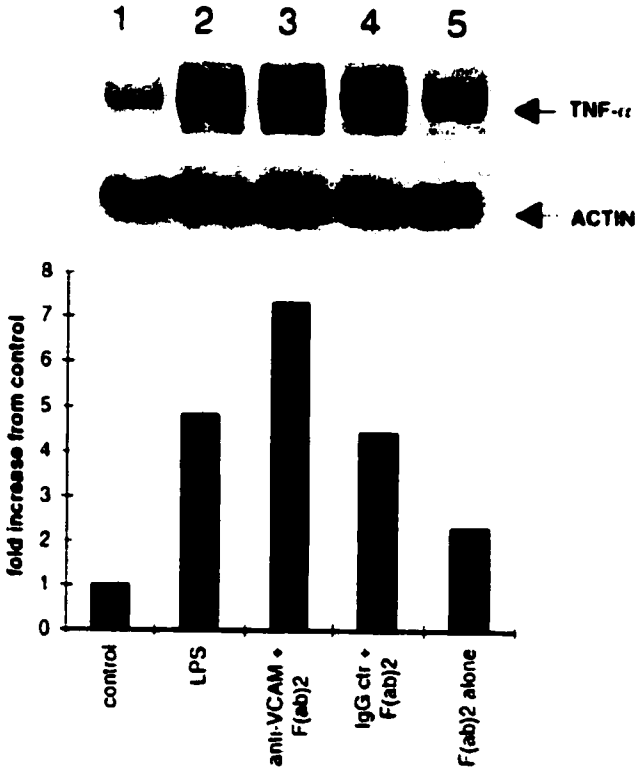


Figure 6: The cross-linking of VCAM-1 on the surface of microglia results in increased TNF-α transcript levels. Microglia were exposed to the various conditions listed here as described in the text, and semi-quantitative RT-PCR for TNF-α and actin mRNA was performed. Representative blots are shown and the level of transcripts for TNF-α or actin in each treatment group was obtained using a phosphoimager. The ratio of TNF-α to actin mRNA level for each group was obtained, and the ratio was then expressed as a percentage of untreated controls (lane 1), with the level of untreated control being set at one fold. Lipopolysaccharide (LPS, 5 μg/ml) was used as a positive control to stimulate TNF-α production (lane 2). Note that $F(ab)_2$ fragment alone (lane 5) or IgG isotype control plus $F(ab)_2$ (lane 4) stimulated TNF-α transcription, likely the result of non-specific interaction of this fragment with microglia or due to the presence of contaminating LPS in the antibody solution . Nonetheless, in the presence of anti-VCAM-1 and $F(ab)_2$ fragment (lane 3) TNF-α was clearly increased.

3.3. Discussion

The infiltration of T cells into the CNS is considered a key event in the pathogenesis of MS or EAE. In EAE, chronologic studies have demonstrated that antigen-specific T cells home to the CNS early in the immune response, presumably aided by chemotactic gradients provided by chemokines, and localize to the perivascular space (Sedgwick et al, 1987; Cross et al, 1993). Following the initial wave of antigen-specific T lymphocytes, there is an

enhanced recruitment of a large number of non-antigen-specific T cells which traverse into the CNS parenchyma (Sedgwick et al, 1987; Cross et al, 1993); indeed, the later arriving T cells need not even be in activated state (Oksaranta et al, 1995). Of interest, the antigen-specific T cells in adoptive transfer EAE experiments constitute a minority population (less 2% of the total cell infiltrate), and furthermore, the clinical signs of EAE correlate temporally with the arrival of the non-antigen-specific T cells (Cross et al, 1993).

The entry of T cells into the parenchyma of the CNS places them in close proximity to microglia, the resident macrophage of the CNS, and a source of many inflammatory cytokines, including TNF-α. Of note, TNF-α, as mentioned previously, can be toxic to oligodendrocytes and can produce demyelination. Understanding the mechanism by which TNF-α is generated within the CNS can thus impact upon the rational treatment of inflammatory demyelinating diseases that include MS.

How is TNF-α generated by the microglia cells? Stimulation by soluble molecules, such as lipopolysaccharide (LPS) or IFN-γ is one mode (Meda et al, 1995), although LPS has not been demonstrated to have a physiological relevance in MS, and the adult human microglia tends to be a poor source of TNF-α in response to IFN-γ (Becher et al, 1996). In this chapter, we demonstrate that the contact of microglia with T cells is another mechanism of TNF-α production by microglia. Our results suggest that the T lymphocyte utilizes an α4 integrin to interact with microglia to induce TNF-α production. Specificity is revealed by the inability of a functional blocking antibody to LFA-1, another T cell integrin, to affect TNF-α production by T cell-microglia interactions. It is likely that other membrane molecules are involved in this process since the inhibition of TNF-α production by anti-CD49d was incomplete and required high concentrations. Other candidates include the

membrane-bound TNF-α (Suttles et al, 1994) and CD40 (Stout et al, 1996; Grewal et al, 1996) which have been suggested to be important in the T cell-mediated activation of peripheral macrophages. The possible ligand for VLA-4 on microglia is VCAM-1, and the cross-linking of VCAM-1 alone by an antibody is sufficient to trigger TNF-α transcription.

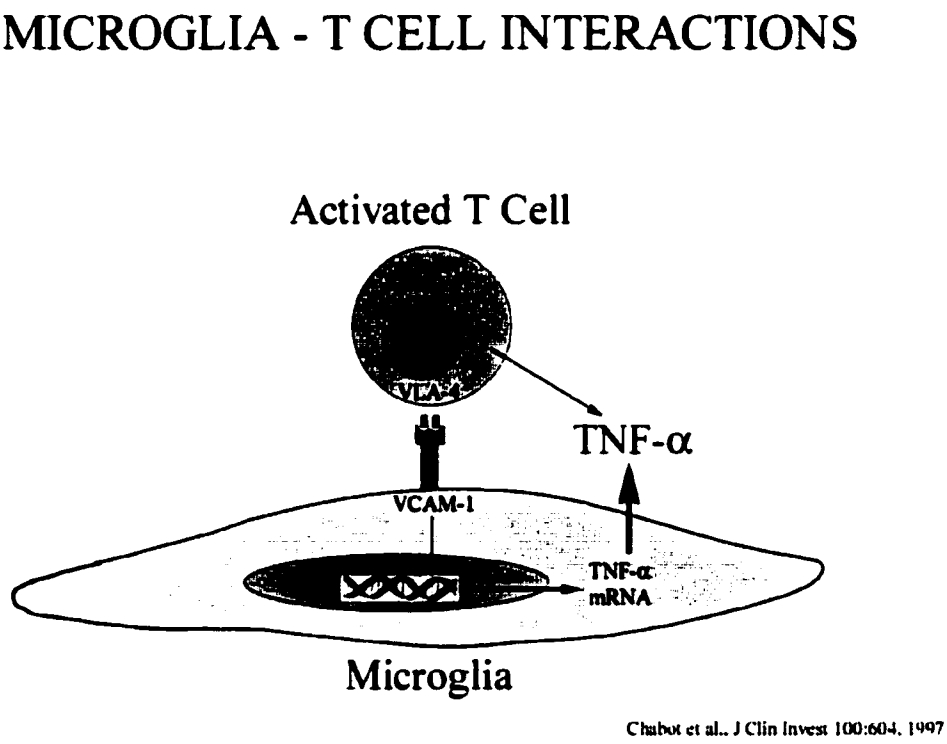


Figure 7: Schematic representation of results obtained for Objective 1.

The finding that the TNF-α generated in T cell:microglia co-culture requires α4 integrin has relevance to the MS and EAE disease processes. In animal experiments, the treatment with a monoclonal antibody to VLA-4 prevents the development, or suppresses and reverses, the clinical signs of EAE (Yednock et al, 1992; Kent et al, 1995). Furthermore, the expression of VLA-4 on proteolipid protein-specific (Kuchroo et al, 1993), or myelin basic protein-specific (Tanaka et al, 1993), T cell clones has been associated with

encephalitogenecity. T cell clones that express low levels of α4 integrins are non-encephalogenic (Baron et al, 1993).

In summary, the results of this chapter are relevant to MS where T cells infiltrate into the CNS to be in close proximity to microglia, and where TNF-α is known be pro-inflammatory and to be toxic to oligodendrocytes. Finally, while the focus of this work has been MS, the results have relevance to other disease states where T cell-macrophage/microglia interactions may occur and where TNF-α is produced to be pathogenic; these disorders include Crohn's disease, rheumatoid arthritis, cancer and even AIDS (Vassalli, 1992).

CHAPTER FOUR

Mechanisms of IL-10 Production from Microglia-T cells Interactions

(Chabot S., Williams G., Hamilton M, Sutherland G., and V.W. Yong. 1999. Mechanisms of IL-10 production in human microglia-T cell interactions. J. Immunol. 162: 6819)

4.1 Introduction

Multiple Sclerosis (MS) is an inflammatory disease of the central nervous system (CNS) which leads to demyelination and loss of neurological functions. The infiltration into the CNS of activated T lymphocytes, the majority of which do not appear to be antigen (Ag)-specific, is considered a key event in the pathogenesis of MS or experimental allergic encephalomyelitis (EAE), an animal model of MS. The mechanisms through which T cells play an etiologic role in MS remain unclear, although once infiltrated, T cells are found in close proximity to the macrophage-like cells of the CNS, the microglia. We have previously reported that T lymphocytes interact with microglia to generate the production of TNF-α through a mechanism which may involve VLA-4 on T cells with VCAM-1 on microglia (Chapter 3 / Chabot et al, 1997). This result is of pathological relevance since TNF-α has been reported to cause apoptosis of oligodendrocytes (Selmaj and Raine, 1988; Louis et al, 1993; D'Souza et al, 1995), the cells that are lost in MS. Of clinical significance is the observation that interferon-beta-1b (IFNβ-1b), a recombinant and modified form of human IFNβ that is effective in the treatment for MS (IFNβ MS Study group, 1995), inhibits TNF-α production likely through the downregulation of VLA-4 on the surface of T cells (Chapter 3 / Chabot et al, 1997).

Interleukin-10 (IL-10) is an 18kDa cytokine produced by a variety of cells including monocytes/ macrophages, T cells, B cells and mast cells. In the CNS, potential sources of IL-10 include the microglia (Williams et al, 1996) and astrocytes (Mizuno et al, 1994). IL-10 has important anti-inflammatory properties. First, IL-10 inhibits the production of pro-inflammatory cytokines by many cell types, including those of the mononuclear phagocytic lineage; indeed IL-10 was shown to inhibit the production of TNF-α and IL-12 produced by monocytes, macrophages and microglia (Bogdan et al, 1991; de Waal Malefyt et al, 1991; Brandtzaeg et al, 1996; Koch et al, 1996; Aloisi et al, 1997). Also, IL-10 plays a role in causing T cells to undergo anergy (inactivation or unresponsiveness) (Akdis et al, 1998). Other anti-inflammatory functions of IL-10 include its inhibitory effect on the process of antigen presentation. Treatment of macrophages/microglia with IL-10 downregulated the expression of molecules essential for the presentation of antigens, such as MHC class II (de Waal Malefyt et al, 1991) and the co-stimulatory molecules B7-1 and B7-2 (Iglesias et al, 1997). Finally, the role of IL-10 as an anti-inflammatory molecule is supported by the phenotype of IL-10-deficient mice: these mice develop chronic colitis, which appears to be mediated by the pro-inflammatory T-helper 1 cells (Kuhn et al, 1993; Davidson et al, 1996; Berg et al, 1996).

Given its anti-inflammatory roles, the production of IL-10 within the CNS will likely have a favorable impact on inflammatory diseases of the CNS. Indeed, recent evidence suggests that the induction of IL-10 production may partly account for the therapeutic effect of interferon-β (IFNβ) in Multiple Sclerosis (MS) (Yong et al, 1998) since patients treated with IFNβ have elevated IL-10 levels in their serum (Byskosh et al, 1996; Rudick et al, 1996) and cerebrospinal fluid (CSF), even after 2 years of treatment,

which correlated with a favorable therapeutic response (Rudick et al, 1998). In experimental allergic encephalomyelitis (EAE), an animal model of MS, the expression of IL-10 in the brains of mice afflicted with the disease is elevated during the recovery phase of the disease (Kennedy et al, 1992). IL-10 was shown to prevent EAE in rats (Rott et al, 1994) although this was not confirmed (Cannella et al, 1996). Nonetheless, in mice genetically deficient for IL-10, the development of EAE following immunization with myelin oligodendrocyte glycoprotein was accelerated compared to wild-type controls, and these mice did not spontaneously recover from EAE unlike the wild-type controls (Samoilova et al, 1998). Another group demonstrated that IL-10 deficient mice were more susceptible and developed a more severe EAE when compared to IL-4 deficient or wild-type mice; furthermore, IL-10 transgenics were resistant to the development of EAE (Bettilli et al, 1998).

The mechanism by which IL-10 is produced within the CNS is unclear. We postulated that T lymphocytes could be an important trigger of IL-10 production by microglia, since the infiltration of T cells into the CNS is a key pathogenic event in several neuro-inflammatory disorders including MS. In this study we investigated *whether* and *how* IL-10 is generated from the interaction of T lymphocytes with microglia *in vitro*. This chapter demonstrates that IL-10 is produced as a result of human microglia-T cell interactions, and that this is due to a contact-dependent mechanism involving the B7 molecules, CD23 and CD40.

4.2. Results

4.2.1 *IL-10 is produced in microglia-T cell co-cultures*

Microglia or T cells in isolation secrete negligible amounts of IL-10 into the conditioned medium. In contrast, their co-culture resulted in significant levels of IL-10 (Figure 8A). As previously reported (Chapter 3/Chabot et al, 1997), TNF-α was also produced in microglia-T cell co-cultures, and was assayed so as to serve as a positive control for microglia-T cell interaction.

A time course assay was performed in order to determine the temporal production of IL-10 compared to TNF-α in human microglia-T cell co-cultures (Figure 8B). TNF-α levels were elevated by 4 hours after microglia and T cells were co-cultured, and became significantly elevated ($p<0.001$) by 6 hours after co-culture when compared to microglia alone. On the other hand, IL-10 levels, which was first detected 6 hours after co-culture, did not become elevated above control levels until 24 hours after co-culture ($p<0.001$). Thereafter, the production of both cytokines reached levels of saturation.

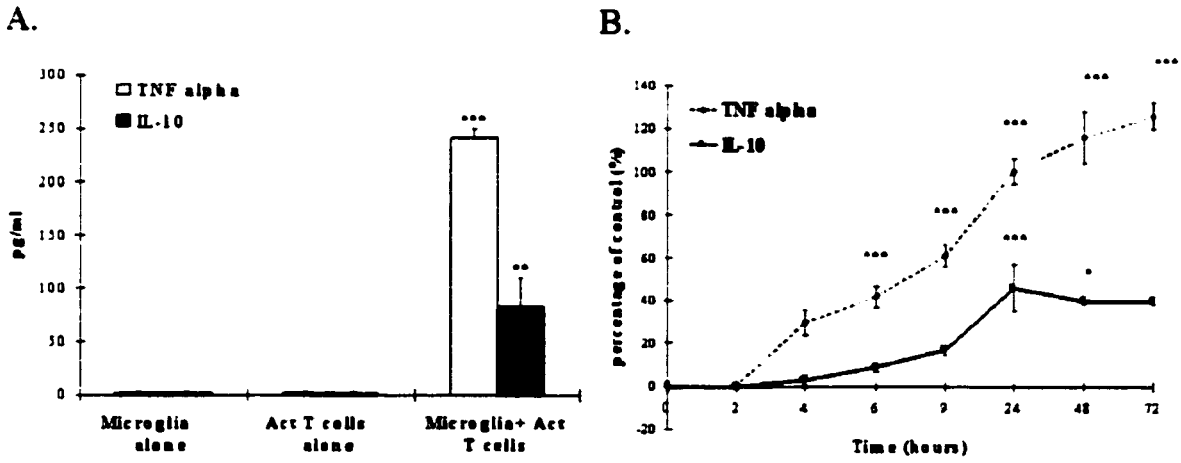


Figure 8. IL-10 is produced from human microglia-T cells interaction.
A. While microglia or activated (act) T lymphocytes in isolation secrete negligible amounts of IL-10 or TNF-α into the culture medium, their co-culture for 24h resulted in significant IL-10 and TNF-α production. Values are mean of triplicates ± SEM. These results are reproduced in 9 other experiments involving different human T lymphocytes and microglia preparations. **B.** The increase in TNF-α levels precedes that of IL-10 protein in microglia-T cell co-cultures compared to microglia alone. In order to standardize comparisons, all values are expressed as % of TNF-α levels at 24h of co-culture. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$)

4.2.2 *Source of IL-10 in microglia-T cell co-cultures*

Since T cells and microglia are both potential producers of IL-10 (Abbas et al, 1997; Sheng et al, 1995), intracellular staining for IL-10 was performed to address which cell type was responsible for the production of IL-10 in microglia-T cell co-cultures. T cells (CD3+) alone (Figure 9A) and microglia (CD14+) alone (Figure 9B) did not stain positive for IL-10 supporting the ELISA results shown in Figure 8A. However, when cells were co-cultured, both cell types were found to be positive for IL-10. In the case of T cells, 23% of CD3+ cells present in the co-culture were positive for IL-10 staining (Figure 9C). On the other hand, most microglia were found to produce IL-10 since 89% of CD14+ cells were positive for the IL-10 staining (Figure 9D). No antibody cross-reactivity was detected since all IgG isotype controls stain were negative.

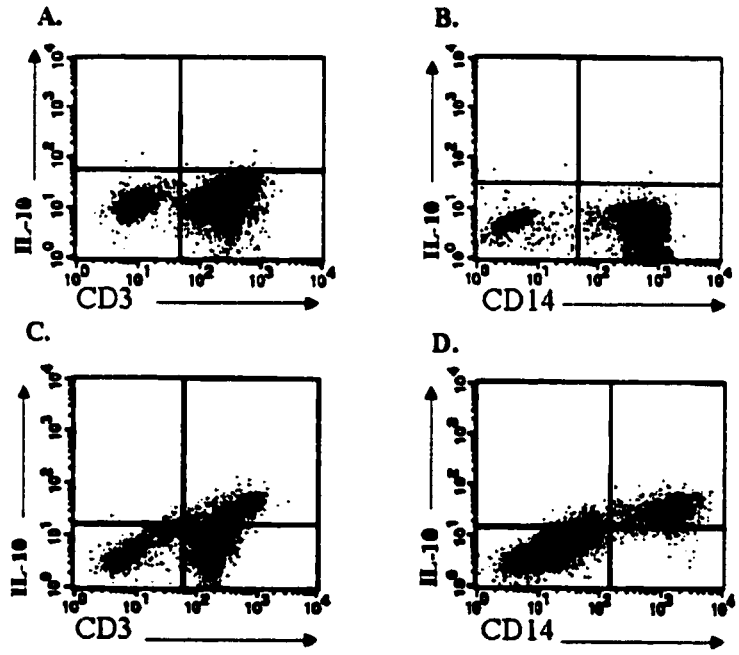


Figure 9. Both microglia and T cells produce IL-10. T cells (CD3+) and microglia (CD14+) alone do not produce IL-10 as shown in A and B respectively. However, when both cell types are co-cultured, 20% of gated CD3+ T cells (**C.**) and 89% of gated CD14+ microglia (**D.**) stain positive for IL-10.

Semi-quantitative RT-PCR, used to determine levels of mRNA for IL-10 in T cells and microglia was another approach used to determine the source of IL-10. Loosely adherent T cells were separated from microglia by several washes of culture medium and collected as previously described (Chapter 3 / Chabot et al, 1997). The removal of T cells was verified by microscopy. Total RNA from the T cells and the adherent microglia was collected 6 hours after the cells were co-cultured, a time point at which T cells remain loosely adherent, and at which levels IL-10 mRNA become elevated before protein synthesis take place several hours later. Results obtained from RT-PCR analyses (n=3) confirmed the results obtained in Figure 9 since they revealed that both cell types produced IL-10 following their co-culture (data not shown).

4.2.3. *IL-10 production in microglia-T cell co-cultures is not dependent on TNF-α*

Because the increase of TNF-α resulting from the interaction of microglia and activated T cells occurs prior to that of IL-10 (Figure 8B), and that TNF-α was shown to enhance the production of IL-10 in human monocytes (Dafterian et al, 1996), we investigated whether the production of IL-10 observed in microglia-T cell co-cultures was dependent on TNF-α. TNF-α is initially produced as a 26 kDa pro-form and is converted to its 17 kDa secreted form by TNF alpha converting enzyme (TACE), a member of the adamylysin subfamily of metalloproteinase (Black et al, 1997). The TACE inhibitor BB-94 has been shown to inhibit TNF-α secretion (Gearing et al, 1994). So, in order to test whether TNF-α was responsible for the production of IL-10 in microglia-T cell co-cultures, two approaches were used. First, TNF-α secretion was blocked using BB-94 (10 μM), and the neutralization of both secreted and transmembrane TNF-α was

performed using an antibody against TNF-α. Treatments with both BB-94 and anti-TNF did not affect the levels of IL-10 secreted, suggesting that TNF-α is not responsible for the production of IL-10 in microglia-T cell co-cultures (Figure 10A). As expected, BB-94 completely inhibited TNF-α secretion, but did not inhibit TNF-α mRNA transcript levels (Figure 10B) confirming that its effect on TNF-α secretion is not due to non-specific cytotoxicity. As a negative control, TIMP1, a natural inhibitor of matrix metalloproteinases (Yong et al, 1998) with no activity on TACE, did not affect the levels of TNF-α or IL-10 secreted into the culture medium (Figure 10A).

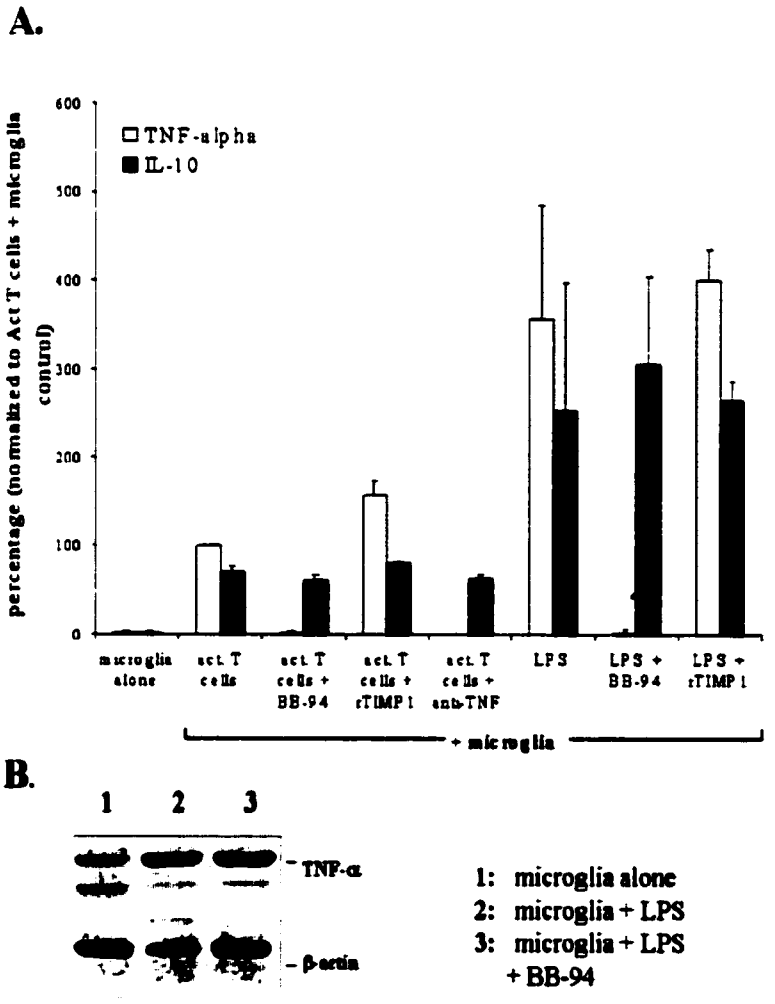


Figure 10. IL-10 levels in microglia-T cell co-culture are not dependent on TNF-α. **A.** The treatment with BB-94 completely inhibited (* $p<0.05$, compared to activated T cells plus microglia controls) the secretion of TNF-α but did not affect IL-10 secretion. Furthermore, treatment with an antibody against secreted and transmembrane TNF-α did not affect IL-10 production. **B.** BB-94 did not affect mRNA level of TNF-α induced by LPS treatment in microglia. The size of TNF-α cDNA product is 444 bp while that for β-actin is 351 bp.

LPS is a potent inducer of TNF-α and IL-10 production (Williams et al, 1996). LPS was used to enhance IL-10 and TNF-α levels in microglia-T cell co-cultures. Again, BB-94 in LPS-treated cells completely blocked TNF-α secretion, but did not affect IL-10 levels (Figure 10A). Collectively, these results demonstrate that the level of IL-10 is not dependent on TNF-α.

4.2.4. *Cognate interactions are necessary for IL-10 production*

To ascertain whether the increase of IL-10 in microglia-T cell co-cultures was due to soluble factors or cell contact interactions, the conditioned medium collected from cultures of activated T cells was added to microglia. Under this condition, IL-10 protein was not detected by ELISA (Figure 11A), suggesting that soluble factors play a minor role, if any, in the induction of IL-10 production in microglia-T cell co-cultures. This data was supported by cell culture insert experiments, in which activated T cells were placed in a culture insert (Becton Dickinson, Bedford, MA), and incubated in close proximity but not contacting the microglia: no IL-10 was generated under this condition. IL-10 was produced only when the two cell types were allowed to contact each other, suggesting that a contact-dependent mechanism is involved in the production of IL-10 in microglia-T cells co-cultures. It is unlikely that IL-10 production generated from this allogeneic interaction between microglia and T cells is MHC-restricted since levels of TNF-α generated from both microglia-T cells allogeneic and syngeneic interactions were shown in our previous study to be similar (Chapter 3 / Chabot et al, 1997).

It has been reported that the production of TNF-α generated from microglia-T cell interactions is partly dependent on the VLA-4/VCAM-1 interaction (Chapter 3 / Chabot

et al, 1997). In this study, we confirm the involvement of VLA-4 in the generation of TNF-α in microglia-T cell co-culture, since an antibody to the alpha chain of VLA-4, anti-CD49d, decreased TNF-α levels (Figure 11B). In contrast, anti-CD49d did not affect IL-10 levels, suggesting that IL-10 production is not VLA-4 dependent (Figure 11B).

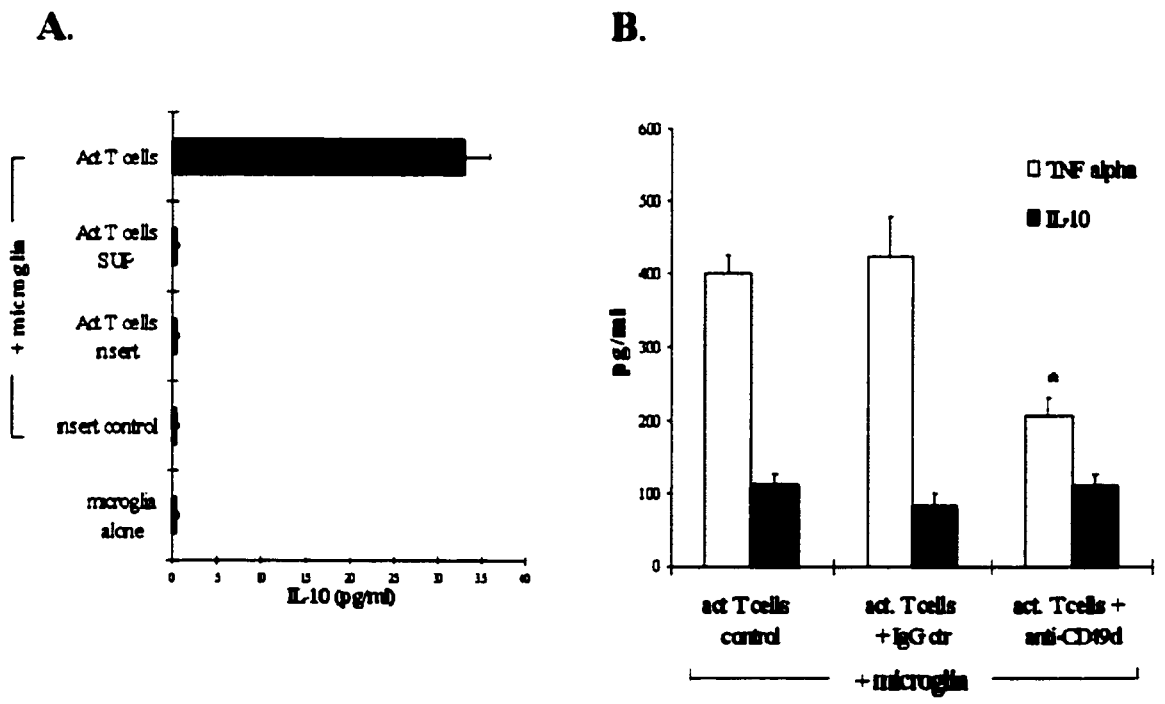


Figure 11. Direct contact between microglia and T cells is necessary for the production of IL-10, but VLA-4-dependent interactions are not involved. A. IL-10 is not generated when the conditioned medium (sup) from activated T cells is added to the microglia, or when activated T cells are placed in a culture insert, in close proximity but not contacting the microglia. **B**. Treatment of T cells with a neutralizing antibody against VLA-4 inhibits TNF-α but not IL-10 production. Of note, treatment of activated T cells with IgG1 isotype control did not affect TNF-α or IL-10 production. Values are mean ± SEM of triplicate experiments. * $p<0.05$ compared to activated T cells plus microglia controls.

4.2.5. *The role of CD40, B7 and CD23 in IL-10 production*

Given that the production of IL-10 in microglia-T cell interaction is cell-contact dependent (Figure 12A), we sought to elucidate the identity of the cell surface molecules involved. We focused on the CD40, CTLA-4 and CD23 pathways, since the respective ligand-receptor pairs are found on microglia and T cells (see below). First, the contribution of CD40/CD40L interactions, known to play a crucial role in macrophage-T cell interactions (Grewal et al, 1998) was studied. CD40 is a molecule expressed on macrophages, B cells, dendritic cells, and endothelial cells (Carson et al, 1998), and recently, murine microglia were also found to express CD40 (Carson et al, 1998). On the other hand, CD40 ligand (CD40L or CD154) is expressed on CD4 + T cells, and to a lesser extent on CD8+ T cells; NK cells can also express CD40L (Grewal et al, 1998).

Flow cytometry analysis confirmed the presence of CD40L on the surface of T cells (20 ± 3 % of total T cell population) 72 hours after their activation. CD40L was found to be expressed by CD4+ T cells (72 ± 9 % of CD40L positive cells) and to a lesser extent on CD8+ T cells (40 ± 11 % of CD40L positive cells; mean of 3 experiments involving 3 different blood donors). The presence of CD40 on the surface of microglia was confirmed by immunocytochemistry (Figure 13B).

Whether CD40/CD40L interaction had a role in IL-10 production in microglia-T cell co-culture was examined by the treatment of microglia with an antibody against CD40. Figure 12A demonstrates that anti-CD40 inhibited the levels of IL-10 detected in the culture media in a concentration-dependent manner. This inhibition was not specific to IL-10 since the production of TNF-α was also inhibited (data not shown). In order to confirm that anti-CD40 treatment alone had no inhibitory effect on IL-10 production,

LPS was used to induce the production of IL-10 by microglia and LPS-treated microglia were treated with anti-CD40 (10 μg/ml). Indeed, LPS-induced levels of IL-10 were not affected by anti-CD40 treatment. Moreover, microglia treatment with IgG isotype control, which served as another control, did not affect levels of IL-10 generated in microglia- T cell co-culture.

Next, we examined the possible role of the co-stimulatory pathway. CD28-CTLA-4/B7, in the production of IL-10 resulting from microglia-T cell interactions. CD28 has been reported to be constitutively expressed on 80% of CD4+ T cells and 50% of CD8+ T cells and becomes upregulated following T cells activation (Abbas et al, 1997). On the other hand, CTLA-4 is found at very low levels on resting T cells, and its expression is up-regulated after activation of T cells. CD28 and CTLA-4 share the same receptors, the B7 molecules (B7-1 and B7-2). Binding through CD28 provides a positive signal for T cell activation while CTLA-4 dependent interactions lead to the inhibition of T cell functions. CTLA-4 has a higher affinity and avidity for B7 molecules than CD28, and its expression is upregulated as cell activation progresses to act as an inhibitor of T cell activation (Saito, 1998; Thompson and Allison, 1997; de Simone et al, 1995). The presence of B7 molecules on human microglia has been shown *in vitro* (Williams et al, 1992), as well as *in vivo* on activated microglia and infiltrating macrophages within active MS lesions (De Simone et al, 1995). B7-2 appears to be expressed constitutively on human microglia and is thought to play a role in the initiation phase of the inflammation (Dangond et al, 1997). In contrast, B7-1 is expressed at low levels, and is believed to be involved in the progression of inflammatory responses as it becomes upregulated during inflammatory conditions such as MS (Satoh et al, 1995).

We confirmed the expression of CTLA-4 and CD28 on activated T cells by flow cytometry. CTLA-4 (14 ± 2.9 % of total cell population) was found to be equally expressed by CD4+ (60 ± 6 % of CTLA-4 positive cells) and CD8+ T cells (51 ± 9% of CTLA-4 positive cells; mean of 4 experiments involving 4 different blood donors). After 72 hours of activation, CD28 was expressed by 77 ± 7.2 % of the total cell population, and CD4+ T cells constituted 65 ± 3 % of all CD28 positive cells while 34 ± 0.5 % of CD28 positive cells were CD8+ T cells (mean of 3 different experiments of 3 different blood donors). The presence of the CD28/CTLA-4 receptor, B7-1 (CD80), on the surface of human microglia was confirmed by immunocytochemistry (Figure 13C). The constitutive expression of B7-2 (CD86) was previously reported by others (Dangond et al, 1997).

To test whether the CD28-CTLA-4/B7 interaction plays a role in the production of IL-10 in microglia-T cell co-cultures, microglia were treated with various concentrations of recombinant human CTLA-4-Fc chimera protein which binds both B7-1 and B7-2 with high affinity. Figure 12B shows that IL-10 levels in T cell-microglia co-cultures were reduced in a concentration-dependent manner by the treatment with CTLA-4-Fc. Levels of TNF-α were also inhibited by CTLA-4-Fc treatment (see Figure 14 below). However, CTLA-4-Fc treatment alone was not toxic to microglia since it did not affect IL-10 induced production of LPS-treated microglia.

CD23 is a molecule that plays an important role in allergy and inflammation. It is the low affinity IgE Fc receptor (Fc epsilon RII) expressed on monocytes/macrophages, but it is also viewed as an adhesion molecule because of its ability to interact with CD21 on B cells, and CD11b or CD11c on activated T cells (Bonnefoy et al, 1997). Since

human microglia were found to be positive for CD23 expression (Figure 13D), the role played by CD23 in the production of IL-10 generated in T cell-microglia co-culture was investigated; also, the cross-linking of CD23 on the surface of macrophages has been shown to induce the production of IL-10 through a mechanism dependent on cAMP (Dugas et al, 1996). CD23-dependent interactions were blocked by treating microglia with anti-CD23 (10 μg/ml); this inhibited levels of IL-10 generated in microglia-T cell interactions. Importantly, the inhibition by anti-CD23 was found to be specific for IL-10 since TNF-α levels were not affected. (Figure 12C). Treatment with anti-CD23 on microglia alone did not affect its cytokine levels or its morphology.

In order to determine whether CD4+ or CD8+ T cells were responsible for IL-10 production when co-cultured with microglia, purification of T cell subpopulations was performed using magnetic beads. Co-culture of microglia with either purified CD4+ or CD8+ cells triggered the production of IL-10 (Figure 12D). Moreover, treatment with either anti-CD40 (5 μg/ml), CTLA-4-Fc (5 μg/ml) and anti-CD23 (5 μg/ml) had a similar inhibitory effect on CD4+ and CD8+ dependent IL-10 production (Figure 12D).

The combination of anti-CD40 (5 μg/ml) and CTLA-4-Fc (5 μg/ml) treatments was found to augment each other's activity in inhibiting the production of IL-10 (Figure 14A). Indeed, the level of IL-10 in the culture medium approached the negligible amount found in control microglia culture. Additional blockage with anti-CD23 (i.e. anti-CD40 + CTLA-4-Fc + anti-CD23) did not further reduce the production of IL-10 (Figure 14A).

TNF-α level from microglia-T cells co-cultures was also significantly blocked in an additional manner by the co-administration of anti-CD40 (5 μg/ml) and CTLA-4-Fc (5 μg/ml) (Figure 14B).

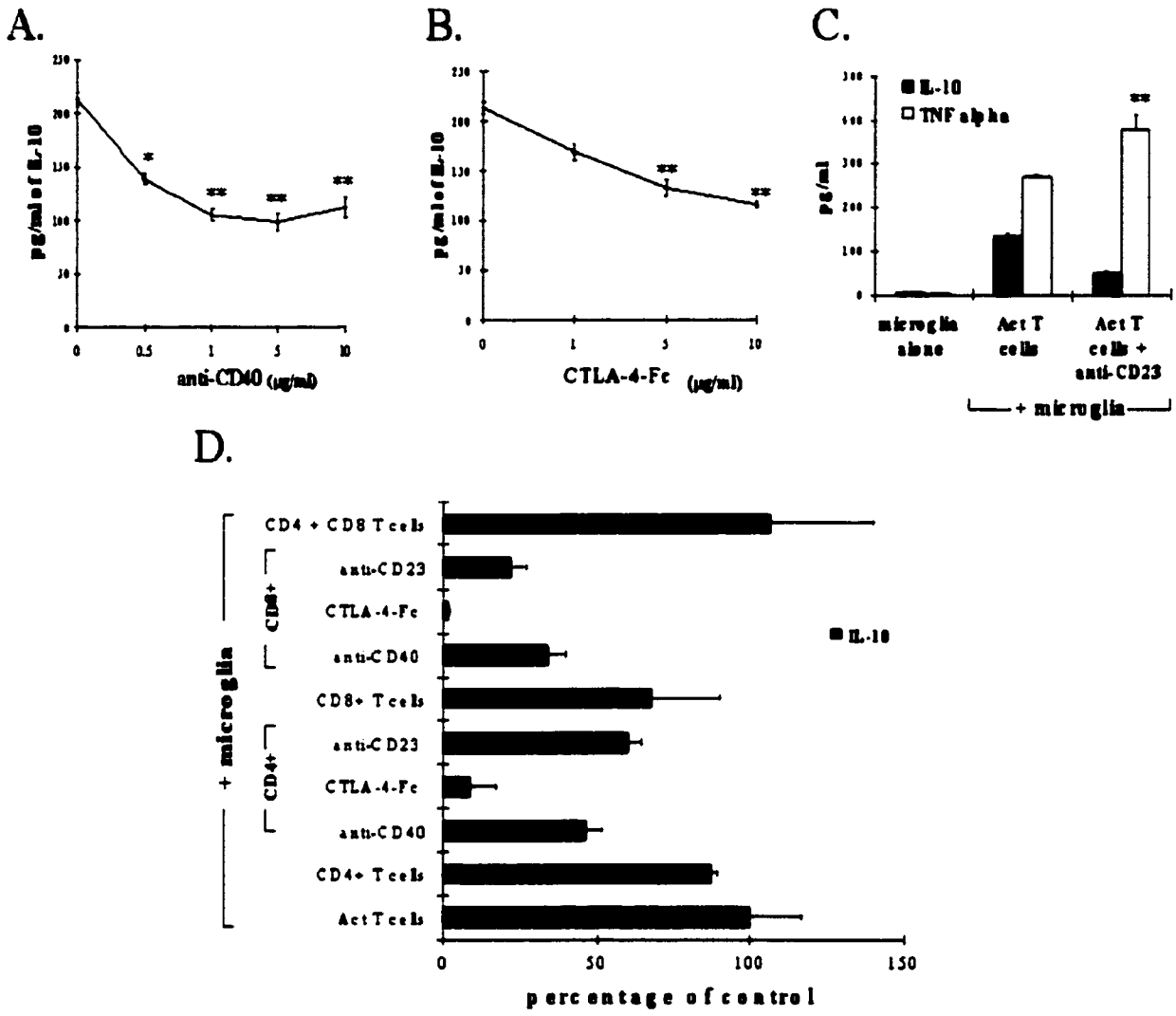


Figure 12. Anti-CD40, CTLA-4-Fc and CD23 blocksIL-10 levels generated in microglia-T cell co-cultures. Treatment of microglia with anti-CD40 (**A**) or CTLA-4-Fc (**B**) decreases IL-10 production in T cell microglia co-culture in a concentration-dependent manner. Anti-CD23 treatment (**C**) inhibited the production of IL-10 but not that of TNF-α. IL-10 levels generated from microglia co-cultured with purified CD4+ or CD8+ T cells are affected in a similar fashion by treatments of anti-CD40, CTLA-4-Fc and anti-CD23 (**D**). Values are mean of triplicates analyses ± SEM. *p<0.05; ** p<0.01 compared to their respective controls.

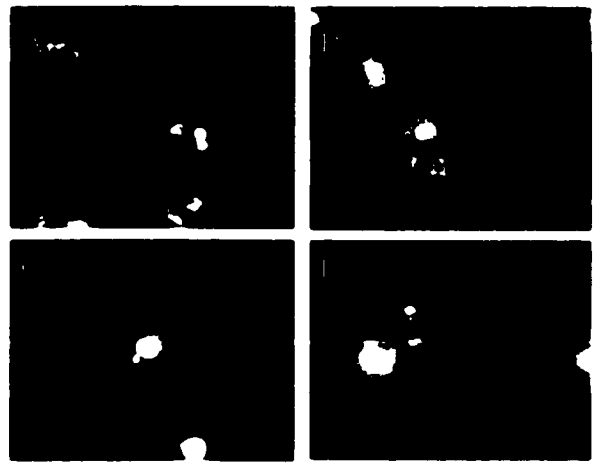


Figure 13. Microglia express CD40, B7-1 and CD23 on their surface. Non-specific immunoreactivity of microglia cells, obtained with secondary antibody but without primary antibody incubation, is shown in (**A**). Positive surface staining of CD40, B7-1 and CD23 are shown in **B**, **C**, **D** respectively. Note that all stainings were performed on live cells. X2000 magnification.

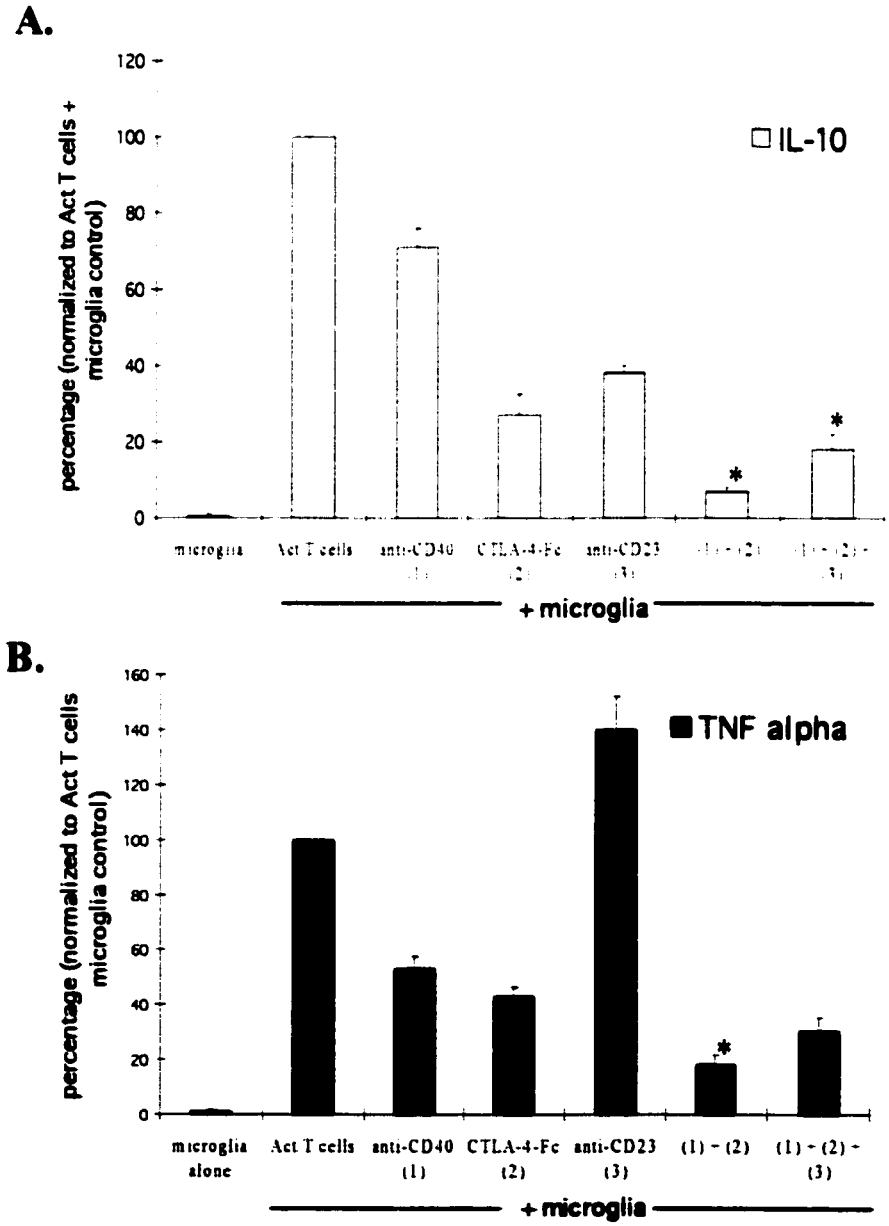


Figure 14. Combined inhibitory effect of anti-CD40 and CTLA-4-Fc on IL-10 and TNF-α production generated from T cell-microglia interaction. The combination of anti-CD40 (1) with CTLA-4-Fc (2) [(1) + (2)] inhibited levels of IL-10 (**A**) as well as TNF-α (B) to a greater extent than when either was used alone. Anti-CD23 did not attenuate further the inhibition of IL-10 produced by anti-CD40 and CTLA-4-Fc in combination.

4.2.6. *Morphological changes of microglia-T cell co-cultures*

Microglia acquire various morphologies *in vitro* as well as *in vivo*. In general, cultured resting human microglia tend to be bipolar (elongated) or ramified (Figure 15A), and become ameoboid (rounded) when they are activated (Williams et al, 1992). On the other hand, in culture, activated T cells are found as single cells or as homotypic aggregates (Figure 15B). When both microglia and T cells were cultured together, aggregates of T cells were found attached to the microglia and bipolar i.e. ramified microglia became ameoboid in shape (Figure 15C). However, in the presence of anti-CD40 or CTLA-4-Fc, microglia retained their bipolar/ramified morphology even though T cells were still adherent on microglia (Figure 15D). It is noteworthy though that less T cells were clustered (i.e. activated) around microglia cells in cultures treated with anti-CD40 or CTLA-4-Fc (Figure 15D), as it is likely to be the result a decrease in co-stimulatory processes necessary for further T cell activation in which B7 and CD40 play an important role. Taken together, these morphological results confirm the cytokine data that microglia become activated when in contact with T cells, but that this activation is attenuated by anti-CD40 or CTLA-4-Fc.

In contrast to anti-CD40 or CTLA-4-Fc, the anti-CD23 antibody, which reduced IL-10 but not TNF-α, did not fully prevent the ameoboid transformation of microglia in contact with T cells; indeed a range of morphology from ramified to amoeboid was observed .

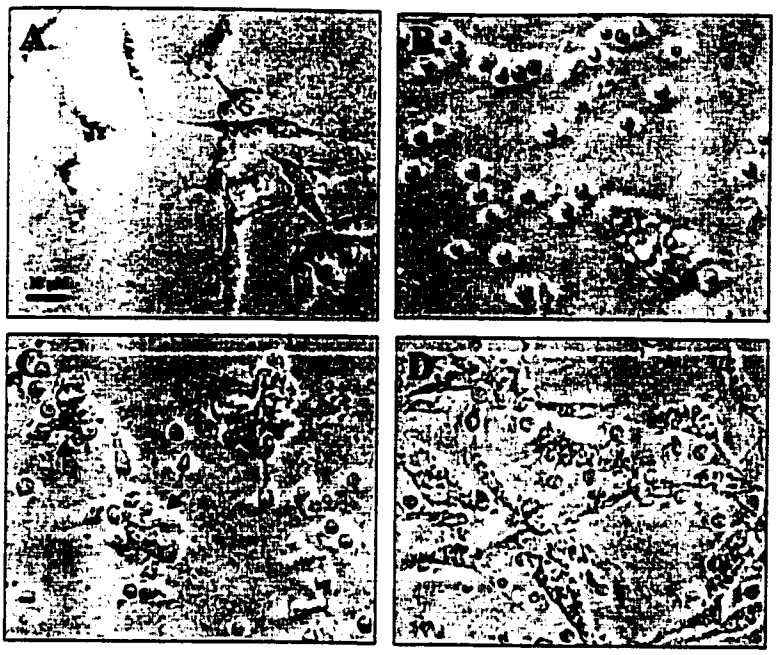


Figure 15. Morphological features of microglia-T cell interaction. A. Cultured resting microglia are bipolar or ramified in morphology. B. Activated T cells alone are found as single cells or as homotypic aggregates. **C.** When co-cultured, T cells aggregate on the surface of microglia which retract their processes to become amoeboid (rounded) in appearance, a morphological change characteristic of microglia activation. Ameoboid microglia are found under the clusters of T cells and are indicated by arrows. **D.** When T cell-microglia interaction between microglia and T cells is blocked in this case with a combination of anti-CD40 and CTLA-4-Fc, T cells still associate with microglia, but microglia appear to remain unactivated since they do not undergo morphological change to an amoeboid shape. Of note, similar results are obtained when microglia are treated with anti-CD40 and CTLA-4 Fc only. The scale in panel A represents 10 μm and is the same in all 4 panels.

4.3 Discussion

IL-10 is recognized as a potent anti-inflammatory cytokine due to its ability to inhibit the production of pro-inflammatory cytokines and inflammatory mediators (Bogdan et al, 1991; Aloisi et al, 1997), antigen presentation (de Waal Malefyt et al, 1991; Iglesias et al, 1997), Th1 differentiation, T cell activation (Akdis et al, 1998), and the production of specific antibody (Choe et al, 1998). Thus, it is likely that the

production of IL-10 is critical for shutting down inflammatory reactions involved in chronic neuro-inflammatory diseases such as MS.

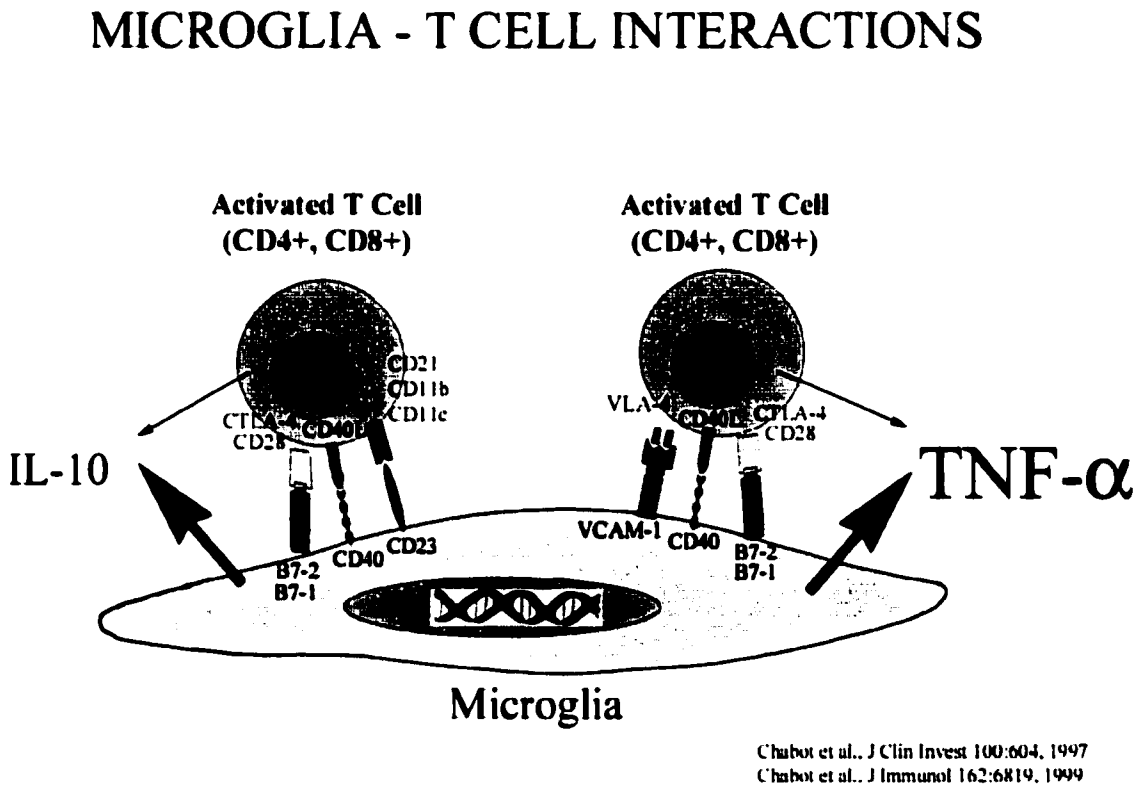


Figure 16. Summary of Results.

The mechanisms involved in the regulation of IL-10 expression are not very well understood, although recombinant HIV-1 Nef protein, recombinant IFNβ, and LPS are known to be inducers of IL-10 production (Williams et al, 1996; Brigino et al, 1997). Furthermore, the production of IL-10 by macrophages appears to be induced through the Fcγ receptor (Stout et al, 1996).

This chapter investigated novel mechanisms through which IL-10 may be generated, particularly in the context of the CNS. Activated T lymphocytes infiltrate the

CNS during neuro-inflammation and are then found in close proximity to the microglia. Results show that the interaction of microglia with T cells leads to the production of IL-10, and that blockade of the CD40/CD40L, CD28-CTLA-4/B7, and CD23 pathways results in the inhibition of IL-10 levels, suggesting that these pathways play a role in the anti-inflammatory response. Importantly, combinational blockade of the CD40/CD40L and CD28-CTLA-4/B7 pathways reduced IL-10 production by microglia-T cell interactions almost down to the negligible levels seen with microglia or T cells in isolation, highlighting the important contribution of these two pathways in regulating IL-10 levels. While anti-CD23 also reduced IL-10 levels, its addition to the anti-CD40 and CTLA-4-Fc combination did not further augment the effect of the latter.

Some selectivity of ligand-receptor pairs in microglia-T cell interactions was revealed by the results of this chapter. While the CD40/CD40L and CD28-CTLA-4/B7 pathways regulate both IL-10 and TNF-α, the VLA-4/VCAM-1 interaction was specific for TNF-α; in contrast, the CD23 system affected IL-10 but not TNF-α (Figure 16).

It is well established that the interaction of CD40 with its ligand CD40L plays an important role during inflammation and cell-mediated immunity. Of relevance to neuro-inflammation CD40 expression was found to be elevated in the brains of multiple sclerosis patients and in mice undergoing chronic EAE, and this elevation correlated with disease activity, suggesting that CD40/CD40L interactions may play a role in the pathogenesis of these diseases (Gerritse et al, 1996; Issazadeh et al, 1998). The interaction of CD40L with CD40 has been shown to induce the production of cytokines such as TNF-α and IL-12. In addition, Stout et al (1996) reported that T cells isolated from CD40L-deficient mice fail to induce macrophages to produce TNF-α. While these

studies have shown that the CD40/CD40L interaction plays an important role in the pro-inflammatory process, its role during the anti-inflammatory or Th2 type response has not been well characterized. The results of this study provide the first direct evidence that CD40/CD40L interaction plays a role during the anti-inflammatory response by regulating IL-10 production.

This study also demonstrates a role for the CD28-CTLA-4/B7 pathway in regulating IL-10 production in microglia-T cell co-culture, since inhibition of the B7 dependent interactions leads to a decrease in IL-10 production. Other laboratories have provided evidence for the CD28-CTLA-4/B7 pathway in the regulation of IL-10 levels by other cell types. First, blockade of this pathway using CTLA-4-Fc was shown to inhibit the *in vivo* production of IL-10 from activated lung CD3+ T cells by 70-80% (Tsuyuki et al, 1997). Second, the production of IL-10 in vitro by anti-CD3 activated CD4+ T cells was shown to occur only when CD28 and CD40L were cross-linked simultaneously (Blotta et al, 1996).

A specific role for CD23 in the production of IL-10 generated from microglia-T cells interations is also suggested by the results of this study since anti-CD23 treatment specifically inhibited IL-10 but not TNF-α. This study did not address the nature of the ligands for CD23, but CD11b and CD11c are obvious candidates since they are found on activated T cells. This present report is the first one to show that CD23 is expressed by cells of the CNS, namely the microglia, which suggest a novel role for CD23 in the regulation of immune functions of the CNS.

As TNF-α and IL-10 are both generated in response to microglia-T cell interactions, and given that there is selectivity in the ligand-receptor pairs in regulating

their expression as the results of this study indicate, it is of interest to determine whether the production of TNF-α and IL-10 can be selectively regulated. In the context of MS the elevated secretion of IL-10 is likely beneficial given its anti-inflammatory role while the generation of TNF-α may exert deleterious effect given that this is a pro-inflammatory cytokine that can also induce apoptosis of oligodendrocytes (Selmaj et al, 1988; Louis et al, 1993; D'Souza et al, 1995).

In summary, the results from this study demonstrate that IL-10 is produced as a consequence of direct microglia-T cell interaction, an observation that is relevant to the regulation of an anti-inflammatory response within the CNS.

CHAPTER FIVE

PMA/IFNγ-Treated U937 Cells is a Model of Human Adult Microglia

5.1 Introduction

Microglia are the immune effector cells of the CNS. They serve specific functions in the CNS response to injury, in the defense of the CNS against pathogens, and in the removal of tissue debris during normal development. The origin of microglia is still a controversial issue, but substantial evidence supports the theory that microglia are of mesodermal origin, and are related to cells of the monocyte/macrophage lineage which derive from bone marrow cells (reviewed in Cuadros and Navascues, 1998). When activated, functions of microglia are similar to those of macrophages, which include antigen presentation, phagocytosis, and secretion of soluble molecules involved in inflammation. Phenotypically, it is difficult to distinguish microglia from infiltrating macrophages, since they express the same markers. These include MHC class I and II, CD4, CD11a (LFA-1), CD11b (CR3 complement receptor/Mac-1), CD11c (CR4, p150.95), CD14 (LPS receptor), CD45, CD64 (FcγRI), CD68, F4/80, and vimentin (Streit, 1995; Rezaie and Male, 1999). Given their origin, their functional and phenotypical resemblance with macrophages, microglia are often considered as the macrophages of the CNS.

In order to act as fully competent macrophages, microglia need to be activated. Several mechanisms of microglia activation have been described. It is well known that soluble cytokines that are released from neighboring cells can act as activators of

macrophage effector functions. For example, interferon (IFN)-γ, produced by T lymphocytes and NK cells, can induce microglia activation by triggering the production of pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α and IL-1β (Meda et al, 1995; Liu et al, 1998). Another mechanism of microglia activation involves the bacterial cell wall component lipopolysaccharide (LPS). LPS-binding protein (LBP), found in the serum, is known to regulate the activation of microglia by facilitating the binding of LPS to CD14 (Amura et al, 1998). *In vitro*, treatment with a combination of LPS and IFNγ induces the production of reactive nitrogen oxides (NO, $ONOO^-$), reactive oxygen intermediates (superoxide anion, H_2O_2), and enzymes, such as lysozyme, cathepsin B/L, and acid hydrolases (Banati et al, 1993; Boje et al, 1992; Colton et al, 1987; Liu et al, 1996). This has also been shown in vivo. For instance, after injecting a mixture of LPS and IFNγ in the rat hippocampus, microglia are activated as shown by morphological changes and by an increase in IL-1β and iNOS immunostaining (Hartlage-Rubsamen et al, 1999). Finally, cognate interactions of microglia with activated T lymphocytes can also result in microglia activation. We have previously demonstrated that cytokine production, in particular TNF-α and IL-10, is induced from interactions between human adult microglia and activated T lymphocytes, which is due to a contact-dependent mechanism involving molecules such as CD40 and B7 (Chabot et al, 1997; 1999). Similarly, the ligation of microglial CD40 by CD40L was shown to induce the production of TNF-α (Tan et al, 1999).

The myelomonoblast-like human histiocytic lymphoma cell line U937 (Sundstrom et al, 1976) is used as a model for human macrophages when treated with phorbol-12-myristate-7-acetate (PMA) (Larrick et al, 1980; Hewison et al, 1992; Joyce

and Steer, 1992). There is evidence that cellular differentiation of U937 cells into macrophages induced by PMA is the result of growth inhibition due to the effects of PMA on the cell cycle machinery (Vrana et al, 1998). PMA-treated U937 cells acquire macrophage functions including the capacity to produce superoxide anion, and to respond to formyl-methionyl-leucyl-phenylalanine (fMLP) (Joyce and Steer, 1992). In this study, we investigated whether the U937 cell line can be a model of microglia, particularly in its cognate interactions with activated T cells to produce cytokines, and whether the mechanisms involved are similar to those identified in microglia-T cell interactions. If so, then the U937 cell line constitute a valuable and limitless resource to better understand the biology of microglia, which are available for study only in limited amounts.

5.2. Results

5.2.1. *Morphology of PMA/IFNγ-treated U937 cells cultures*

Under resting condition, the U937 line consists of floating cells which are uniformly round (Figure 17A). Following 48 hours of treatment with PMA, U937 cells become adherent as they are differentiating (Figure 17B). When PMA-treated U937s receive an additional treatment of IFNγ for a period of 24 hours, cells become further differentiated, as shown by the extension of processes and a more ramified morphology (Figure 17C). The morphology of PMA/IFNγ-treated U937 cells closely resembles that of human adult microglia in culture (Figure 17D). As a result of this morphological resemblance, all subsequent experiments utilized PMA/IFNγ-treated U937 to determine if this could constitute a model of microglia.

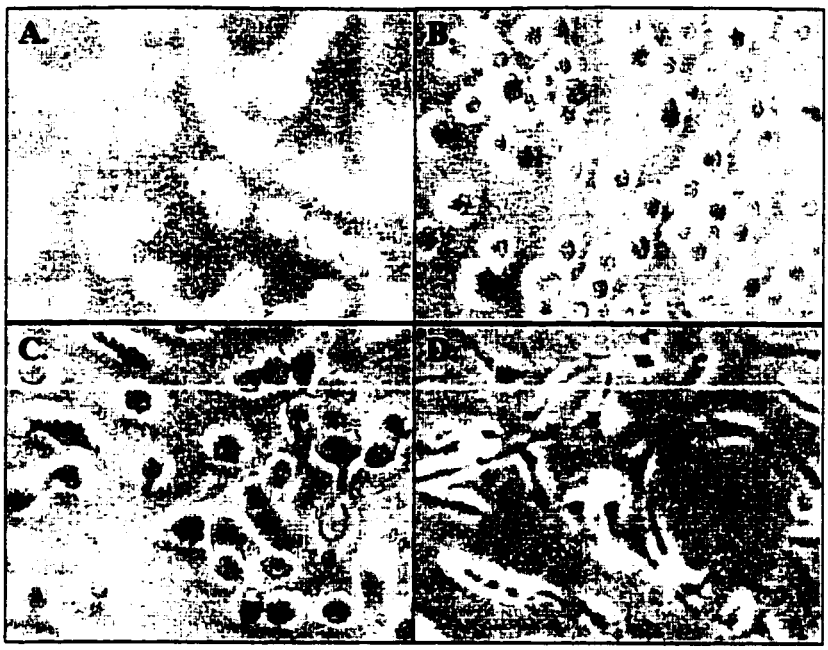


Figure 17. Morphological features of U937 cultures, and effect of PMA and IFN-γ treatment. Untreated U937 cells are rounded in morphology, phase bright, and remain floating (**A**); since cells are floating, it is difficult to photograph with good contrast. Treatment with PMA induces the adherence of U937 cells (**B**). Further treatment with IFNγ for 24h results in the morphological ramification of some of the PMA-treated U937 cells (**C**). With increasing length of culture period, the majority of the PMA/IFNγ cells will become bipolar, akin to the human adult microglia photographed at 7 days in culture (**D**).

5.2.2. *U937-T cells co-cultures result in the production of inflammatory cytokines*

Protein levels of pro- and anti-inflammatory cytokines in the conditioned medium of PMA/IFNγ-treated U937 cells co-cultured with OKT3-activated T cells were measured by ELISA. Results were compared to those from microglia-T cell co-cultures. In isolation, PMA/IFNγ-treated U937 cells (U), microglia (M) or activated T cells (T) secrete negligible amounts of TNF-α, IL-12, and IL-4 (Figure 18). In the case of IL-10, none was found in the conditioned medium of T cells or microglia alone, but PMA/IFNγ-treated U937 cells secrete a detectable amount (40-100 pg/ml). In U937-T cell (U+T) or

microglia-T cell (M+T) co-cultures, levels of TNF-α, IL-12, IL-10 and IL-4 are significantly enhanced when compared to either cell population in isolation. It was noted that the production of TNF-α, IL-10 and IL-4 tended to be higher in co-cultures of T cells with U937 cells, in comparison with microglia-T cell interactions. Quantitatively, of all cytokines tested in co-cultures, levels of TNF-α increase the most, with concentration values above 400 pg/ml (sometimes reaching 3 ng/ml). The second most abundant cytokine is IL-10 with concentrations ranging from 100 to 800 pg/ml. Much lower amounts of IL-4 (50-100 pg/ml) and IL-12 (10-20 pg/ml) are produced in interactions between T cells and microglia or PMA/IFNγ-treated U937 cells (Figure 18).

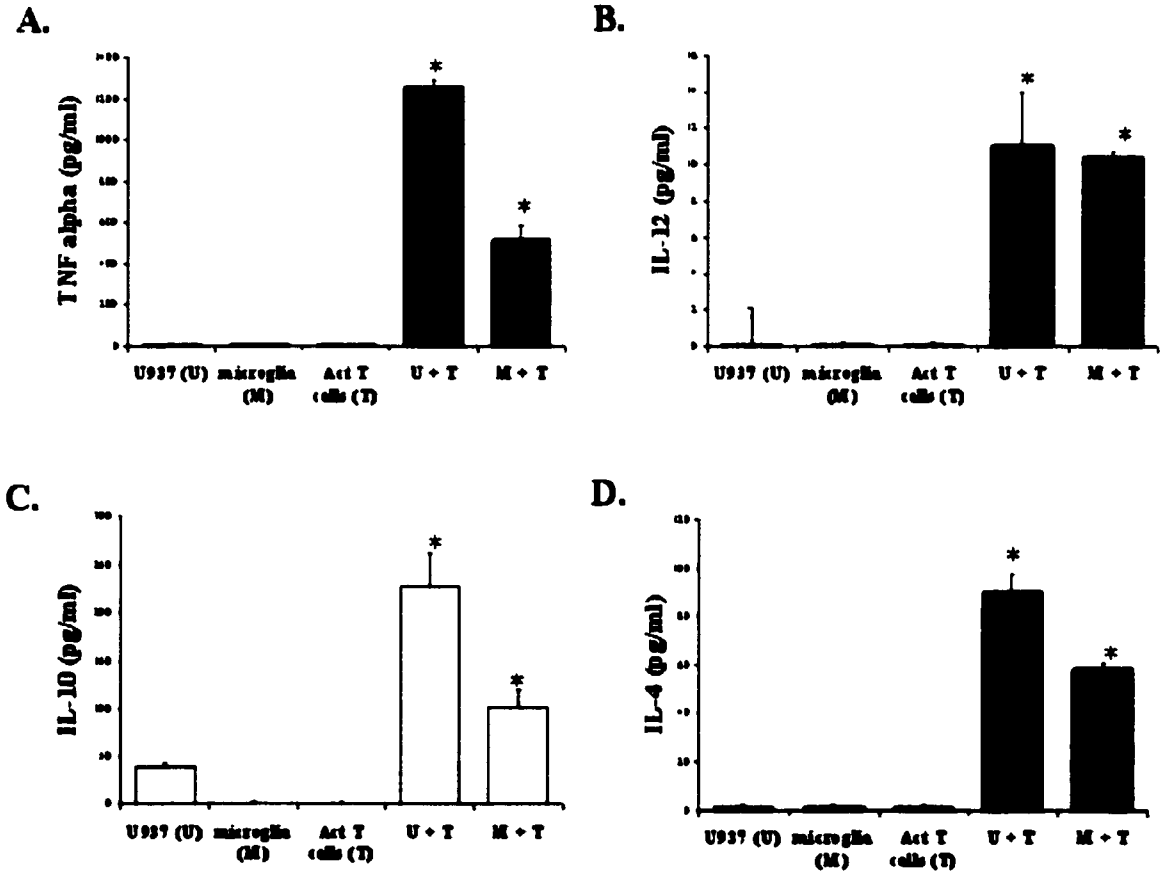


Figure 18. Cytokine production in PMA/IFNγ-treated U937-T cells co-cultures. PMA/IFNγ-treated U937, human adult microglia or OKT3-activated T cells produce undetectable levels of TNF-α (**A**), IL-12 (**B**), and IL-4 (**D**) under resting condition. PMA/IFNγ-treated U937 cells produce low, but significant, levels of IL-10 (**C**), while microglia or activated T cells produce negligible amount. Following co-culture of PMA/IFNγ-treated U937 cells and activated T cells, protein levels of all cytokines tested are significantly increased. Values are mean of triplicates analyses ± SEM. *$p<0.05$ compared to U937, microglia or T cells alone.

5.2.3. *Contact is necessary for cytokine production in U937-T cell co-cultures*

To ascertain whether the increase in cytokine levels generated from U937-T cell co-cultures, specifically TNF-α and IL-10, was due to soluble factors or cell contact interactions, two culture conditions were introduced. First, the conditioned medium (SUP) collected from cultures of activated T cells was added to PMA/IFNγ-treated U937 cells. Under this condition, TNF-α (Figure 19A) and IL-10 (Figure 19B) protein levels remained unchanged from control levels (U937 or Act T alone), suggesting that soluble factors play a minor role, if any, in inducing the production of cytokines in U937-T cell co-cultures.

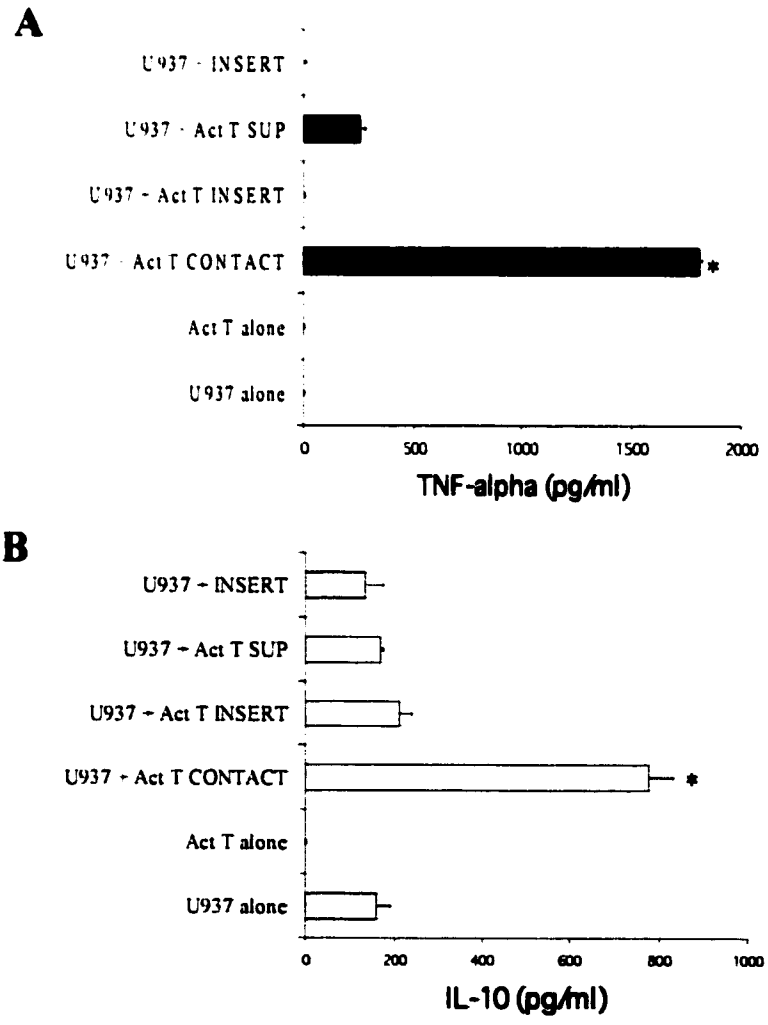


Figure 19. Direct contact between PMA/IFNγ-treated U937 cells and activated T cells is necessary for the production of TNF-α and IL-10. Conditioned medium of activated (Act) T cells cultures (SUP) added to PMA/IFNγ-treated U937 cells does not affect TNF-α (**A**) or IL-10 (**B**) production. Similarly, control levels of TNF-α and IL-10 remain unchanged when T cells are placed in a cell culture insert (INSERT). Significant increase of TNF-α and IL-10 levels are found only when PMA-treated T cells and activated T cells make contact in co-cultures. Values are mean of triplicates analyses ± SEM. *$p<0.05$ compared to their respective controls.

As a second approach, activated T cells were placed in cell culture inserts (INSERT), where they were in close proximity, but not contacting PMA/IFNγ-treated U937 cells. No change in TNF-α (Figure 19A) and IL-10 (Figure 19B) levels was detected when compared to control levels under this condition, suggesting that cell-cell contact between PMA/IFNγ-treated U937 and activated T cells is necessary for cytokines to be produced. Finally, when the two cell types were allowed to contact each other (U937 + Act T), significant levels of TNF-α and IL-10 were produced. Taken together, these results indicate that a contact-dependent mechanism is involved in cytokine production generated from U937-T cells co-cultures.

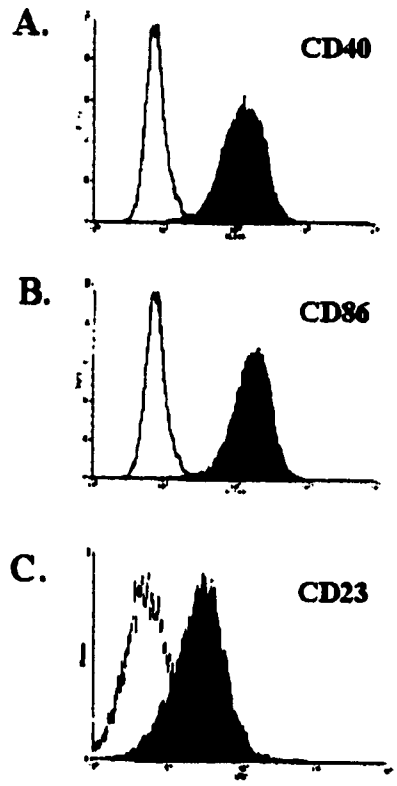


Figure 20. CD40, CD86 and CD23 expression on PMA/IFNγ-treated U937 cells. Flow cytometry analysis demonstrates that CD40 (**A**), CD86 (**B**), and CD23 (**C**) are expressed on the surface of U937 cells treated with PMA and IFNγ (black histogram). The IgG isotype staining controls are represented by the clear histogram. These results are representative of 3 separate experiments.

5.2.4. *PMA/IFNγ-treated U937 cells express VCAM-1, CD40 and CD23*

We have reported that human adult microglia express VCAM-1, CD40, CD86 (B7.2) and CD23, and that these molecules play a role in cytokine production generated from microglia-T cell interactions (Chapter 3 / Chabot et al, 1997; Chapter 4 / Chabot et al, 1999). To ensure that PMA/IFNγ-treated U937 cells also express those molecules, flow cytometry analyses were performed. Figure 20 demonstrates that CD40, CD86 and CD23 are expressed on the surface of PMA/IFNγ-treated U937 cells as shown by an increase in fluorescence when compared to IgG isotype control. We have previously demonstrated the presence of VCAM-1 on the surface of PMA/IFNγ-treated U937 cells (Chapter 6).

5.2.5. Effects of blockade of B7, CD40, and CD23 pathways on cytokine production in U937-T cell co-cultures

We have reported that the production of TNF-α generated from microglia-T cell co-cultures depends on interactions that involve at least 3 arms, namely VLA-4-VCAM-1, CD40L-CD40, and CD28/CTLA-4-B7. In the case of IL-10 production, CD40L-CD40, CD28/CTLA-4-B7 and CD23-CD11b/CD11c interactions were shown to be involved (Chabot et al, 1997; 1999). In the present study, we tested whether some of these interactions play a role in the production of TNF-α and IL-10 generated from U937-T cells interactions. To block the CD28/CTLA-4-B7 interaction, U937 cells were treated with recombinant human CTLA-4-Fc chimera protein, which binds both B7-1 and B7-2 with high affinity. Figure 21 shows that both TNF-α and IL-10 levels in T cell-U937 co-cultures were significantly reduced by such a treatment. Finally, CD23-dependent

interactions were blocked by treating with anti-CD23; this inhibited levels of IL-10 generated in PMA/IFNγ-treated U937-T cell interactions (Figure 21B). Importantly, as we reported for microglia (Chapter 4 / Chabot et al, 1999), the inhibition by anti-CD23 was found to be specific for IL-10 since TNF-α levels were not affected. (Figure 21A). To serve as a control, U937 cells were treated with IgG_1 isotype control. This treatment did not affect levels of TNF-α or IL-10 generated in U937- T cell co-cultures (Fig 21).

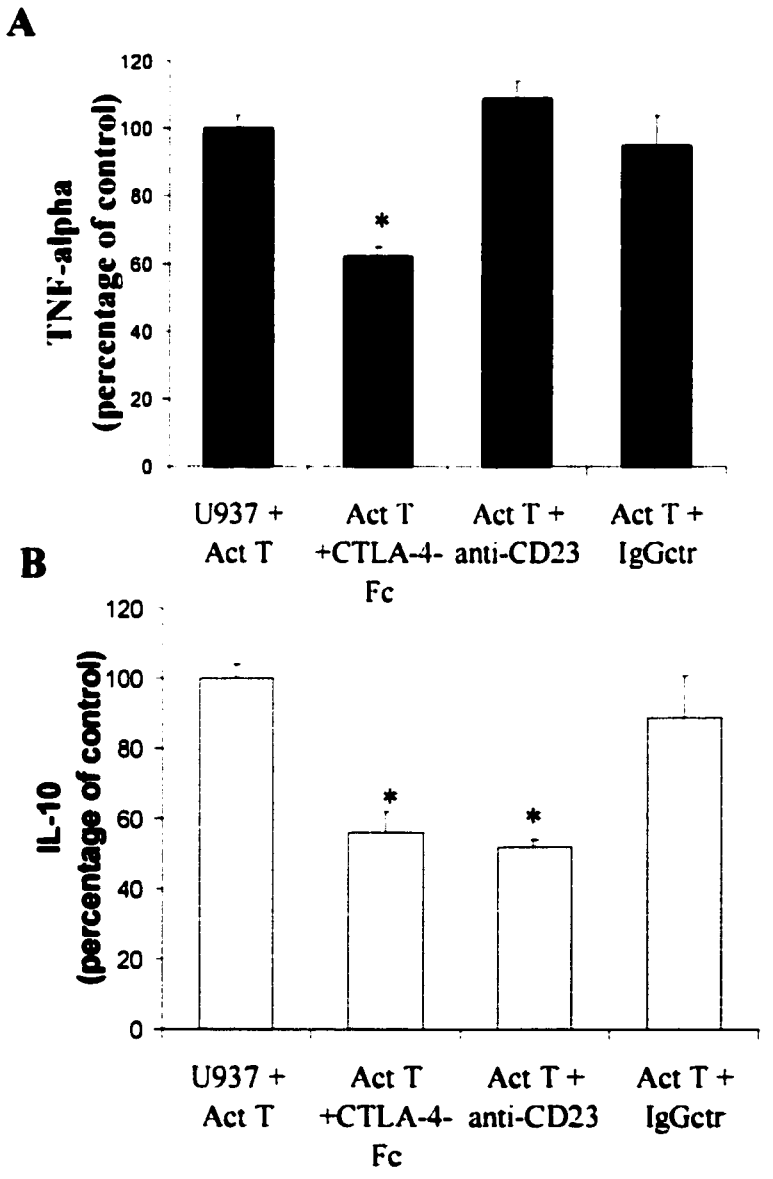


Figure 21: CTLA-4-Fc and CD23 affect TNF-α and IL-10 levels generated in PMA/IFNγ-treated U937-T cell co-cultures. Treatment of microglia with CTLA-4-Fc decreases TNF-α (**A**) and IL-10 (**B**) production in PMA/IFNγ-treated U937-T cell co-cultures, while anti-CD23 treatment inhibits the production of IL-10 but not that of TNF-α. Values are mean of triplicates analyses ± SEM. *$p<0.05$ compared to their respective controls.

5.2.6. *Morphological changes of PMA/IFNγ-treated U937-T cell co-cultures*

We have previously described morphological changes of microglia-T cell co-cultures (Chapter 4/Chabot et al, 1999). Microglia acquire various morphology *in vitro* as well as *in vivo*. In general, cultured resting human microglia tend to be bipolar (elongated) or ramified, and become ameoboid (rounded) when they are activated (Wiliams et al, 1992). On the other hand, in culture, activated T cells are found as single cells or as homotypic aggregates. When both microglia and T cells are cultured together, aggregates of T cells are found attached to the microglia and bipolar ramified microglia become ameoboid in shape (Chapter 4/Chabot et al, 1999).

Morphological changes observed in PMA/IFNγ-treated U937-T cell co-cultures resemble that observed in microglia-T cell-cultures. While the majority of PMA/IFNγ-treated U937 cells are bipolar in shape, their exposure to activated T cells cause cells to retract their processes and acquire an amoeboid morphology.

5.3. Discussion

Microglia are the immune effector cells of the CNS. They are thought to be the first cell type to respond to an insult inflicted upon the CNS, and, in this regard, have been referred to as "sensors of pathology" (Kreutzberg et al, 1996). When activated, microglia acquire macrophage-like functions including phagocytosis, activation of the respiratory burst, antigen presentation, and production of inflammatory mediators such as cytokines. Besides their immune functions, microglia may also regulate neurotrophism and regeneration of the CNS to an injury, but there is controversy whether activated microglia exert protective or cytotoxic functions. Both neurotrophic and neurotoxic

properties of microglia have been demonstrated. For example, it was shown that the conditioned medium of rat microglia promotes the survival and neurite extension of neurons (Nagata et al, 1993; Chamak et al, 1994), and that microglia can produce neurotrophic factors, such as nerve growth factor (NGF), neurotrophin-3 (NT-3), brain-derived neurotrophic factor (BDNF) and glial cell-derived neurotrophic factor (GDNF) (Mallat et al, 1989; Heese et al, 1998; Elkabes et al, 1996; Batchelor et al, 1999). On the other hand, other investigators demonstrated using neuronal/microglia co-cultures that microglia have neurotoxic functions by inducing neuronal injury when treated with LPS or IFNγ/β-amyloid peptide (McMillan et al, 1995; Meda et al, 1995). Thus, the biology of microglia requires further characterization.

Table 2.
Comparison of PMA/IFNγ-treated U937 and human adult microglia responses

Parameters tested	*PMA/IFNγ-treated U937*	*Human Adult Microglia*
Basal expression of cytokines		
TNF-α	-	-
IL-10	+	-
IL-4	-	-
IL-12	-	-
Interaction with Activated T cells		
TNF-α production	Significantly increased	Significantly increased
IL-10 production	Significantly increased	Significantly increased
IL-4 production	Significantly increased	Significantly increased
IL-12 production	Significantly increased	Significantly increased
Morphological changes	Bipolar/ramified to amoeboid	Bipolar/ramified to amoeboid
Expression of Adhesion Molecules		
VCAM-1	+	+
CD40	+	+
CD86	+	+
CD23	+	+
Effects of blocking reagents on cytokine production		
CTLA-4-Fc (TNF-α/IL-10)	Blocks	Blocks
Anti-CD23 (IL-10 only)	Blocks	Blocks

+ denotes the presence while - represents the absence of the parameter being determined

To better understand the biology of microglia, an *in vitro* model is helpful. Several groups now utilize microglia cultured from different species. Human adult microglia have been isolated from resected brain specimens of patients undergoing surgery to treat intractable epilepsy (Williams et al, 1992; Yong and Antel, 1997). Due to the limited supply of brain specimens, it would be advantageous to identify human cell lines that mimic the properties of microglia. Such cell lines would allow limitless biochemical, molecular and immunological studies to be performed to facilitate our understanding of microglia biology.

The results of this chapter demonstrate that the pro-monocytic cell line, U937, can serve as a model of human adult microglia cells when cells are sequentially treated with PMA and IFNγ (Table 2). PMA/IFNγ-treated U937 cells differentiate and acquire a microglia-like morphology, and express VCAM-1, CD40, CD86 and CD23, as has been shown on microglia. Like microglia, PMA/IFNγ-treated U937 cells can interact with activated T lymphocytes to produce inflammatory cytokines, such as TNF-α and IL-10. As described for microglia-T cell interactions (Chapter 4/Chabot et al, 1999), B7 co-stimulatory molecules regulate TNF-α and IL-10 production generated from U937-T cells interactions, while CD23 is selectively involved in the production of IL-10. By demonstrating that microglia and PMA/IFNγ-treated U937 cells are similar, we provide supporting evidence that microglia are closely related to macrophages. Indeed, microglia are often referred as the macrophages of the CNS due to a number of reasons. First, there is substantial evidence to support the theory that, like macrophages, microglia are of mesodermal origin, and that they derive from bone marrow cell precursors (reviewed in Rezaie and Male, 1999). Second, microglia and macrophages perform similar functions, including

phagocytosis, antigen presentation, and secretion of inflammatory molecules. Third, molecules serving as markers of microglia are also expressed on macrophages, which makes distinguishing them *in vivo* difficult, on the basis of antigenic properties.

Cytokines are important regulators of inflammation. Although most cells can produce cytokines, macrophages are a major source. Mechanisms involved in cytokine production by macrophages are not entirely understood, but cognate interactions with activated T cells appear to be involved (Stout and Suttles, 1997). For example, it was shown that contacts of macrophages with T cells induce macrophage effector functions, such as TNF-α and IL-1β synthesis, and nitric oxide production, and that this was mediated by CD40-CD40L interactions (Stout et al, 1996; Wagner et al, 1994; Tao and Stout, 1993). Membrane TNF-α was also shown to contribute to T cell contact-dependent signaling of macrophages by mediating the production of TNF-α (Suttles et al, 1994). The results of the present study further demonstrate that cognate interactions between PMA/IFNγ-treated U937 and OKT3-activated T cells results in the production of cytokines, a hallmark of macrophage activation. Both pro- (TNF-α, IL-12) and anti-inflammatory (IL-10, IL-4) cytokines are generated from PMA/IFNγ-treated U937-T cell interactions, suggesting for the first time that contact-dependent signals by activated T cells regulate not only pro-inflammatory, but also anti-inflammatory, functions of macrophages.

It should be noted that an immortalized microglia cell line has been derived from the fetal human brain (CHME) (Atanassov et al, 1995; de Gannes et al, 1998). In order to determine whether the CHME cell line could serve as a model of human adult microglia, we have performed experiments similar to those using PMA/IFNγ-treated U937 cells. In contrast to U937-T cells interactions, we found that the interaction of CHME cells with

activated T lymphocytes does not generate the production of TNF-α or IL-10. To date, the PMA/IFNγ-treated U937 cell line is the best model for human adult microglia that we have identified.

In summary, this chapter shows that U937 cells sequentially treated with PMA and IFNγ interact with activated T lymphocytes in a manner similar to that described for human adult microglia. I propose that PMA/IFNγ-treated U937 cells can serve as a model of microglia.

CHAPTER SIX

VCAM-1 is a Signaling Receptor Involved in Macrophage Activation

6.1 Introduction

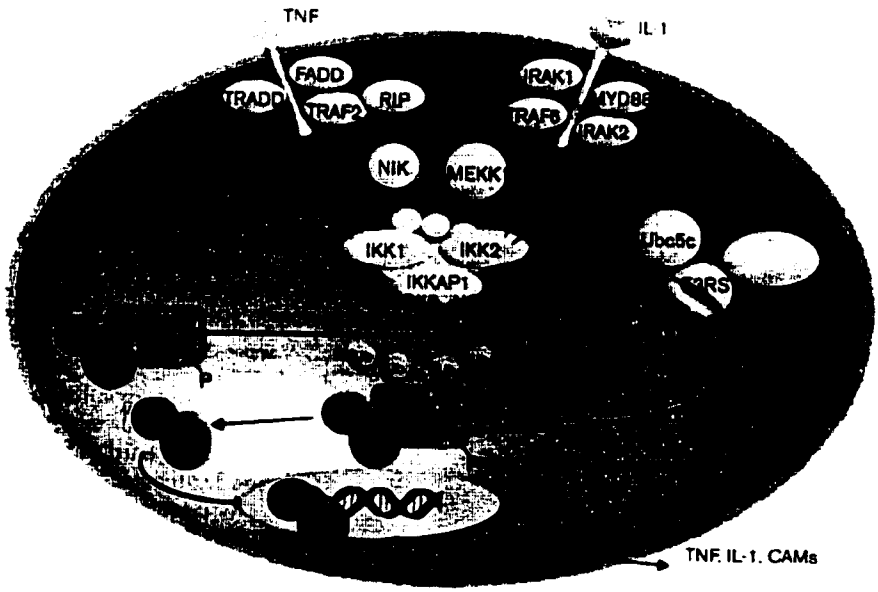
Vascular cell adhesion molecule-1 (VCAM-1), a member of the immunoglubulin (Ig) superfamily proteins, is an adhesion molecule expressed on endothelial cells that plays an important role in the recruitment of leukocytes to sites of inflammation. VCAM-1 is also expressed on other cell types such as follicular dendritic cells (Huang et al, 1995), macrophages (Walton et al, 1994), macrophage-like cells, including microglia (Chabot et al, 1997), and astrocytes (Lee and Benveniste, 1999). The functional significance of VCAM-1 expression on these cells is unclear. To date, three ligands of VCAM-1 have been identified. These are the $\alpha 9\beta 1$ integrin, which is selectively expressed on neutrophils (Taooka et al, 1999), and $\alpha 4\beta 1$ (VLA-4) and $\alpha 4\beta 7$ integrins, expressed on most leukocytes, including lymphocytes, monocytes, basophils, eosinophils and neutrophils (reviewed in Foster, 1996; Reinhartd et al, 1997). Due to this broad range of cellular expression, it is likely that cell-cell interactions involving VCAM-1 and its ligands regulate not only leukocyte migration, but also various other immune responses, such as antibody production, T cells activation, allergic reactions, and macrophage activation. Indeed, in a previous study, we demonstrated that blockade of the VCAM-1/VLA-4 pathway during human microglia-T cell interactions reduced the microglial production of TNF-α (Chapter 3 / Chabot et al, 1997), but not IL-10 (Chapter

4 / Chabot et al, 1999). These results suggest that VCAM-1 expressed on microglia may serve to initiate a signaling role in regulating TNF-α production.

TNF-α elicits a wide spectrum of systemic and cellular responses, including fever, shock, tissue injury, tumor necrosis, anorexia, induction of other cytokines and immunoregulatory molecules, cell proliferation, differentiation, and apoptosis (reviewed in Tracey and Cerami, 1993). Moreover, TNF-α has been implicated in many inflammatory conditions, such as Crohn's disease, rheumatoid arthritis, septic shock, cachexia, AIDS and multiple sclerosis (reviewed in Beutler, 1999). Since the development of therapy for these diseases may be aided by reducing TNF-α expression, it is important to understand the mechanisms involved in TNF-α production. At the molecular level, the inducibility of TNF-α gene transcription is tightly regulated, and was shown to be dependent on the transcription factor nuclear factor κB (NF-κB) (Shakhov et al, 1990; Drouet et al, 1991).

Under resting conditions, NF-κB exists in the cytoplasm of the majority of cell types as homodimers or heterodimers of structurally related proteins belonging to the NF-κB family, which includes Rel A (p65), c-Rel, RelB, NF-κB1 (p105/p50), and NF-κB2 (p100/p52). In its inactive state, NF-κB is bound to inhibitory proteins of the IκB family, namely IκB-α, IκB-β, IκB-γ, or IκB-ε. The binding of IκB family members regulates the subcellular localization of NF-κB by masking its nuclear localization signal (NLS) located near the carboxyl terminus of the Rel-homology domain (Mercurio and Manning, 1999). In response to a multitude of stimuli associated with stress and injury, the activation of NF-κB is achieved through the degradation of IκB proteins which allows the translocation of NF-κB to the nucleus (Figure 22). The degradation of IκB proteins is

tightly regulated by the activation of the IκB kinase-1 (IKK-1 or IKK-α) and IκB kinase-2 (IKK-2 or IKK-β) (Regnier et al, 1997; Mercurio et al, 1997; DiDonato et al, 1997; Zandi et al. 1997; Woronicz et al, 1997), which phosphorylates IκB proteins at specific amino terminal serine residues. For example, in the case of IκB-α, Ser32 and Ser36 becomes phosphorylated (Ghosh et al, 1990). Phosphorylated IκB proteins are then ubiquitinated by an E3 ubiquitin ligase (Yaron et al, 1997), targetting them for their subsequent degradation by the 26S proteosome (Chen et al, 1995). When IκBs are degraded, the NLS of NF-κB is exposed, which permits its interaction with the nuclear import machinery and its subsequent translocation to the nucleus (Henkel et al, 1992). Once in the nucleus, NF-κB acts as a transcription factor by binding to the κB enhancer region located in the promoter region of target genes, including TNF-α, IL-1, E-Selectin, and VCAM-1 (Ziegler-Heitbrock et al, 1993; Whitley et al, 1994; and Neish et al, 1995).



Adapted from Mercurio et al, 1999
Current Opinion in Cell Biology

Figure 22: Schematic representation of NF-κB pathway.

In this study, we have used the NF-κB cascade to address if VCAM-1 could serve a signaling role for TNF-α production by mononuclear phagocytes. We report that the ligation of VCAM-1 on the surface of differentiated U937 cells, a model for human macrophages, leads sequentially to the phosphorylation of IκB-α at serine32, the degradation of IκB-α, the translocation of NF-κB into the nucleus and the induction of TNF-α gene transcription. These findings indicate a novel functional role for VCAM-1 in macrophage activation as a signaling receptor.

6.2. Results

6.2.1. *VCAM-1 is expressed on the U937 macrophage cell lines*

To verify the presence of VCAM-1 on the surface of U937 cells, flow cytometry analyses were performed. Under resting conditions, the VCAM-1 surface expression on U937 cells remained low (Figure 23A). However, following 48 hours of treatment with PMA, a potent activator of protein kinase C (PKC), an increase in VCAM-1 surface expression levels was observed (Figure 23B). Moreover, treatment with 100 IU/ml of IFN-γ for 24 hours further enhanced this PMA-induced level of VCAM-1 surface expression (Figure 23C). Thus, all VCAM-1 cross-linking experiments were performed on cells pre-treated sequentially with PMA for 2 days, and IFN-γ for another day.

Immunocytochemistry was used as another approach to detect VCAM-1 expression on U937 cells. Following IFN-γ treatment of PMA-treated U937 cells, an increase in VCAM-1 staining was observed. Pre-absorption of the VCAM-1 antibody with recombinant VCAM-1 attenuated the VCAM-1 positive signal, which demonstrates the specificity of the VCAM-1 antibody used in our experiments.

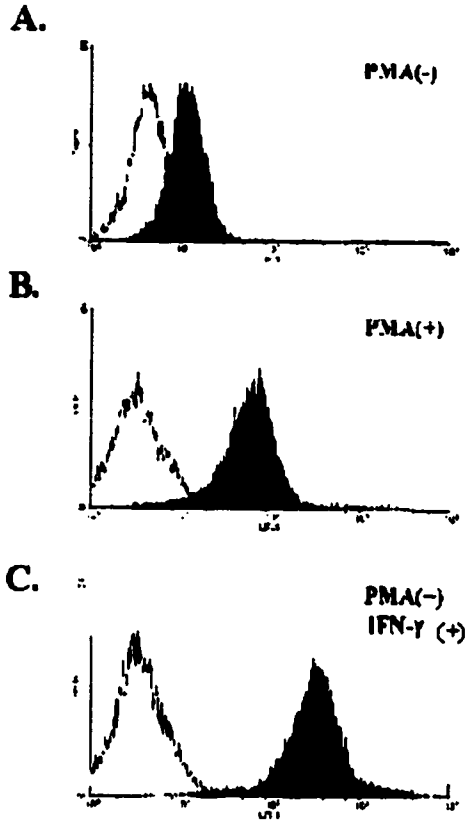


Figure 23: VCAM-1 is expressed on the surface of U937 cells. VCAM-1 is expressed at low levels on the surface of unmanipulated U937 cells (A). After 2 days of treatment with PMA, VCAM-1 surface expression is increased (B), and this is further elevated by treatment with IFN-γ (C). These histograms are representative of results obtained in 3 separate experiments.

6.2.2. *Ligation of VCAM-1 leads to the phosphorylation and degradation of IκB-α*

IκB-α is a 35-37 kDa protein that inhibits the p50/p65 NF-κB complex or Rel protein (Kerr et al, 1991; Davis et al, 1991; Haskill et al, 1991); this isoform is thought to be a stronger inhibitor of NF-κB *in vivo* than IκB-β (Tran et al, 1997). Using Western blot analyzes to detect the presence of phosphorylated (Ser32) IκB-α, we found an increase of phosphorylated IκB-α by 5-15 minutes of cross-linking VCAM-1 on PMA/IFNγ-treated U937 cells (Figure 24A). In correspondence, lower levels of IκB-α proteins was detected 15-30 minutes after the ligation of VCAM-1 on U937 cells, when compared to non-ligated PMA/IFNγ-treated U937 cells (Figure 24B). These results suggest that the IκB-α protein was degraded following the ligation of VCAM-1. The observation that IκB-α protein level is reestablished to control levels at 60 minutes

indicates that the re-synthesis of IκB-α protein occurs rapidly following VCAM-1 cross-linking, as has been shown in the case of LPS treatment (Legrand-Poels et al, 1997). Indeed, Velasco et al (1997) demonstrated that LPS induced the rapid degradation of IκB-α in peritoneal macrophages, and that this was followed by a recovery of IκB-α protein level 1 hour following LPS administration.

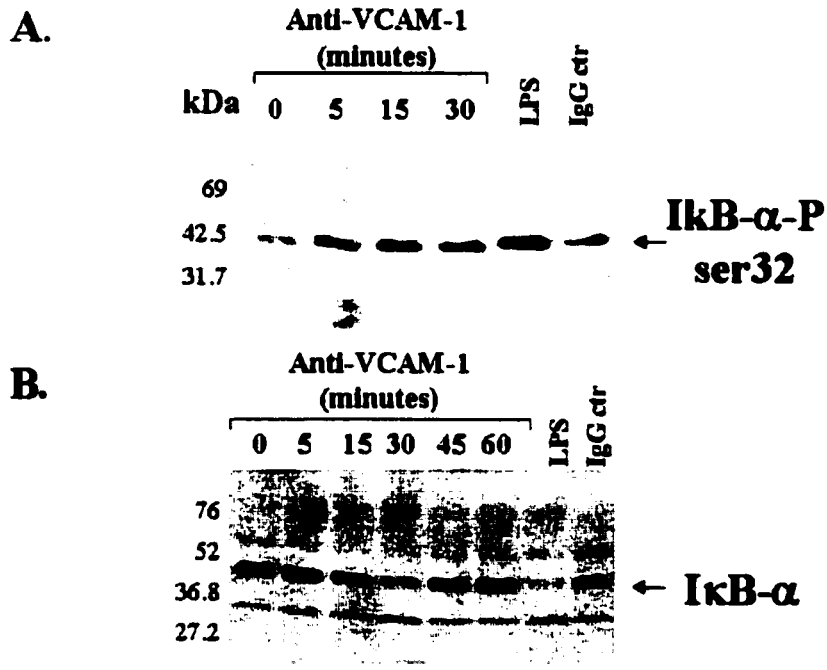
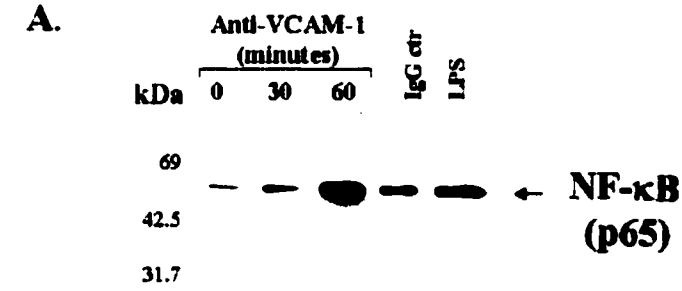


Figure 24: Proteins levels of IκB-α and its phosphorylated form after VCAM-1 cross-linking. Levels of IκB-α-Ser32-phosphorylated protein increase shortly after VCAM-1 cross-linking in U937 cells (A), while the levels of IκB-α protein decrease (B). IgG isotype control (ctr) was used as a negative control while LPS served as a positive control. These blots are representative of results obtained in 3 experiments.

IκB-β, a 45 kDa protein, has been found to regulate the persistent activation of NF-κB (Thompson et al, 1995) and it is constitutively phosphorylated and resynthesized as a hypophosphorylated form (Weil et al, 1997). The presence of IκB-β was detected in U937 by immunocytochemistry and Western Blot analysis, but VCAM cross-linking did

not affect the levels of IκB-β. In addition, LPS treatment did not cause the degradation of IκB-β. These results support those obtained in endothelial cells where LPS-induced NF-κB activation was shown to involve the degradation of IκB-α but not that of IκB-β (Zern et al, 1998). The degradation of IκB-γ was not examined since it is predominantly expressed in lymphoid cells (Inoue et al, 1992), and IκB-ε was not detected in our samples.



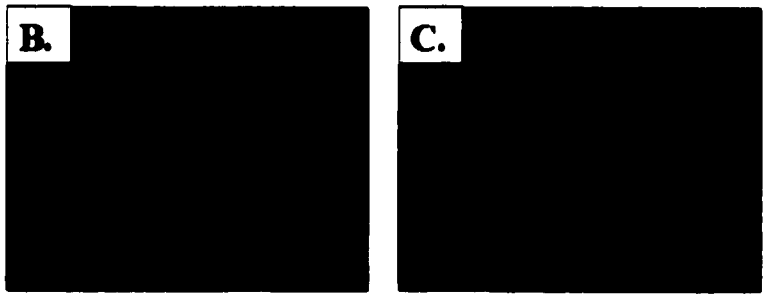


Figure 25: Nuclear translocation of NF-κB following VCAM-1 cross-linking. Increased NF-κB (p65) protein level is found in nuclear extracts of U937 cells 30-60 minutes after ligation of VCAM-1 (A). Immunocytochemistry demonstrates the cytoplasmic localization of NF-κB (p65) in non-ligated U937 (B), and its nuclear localization in anti-VCAM-1 treated U937 cells (C).

6.2.3. *The translocation of NF-κB into the nucleus of macrophages occurs following the VCAM-1 cross-linking*

Because TNF-α transcription was shown to be regulated by p50/p65 heterodimers (Collins et al, 1995), the level of p65 (NF-κB/Rel A) in nuclear extracts of cells was

assayed by Western blot analysis. Sixty minutes after treatment with anti-VCAM, an elevated level of NF-κB (p65) was detected in the nuclear extracts obtained from U937 cells (Figure 25A). In agreement with findings by Frankenberger et al (1994), a constitutive level of nuclear NF-κB (p65) in U937 cells was observed.

Immunocytochemistry was used as another approach to assay for the nuclear translocation of NF-κB following ligation of VCAM-1. Figure 25C demonstrates that NF-κB (p65) localizes in the nucleus of PMA-treated U937 macrophage cells 1 hour following VCAM-1 cross-linking, as compared to its cytoplasmic localization observed in resting conditions of U937 cells (Figure 25B)

To assess the functional ability of translocated nuclear NF-κB to bind to its consensus sequence on DNA, electromobility shift assays were performed. Nuclear extracts were obtained from PMA-treated U937 cells cross-linked with the VCAM-1 antibody, and were exposed to dioxygenin (Dig)-labeled oligonucleotides encoding the NF-κB DNA binding sequence. Dig-positive double bands (identified by the arrow in Figure 26) was detected for NF-κB by immunoblotting, and its intensity was increased in anti-VCAM and LPS-treated cells compared to untreated or IgG_1 control treated cells. The NF-κB-oligonucleotide complex formation in nuclear extracts of anti-VCAM-1 treated cells was abolished by the addition of a 100-fold molar excess of unlabeled oligonucleotides, or by mutant NF-κB oligonucleotides, in which the guanine residue essential for the binding of NF-κB has been replaced by a cytosine residue (Figure 26).

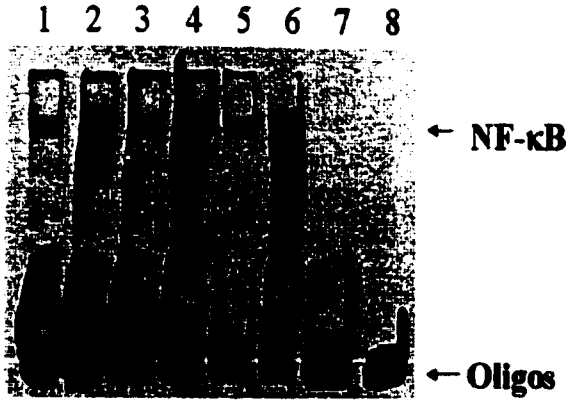


Figure 26: Translocated NF-κB binds to its consensus sequence on DNA. Electrophoretic mobility shift assay (EMSA) shows an increase in NF-κB-oligonucleotide complex (upper 2 arrows) in nuclear extracts of U937 cells cross-linked with anti-VCAM-1 (lane 2) when compared to levels of NF-κB binding in untreated U937 (lane 1). IgG_1 isotype control was used as a negative control (lane 3) while LPS (lane 4) served as a positive control. Mutant oligonucleotide containing the G→C mutation in the NF-κB binding site (lane 5) or 100 fold excess of unlabeled oligonucleotides (lane 6) competed out the NF-κB-oligonucleotide formation detected in nuclear extracts of VCAM-1 cross-linked U937 cells. NF-κB oligos and mutant oligos alone are shown in lanes 7 and 8 respectively. This EMSA data is representative of results obtained in 3 separate experiments.

6.2.4. *Cross-linking of VCAM-1 induces the transcription of TNF-α in macrophages*

In order to test the hypothesis that VCAM-1 cross-linking could lead to the transcription of NF-κB dependent genes, levels of TNF-α mRNA were determined by semi-quantitative RT-PCR. A time course assay demonstrated that TNF-α mRNA levels increased 1 hour following VCAM-1 cross-linking when compared to levels detected in untreated control (Figure 27). As a control, levels of IL-10 transcripts were also measured. No increase of IL-10 mRNA was observed following VCAM-1 cross-linking (data not shown). As has been proposed by others (Frankenberger et al, 1994), the low detectable level of TNF-α mRNA transcripts observed in untreated U937 macrophage

cells may be due to the constitutive nuclear NF-κB which appears to be functionally active in these cells (Figure 26).

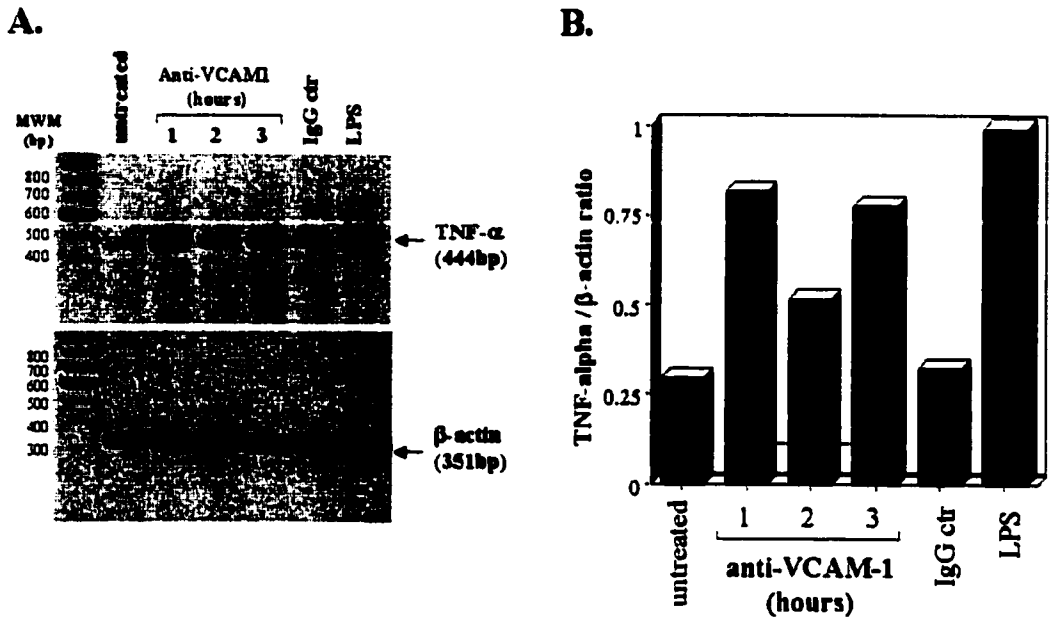


Figure 27: VCAM-1 cross-linking induces TNF-α transcription. RT-PCR analysis demonstrated that TNF-α mRNA levels observed in U937 cells increase 1 hour following VCAM-1 ligation. IgG_1 isotype control (ctr) was used as a negative control, whereas LPS served as a positive control for the induction of TNF-α transcription. Panel A is a typical PCR reaction profile while in panel B, the amount of TNF-α transcript has been normalized to that of β-actin. These results have been reproduced 3 times.

6.3. Discussion

Macrophages play a central role in the regulation of both innate and adaptive immunity due to their ability to produce a wide variety of inflammatory molecules, and because they can act as phagocytic cells and antigen-presenting cells. However, in order for macrophages to perform these functions, their activation is required. The mechanisms involved in macrophage activation are not completely understood, but several activators of macrophages have been described. These include interferon (IFN)-γ, bacterial lipopolysaccharide (LPS), and cognate interactions with activated T lymphocytes. An

important outcome of macrophage activation is the release of TNF-α, which has been implicated in the pathogenesis of various inflammatory diseases. Therefore, a better understanding of the mechanisms involved in macrophage activation and the subsequent production of TNF-α is helpful for the development of novel therapies for these diseases.

The results of this chapter provide two new insights into macrophage biology; first, we reveal for the first time that VCAM-1 on macrophages can signal, and second, this signaling activates the NF-κB pathway which may result in the production of TNF-α (Figure 28). Indeed, taken together, Figures 24 to 27 shows that the event of NF-κB activation occurs in a temporal manner following ligation of VCAM-1. IκB-α initially becomes phosphorylated, followed by its degradation, and then NF-κB is translocated to the nucleus where it initiates the NF-κB-dependent TNF-α gene transcription. The magnitude of the VCAM-1 activation of the NF-kB pathway is comparable to that obtained with the potent macrophage activator, LPS. Collectively, the results suggest a new mechanism by which TNF-α production is regulated in activated macrophages.

VCAM-1 is an adhesion molecule found on endothelial cells, and is best known for its adhesive role in leukocyte recruitment. Given our current results, one may propose that VCAM-1 is also involved in endothelial cells (EC) activation by inducing the NF-κB pathway leading to the production of NF-κB-dependent genes. Indeed, there is evidence to suggest that NF-κB is a crucial element involved in the molecular mechanisms that regulate gene expression during EC activation, which includes cytokines such as TNF-α and IL-1, and adhesion molecules such as E-selectin, intercellular adhesion molecule-1 (ICAM-1) and VCAM-1 itself (Mantovani et al, 1997). VCAM-1 ligation on endothelial cells, through its integrin ligands expressed on infiltrating leukocytes, may result in the

induction of adhesion molecules expression guided by the activation of NF-κB, which futher leads to an increase of leukocyte recruitment and in the overall amplification of the inflammatory response. Indeed, two reports demonstrated that the cross-linking of VCAM-1 on endothelial cells activates the phosphoinositide pathway and triggers Ca2+ mobilization (Ricard et al, 1997), and that it induces a transient increase in cytosolic free calcium concentration ([Ca2+]i). (Lorenzon et al, 1998).

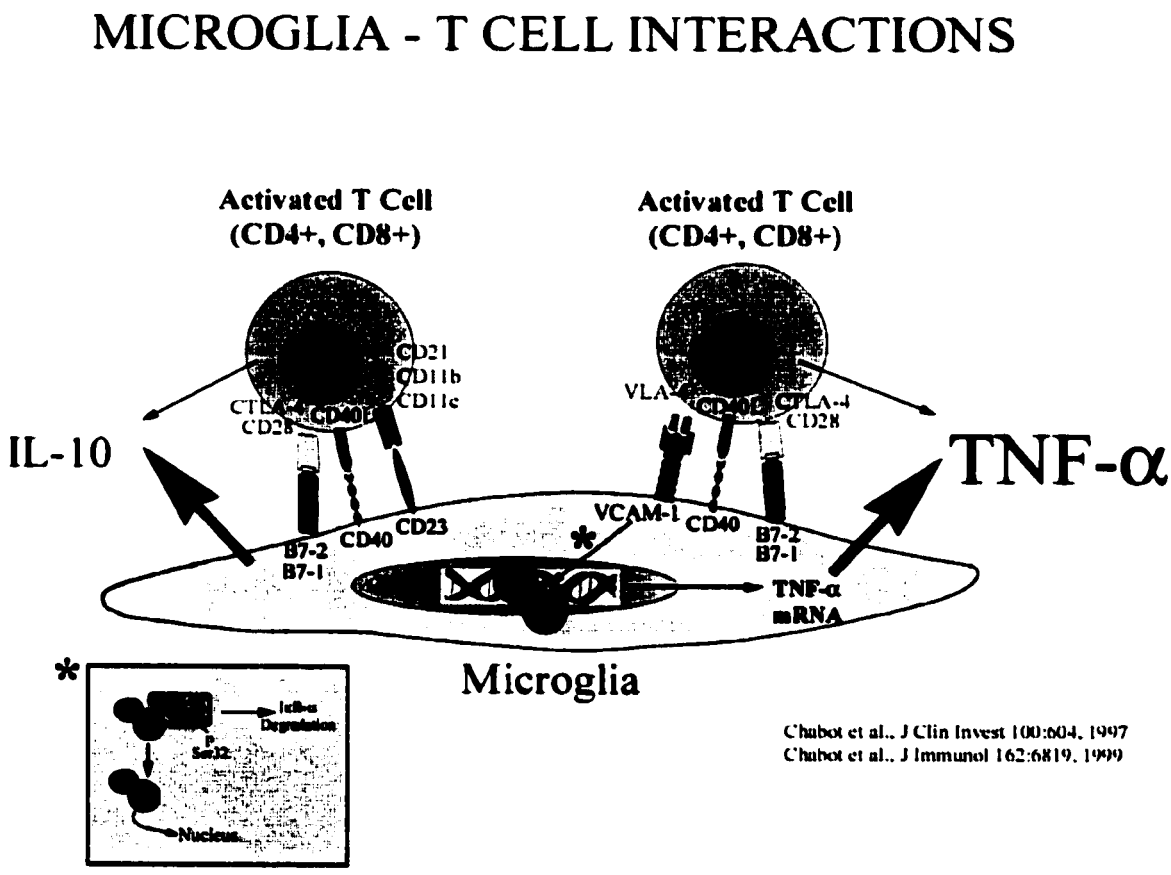


Figure 28: Summary of Results of Objective 4.

The molecular events taking place at the plasma membrane that are associated with VCAM-1-induced signaling remain to be determined. In endothelial cells, two forms of VCAM-1 are expressed, namely a glycophosphatidylinositol (GPI)-anchored form,

and a non-anchored isoform (Kinashi et al, 1995). It is not yet known which form(s) of VCAM-1 is expressed predominantly on macrophages. Because GPI-anchored proteins do not have a membrane-spanning domain, it has been thought that they cannot transmit a signal into the cell. However, recent evidence suggests that a mechanism by which GPI-anchored proteins mediates signal transduction is through the formation of membrane microdomains named caveolae, which helps bring signaling accessory proteins together, such as tyrosine kinases, and G-proteins (Horejsi et al, 1999). Studies performed on other GPI-anchored proteins, which include the LPS receptor CD14, the neutrophil IgG receptor CD16d, LFA-3 (or CD58), CD44, the complement proteins CD55 and CD59, and the receptor tyrosine kinase Ret (GDNF receptor), demonstrate that their ligation on the cell surface results in the activation of signaling pathways (Horejsi et al, 1999; Saarma and Sariola, 1999). Signals induced include a transient increase in calcium influx, and the activation of protein tyrosine kinase (PTKs) and PKC (Illangumaran et al, 1998; Tachado et al, 1997). Because there is evidence to show that some of these signals activate IKK (Trushin et al, 1999), it is possible that through VCAM-1-induced formation of GPI microdomains stimulates PTK and PKC leading to the activation of the NF-κB pathway.

The study of the mechanisms induced from VCAM-1-dependent interactions is important since it involved in a variety of immune responses. The ligands of VCAM-1, namely VLA-4 ($\alpha4\beta1$), $\alpha4\beta7$ and $\alpha9\beta1$, are expressed on most inflammatory cells which includes T lymphocytes, B lymphocytes, eosinophils, basophils and neutrophils (reviewed in Foster, 1996). This suggests that VCAM-1-induced signaling may be implicated in a variety of cell-cell interactions that are involved in an immune response,

which may include antibody production, T cells activation, phagocytosis, leukocyte trafficking and allergic reactions.

Our evidence that VCAM-1 cross-linking induces TNF-α gene transcription is relevant to the important role that TNF-α plays in the regulation of a multitude of systemic and cellular responses (reviewed in Tracey and Cerami, 1993). For example, it is known that TNF-α is involved in the regulation of physiological conditions, such as sleep (Krueger et al, 1998) and fever (Luheshi, 1998) via direct actions on the brain. Two cell types of the CNS have been shown to be a source of TNF-α, namely astrocytes and microglia. Correspondingly, the expression of VCAM-1 by these two types of CNS cells has also been demonstrated (Chabot et al, 1997; Lee and Benveniste, 1999). Therefore, it is possible that VCAM-1 is also implicated in the regulation of sleep and fever, where the interaction between VCAM-1-expressing CNS cells with infiltrating leukocytes could lead to the central production of TNF-α.

Finally, the results of this chapter are also relevant to the understanding of disease conditions in which VCAM-1 is implicated (reviewed in Isobe et al, 1998). The expression of VCAM-1 is increased in a variety of autoimmune diseases, such as multiple sclerosis (MS), rheumatoid synovitis (Masuyama and Kitani, 1996), rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), Sjogren's syndrome, autoimmune thyroid disease, and diabetes mellitus (reviewed in McMurray, 1996). Furthermore, a role for VCAM-1 in transplantation has also been proposed. In a study by Schlegel et al (1995), it was shown that anti-VCAM-1 prevented by 50% the incidence of murine graft-versus-host disease, and significantly increased the survival of transplant recipients. In addition, there is evidence to suggest that VCAM-1 is implicated in the pathogenesis of infectious

diseases. Indeed, it was found that VCAM-1 can act as a cellular receptor for the encephalomyocarditic virus (Huber, 1994) as well as for the malaria-causing organisms plasmodium falciparum (Ockenhouse et al, 1992). The NF-κB-induced signaling by VCAM-1 may be a pathogenic event in these diseases conditions.

In summary, we demonstrate a novel role for VCAM-1 as a signaling receptor in macrophages that lead to the NF-κB-regulated transcription of TNF-α. The observations shed new insights into VCAM-1 in normal physiology and in disease states.

CHAPTER SEVEN

Effects of Interferonβ and Glatiramer Acetate on Cytokine Production from Microglia-T cells Interactions

7.1 Effect of IFNβ

7.1.1 *Introduction*

Multiple Sclerosis is a chronic inflammatory disease of the central nervous system (CNS), which is characterized by demyelination, axonal loss, and neurological impairments. In recent years, the treatment of MS has been significantly improved with the discovery that recombinant interferon-beta (IFNβ) has efficacy in treating the disease. Several multi-center clinical trials demonstrated that two forms of recombinant IFNβ, namely IFNβ-1a (Avonex® and Rebif®) and IFNβ-1b (Betaseron®), decrease the number of relapses in relapsing-remitting MS, and reduce the frequency of lesion formation detected by magnetic resonance imaging (IFN MS Study Group, 1993; IFN MS Study Group and UBC MS/MRI Analysis Group, 1995; Paty et al, 1993; Rudick et al, 1997; Jacobs et al, 1996). Although the mechanisms by which IFNβ is efficacious in MS remain unclear, there is evidence that IFNβ directly modulates the function of immune cells. Several biological activities were shown to be inhibited by IFNβ, including the proliferation of lymphocytes and antigen presentation (reviewed in Yong et al, 1998; Rep et al, 1996). Moreover, there is evidence to suggest that IFNβ affects the ability of leukocytes to migrate by modulating the expression of adhesion molecules, such as very late antigen-4 (VLA-4) (Calabresi et al, 1997), and by inhibiting the production of matrix metalloproteinases (MMPs) involved in the degradation of the basal lamina (Lou et al,

1999; Stuve et al, 1996; Leppert et al, 1996). IFNβ also appears to affect the apoptotic machinery. For instance, Rep and colleagues (1999) reported that IFNβ enhances Fas receptor (CD95) expression on T cells of MS patients, suggesting that IFNβ may induce the apoptotic death and the elimination of autoreactive T cells.

Cytokine dysregulation is a feature of MS. Disease progression in MS correlates with increased levels of pro-inflammatory cytokines, such as TNF-α, IFN-γ, IL-1β and IL-12, and decreased levels of anti-inflammatory cytokines, such as IL-10 and IL-4 (Sharief et al, 1991; Selmaj et al, 1991; Huang et al, 1999; van Bozel-Dezaire et al, 1999). There is much evidence for an effect of IFNβ on cytokine production. Levels of IL-12 (Rohowsky-Kochan et al, 1999), IFN-γ (Gayo et al, 1999) and TNF-α (Brod et al, 1996) were found to be decreased in peripheral blood mononuclear cells (PBMC) from patients treated with IFNβ. In contrast, IFNβ has an enhancing effect on the production of anti-inflammatory cytokines, such as IL-10. It has been reported that IFNβ enhances the secretion of IL-10 by activated T lymphocytes by nearly 4 fold (Rep et al, 1996). In MS patients, levels of IL-10 in the plasma or PBMCs are elevated in patients treated with IFNβ compared to those that are untreated (Rep et al, 1999;Ozenci et al, 1999). Furthermore, elevated IL-10 levels have also been detected in the CSF of MS patients on IFNβ treatment, which was associated with a favorable therapeutic response (Rudick et al, 1998; Monteney et al, 1999). The mechanisms by which IL-10 elevation occurs within the CNS remain unclear.

The infiltration of activated T lymphocytes into the CNS of MS patients is a significant event of MS. The majority of these lymphocytes are non-antigen specific, and they are thought to play an important role in the amplification of local CNS immune

responses (Sedgwick et al, 1987; Cross et al, 1993). Following their extravasation, activated T lymphocytes localize in proximity to microglia, the macrophage-like cells of the CNS. The interaction between activated T lymphocytes and microglia is a potential pathogenic event in MS, because it results in the production of the oligodendrocyte-toxic cytokine, tumor necrosis factor-α (TNF-α) (Chapter 3 / Chabot et al, 1997). It is unclear whether IFNβ can penetrate the blood brain barrier. Thus, the pretreatment of T cells with IFNβ in microglia-T cell interactions represents a realistic model, since it simulates the exposure of T cells to IFNβ in the systemic circulation, and the subsequent entry of these into the CNS parenchyma where they encounter microglia. In this study, we investigated whether IFNβ affects the production of TNF-α and IL-10 from microglia-T cell interactions, thereby providing a mechanism by which IFNβ treatment may result in the production of IL-10 in the CNS.

7.1.2. *Results*

7.1.2A. *IFNβ inhibits TNF-α production*

In Chapter 3, it was shown that TNF-α production from microglia-T cell co-cultures is dependent on VLA-4/VCAM-1 interaction. Because human IFNβ can downregulate the expression of VLA-4 on human peripheral blood monocytes (Soilu-Hanninen et al 1995), we examined whether IFNβ inhibits the production of TNF-α in T cell-microglia co-cultures through its effect on T lymphocytes. Figure 29A demonstrates that the pretreatment of T cells with IFNβ reduced the production of TNF-α in a dose-dependent fashion by T cells when they were co-cultured with microglia. Pretreatment of microglial cells with IFNβ prior to their exposure to T lymphocytes also resulted in a slight diminution

of TNF-α production, but this effect was less marked than when T cells were pretreated with IFNβ.

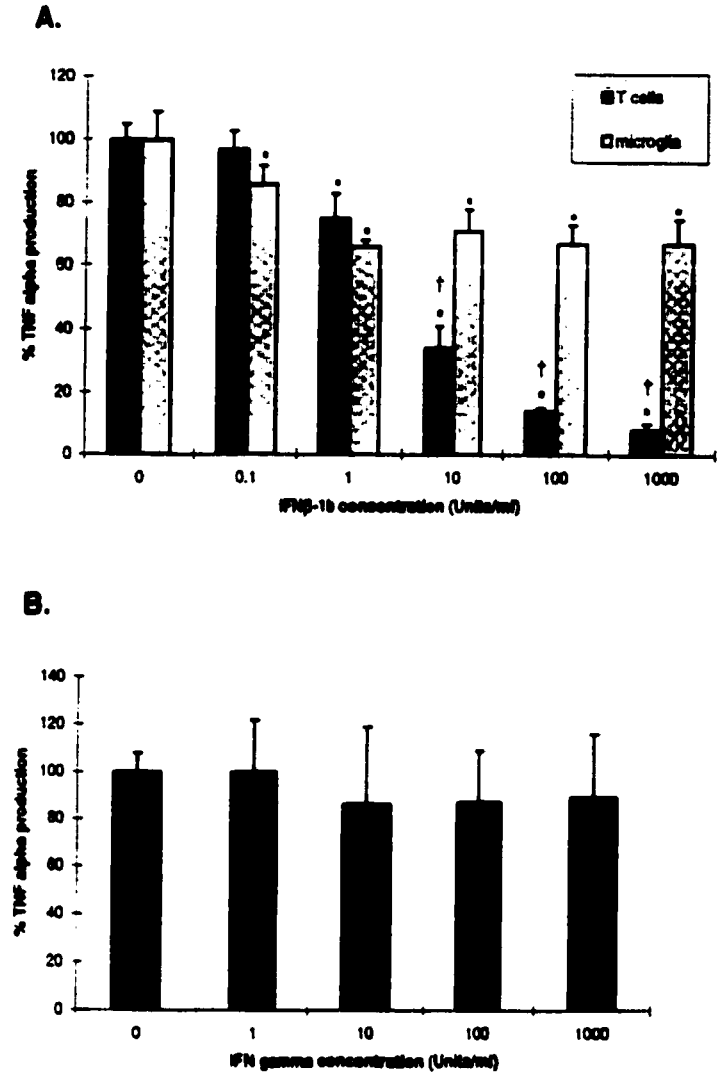


Figure 29. IFNβ-1b reduces the production of TNF-α in T cell- microglia co-culture. **A**: The pretreatment of activated T cells with different concentrations of IFNβ-1b (ranging from 0.1-1000 IU/ml) reduces the production of TNF-α in subsequent T cell-microglia co-cultures. When microglia, but not T cells, are pretreated with IFNβ-1b, the effect on TNF-α production is less marked. *$p<0.05$ compared to their respective controls (i.e. 0 IUnits/ml IFNβ)(one way ANOVA with Duncan's multiple comparisons). †$p<0.001$ compared between T cells and microglia following the same concentration of IFNβ-1b (Student's t-test). **B**: The pretreatment of activated T cells with different concentrations of IFNγ (1-1000 IU/ml) does not affect the production of TNF-α in subsequent T cell-microglia co-cultures. All values in this figure are mean ± SEM of at least 3 samples, and have been normalized to untreated controls.

Interestingly, the effect of IFNβ on T cell:microglia co-cultures is not selective to TNF-α, since IL-6 level is also diminished by IFNβ (content of IL-6 in activated T cells and microglia co-cultures = 3140 pg/ml; in the presence of 100 IU/ml IFNβ, IL-6 level = 2000 pg/ml).

It is known that IFNβ is an anti-mitotic agent for T lymphocytes, but three factors rule out the anti-proliferative effect of IFNβ as being responsible for the decrease in TNF-α production (Noronha et al, 1993; Rudick et al, 1996). Firstly, following the treatment with different concentrations of IFNβ, cell numbers were counted and equal numbers of T cells from each treatment group and controls were then co-incubated with microglia to obtain the results presented here. Secondly, IFNβ at 1000 IU/ml decreased the rate of proliferation of activated T cells by 50% at best (data not shown), while the reduction of TNF-α production at this IFNβ concentration was consistently over 90% (Fig. 29A). Thirdly, the pretreatment of T lymphocytes with another human IFN type, IFNγ, which also reduces T cell proliferation (Weinstock-Guttman et al, 1995) (49% decrease in ^{3}H-thymidine uptake at 100 IU/ml in our study), did not affect the TNF-α production (Fig. 29B).

7.1.2B. *Effects of IFNβ on VLA-4*

VLA-4, expressed on activated T cells, is also called α4β1 or CD49d/CD29. To determine whether the decreased TNF-α production following the treatment of T cells with IFNβ or anti-CD49d were mechanistically related, two approaches were taken. First, we determined whether there would be a synergistic effect, implying different pathways, when T cells were treated with both IFNβ and anti-CD49d prior to their exposure to microglia.

Secondly, we examined whether the treatment of T cells with IFNβ would lead to a reduction of CD49d expression. Figure 30A reveals that while the treatment of T cells with IFNβ or anti-CD49d alone reduced TNF-α production to the same extent, their co-treatment did not further reduce TNF-α levels. Furthermore, western blot analysis demonstrates that the total cellular level of the 80 kDa CD49d is lower in T lymphocytes treated with IFNβ than that in the control cells (Fig. 30B), supporting the postulate that the mechanism by which IFNβ decreases TNF-α production may be by regulating VLA-4 expression on T cells.

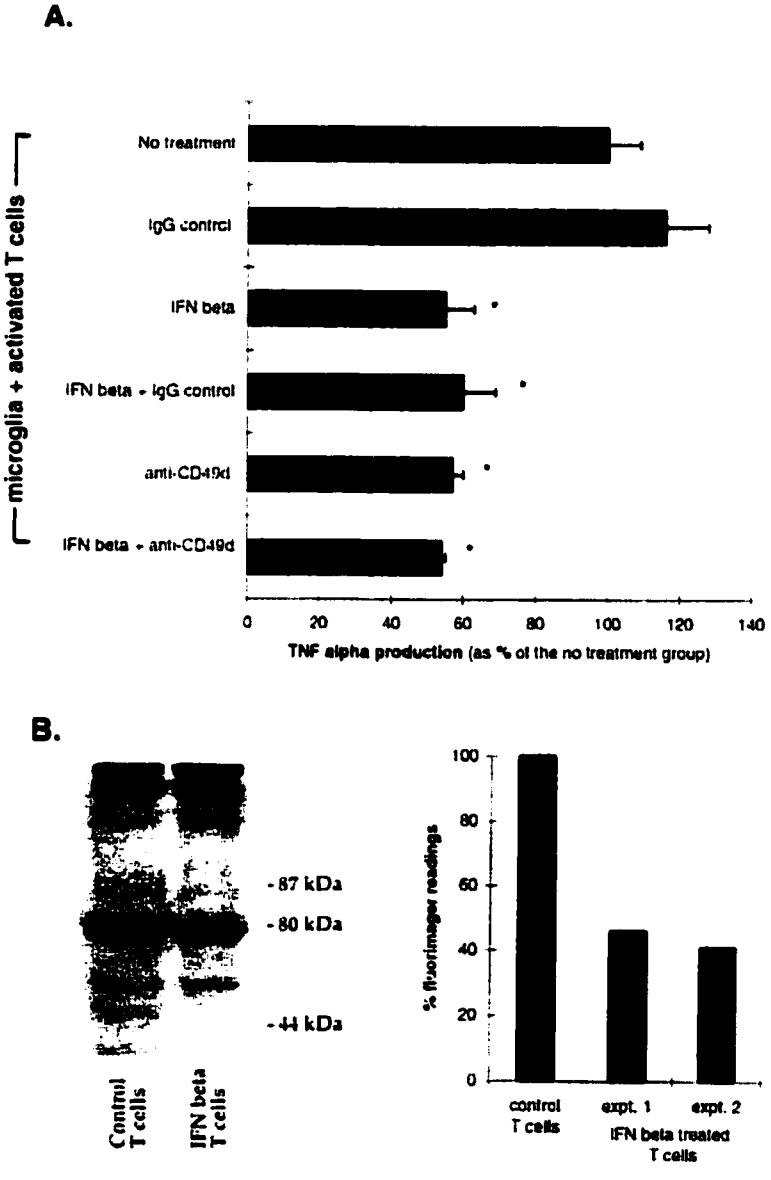


Figure 30. IFNβ-1b reduces TNF-α production by down-regulating the expression level of the α chain of VLA-4, CD49d. **A**: The co-administration of IFNβ-1b (100 IU/ml) with anti-CD49d (50 μg/ml) does not result in a synergistic effect on TNF-α production. Values are mean± SEM of triplicate cultures, normalized to the no treatment group. *$p<0.05$ compared to the no treatment group (one way ANOVA with Duncan's multiple comparisons). TNF-α production by IFNβ-1b treated T cells was not statistically different from that obtained in the presence of anti-CD49d alone, or IFN beta + anti-CD49d. **B**: The protein expression level of the α chain of the VLA-4 integrin, CD49d, on the surface of activated T cells is reduced, compared to control, when T cells (IFN beta T cells) were treated for 72h with IFNβ-1b (100 U/ml).

Of note however, IFNβ did not affect the cell surface level of α4 integrin as assessed by flow cytometry. Using 4 different antibodies to VLA-4 (PS/2 and HP2/1 described above, anti-VLAα4-FITC from Serotec, and anti-VLAα4-PE from Becton Dickinson), the mean intensity fluorescence of control T cells for cell surface (i.e. non-fixed cells) α4 integrin did not differ from that of IFNβ treated cells (results not shown).

7.1.2C. *Effects of IFNβ on cytokine production in microglia-T cell co-cultures*

Activated T cells pretreated with different concentrations of IFNβ were co-cultured with human adult microglia, and after 24 hours of co-culture, levels of TNF-α, IL-1β, IL-6, and IL-10 present in the conditioned medium were measured by enzyme-linked-immunosorbent assay (ELISA). In isolation, adult microglia, anti-CD3 activated T cells, or IFNβ-treated T cells produce undetectable or low protein levels of all cytokines tested, except for IL-6 which is produced constitutively by adult microglia (Figure 31). When T cells are co-cultured with microglia, significant induction of IL-1β, IL-10 and TNF-α occurs. In contrast to these inducible cytokines, the constitutively expressed IL-6 in microglia is not affected by interactions with T cells. The pretreatment of activated T cells with IFNβ inhibits, in a concentration-dependent manner, the production of the pro-inflammatory cytokines, TNF-α and IL-1β, produced from microglia-T cell interaction (Figure 31A, 31B), while increasing those of IL-10 (Figure 31D). IL-6 levels remain unaffected by treatment with IFNβ (Figure 31C), suggesting that IFNβ modulates the production of inducible cytokines and not that of constitutively expressed cytokines.

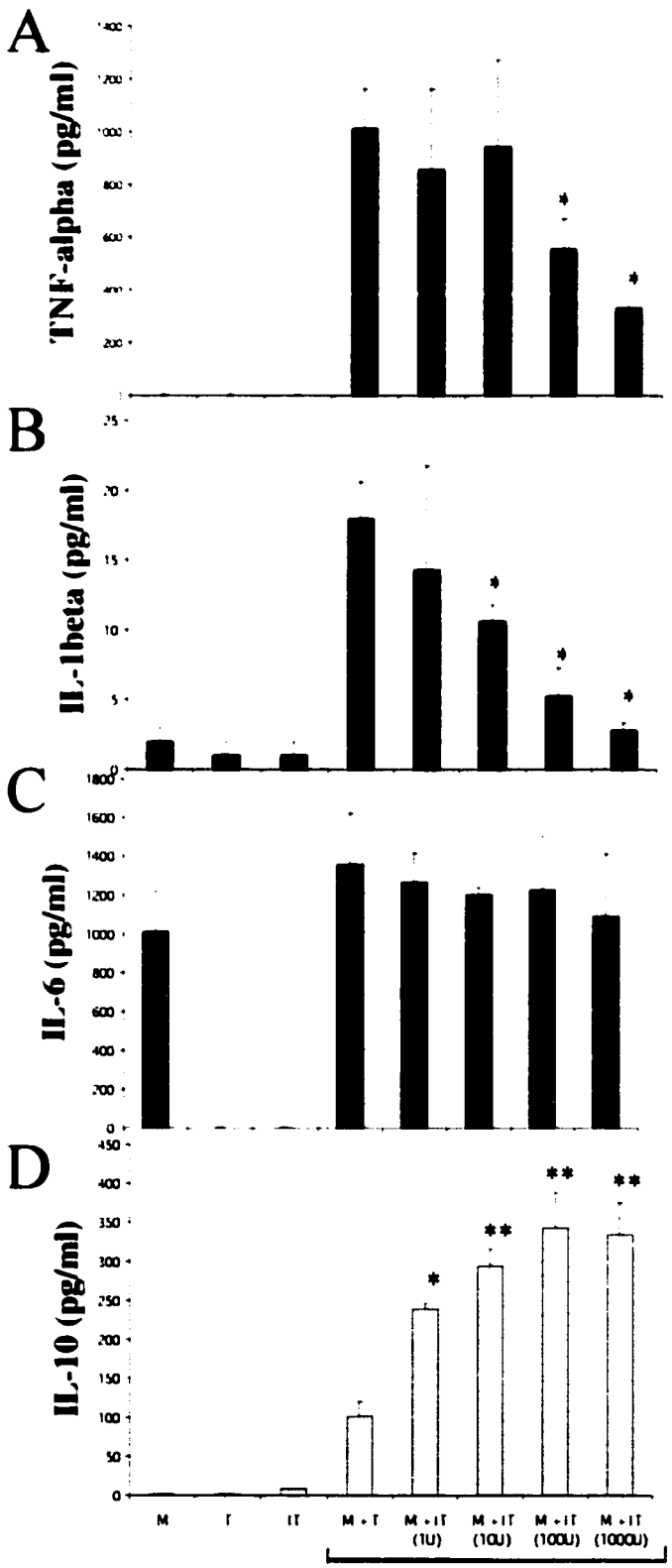


Figure 31. Cytokine levels produced from microglia-T cell interactions and the effect of IFNβ. In isolation, human adult microglia (M), anti-CD3 activated T cells (T) or IFNβ-treated T cells (IT) secrete negligible amount of TNF-α (A), IL-1β (B), and IL-10 (D). In the case of IL-6 (C), constitutive and significant levels are produced by microglia, but not by activated T cells or IFNβ-treated T cells. Co-culture of human adult microglia with activated T cells (M + T) induces the production of TNF-α, IL-1β, and IL-10, but does not alter that of IL-6. Pretreatment of activated T cells for 3 days with various concentration of IFNβ (M+ IT) inhibits in a concentration dependent manner the production of TNF-α, and IL-1β, while enhancing that of IL-10. IL-6 levels remain unchanged from control levels (microglia alone) by such a treatment. Note that the amounts of IL-1β generated from microglia-T cell interaction is low in comparison to TNF-α or IL-6; IL-10 production is intermediate. Values are mean ± SEM of triplicate experiments. * $p<0.05$, ** $p<0.01$ compared to microglia + Act T cell (M+ T) control.

We addressed the time course response of IL-10 increase by IFNβ. Figure 32A reveals that the pretreatment of T cells with IFNβ significantly accelerates the production of IL-10, when compared to levels of IL-10 in control microglia-T cell co-cultures. Indeed, 6 hours of co-culture of IFNβ-treated T cells and microglia is sufficient to obtain significant levels of IL-10 in the conditioned medium, while 24 hours is required to get a sufficient elevation of IL-10 in co-cultures of control T cells and microglia. Another time course study demonstrates that levels of IL-10 increases significantly in the conditioned medium of microglia-T cell co-cultures prior to that of TNF-α when T cells are pretreated with IFNβ (Figure 32B); previously we have reported that in the absence of any drug treatment, TNF-α elevation precedes that of IL-10 in microglia-T cell interactions (Chapter 4 / Chabot et al, 1999).

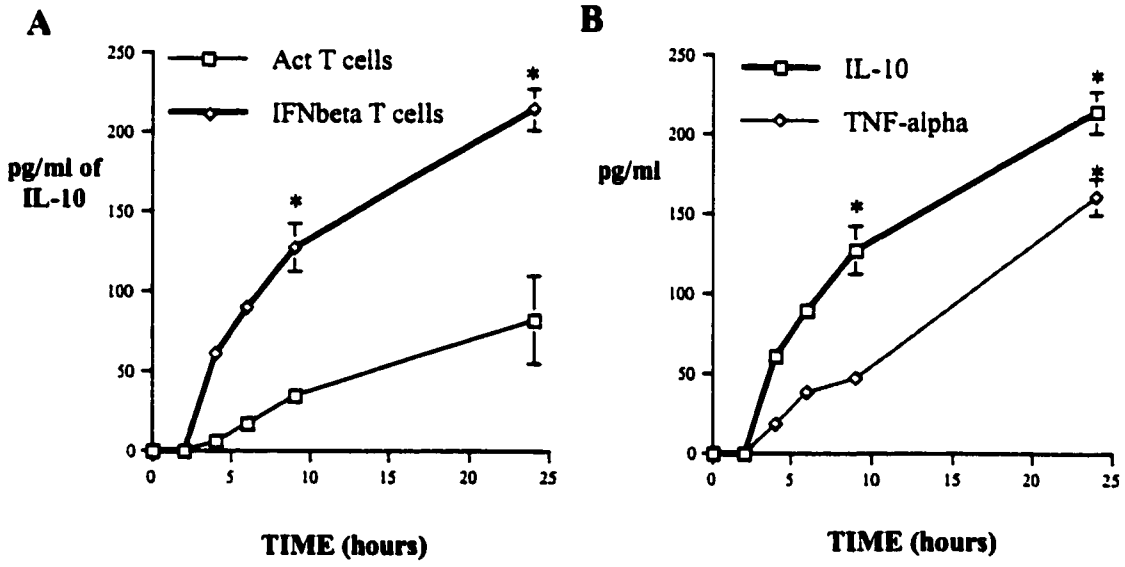


Figure 32. Kinetics of IL-10 and TNF-α production in microglia-T cell interaction, and the effect of IFNβ. Significant levels of IL-10 are detected in the conditioned medium of co-cultures of microglia with IFNβ-treated T cells prior to that found in microglia-activated T cell co-cultures (A), suggesting that pretreatment of T cells with IFNβ potentiates the production of IL-10 generated from their co-culture with microglia. Moreover, IL-10 levels become significantly enhanced in co-cultures of human adult microglia and IFNβ-treated T cells prior to that of TNF-α (B), in reverse to that observed in control microglia-T cell co-cultures (Chabot et al, 1999). Values are mean ± SEM of triplicate experiments. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared to microglia alone.

Taken together, these results indicate that IFNβ treatment accelerates the anti-inflammatory response, as well as inhibiting that of the pro-inflammatory response.

7.1.2D. *IL-10 is produced by both cell types*

Due to the limited supply of human adult microglia, and because a large number of cells are required for flow cytometry analysis of intracellular IL-10, U937 cells treated sequentially with PMA and IFNγ were used as a model of microglia. When co-cultured with activated T lymphocytes, PMA/IFNγ-treated U937 cells share properties of microglia (Chapter 5). Since both T cells and PMA/IFNγ-treated U937 cells are potential producers of IL-10 (Sheng et al, 1995; Yssel et al, 1992), intracellular staining for IL-10 was performed to address which cell type is responsible for the production of IL-10 in PMA/IFNγ-treated U937-T cell co-cultures. As shown in Figure 33, activated T cells or IFNβ-treated T cells (CD3+) alone express low to negligible intracellular IL-10, while a small proportion of PMA/IFNγ-treated U937 cells (CD64+) are IL-10-positive, supporting the ELISA results (see Fig 36 below). However, when cells are co-cultured, a significant increase in CD64+ PMA/IFNγ-treated U937 cells positive for intracellular IL-10 is observed (Figure 33). There is a further increase in cells expressing intracellular IL-10 in PMA/IFNγ-treated U937 cultures exposed to IFNβ-pretreated T cells, and this increase is more predominant in PMA/IFNγ-treated U937 rather than T cells. These results suggest that the microglia is the major producer of IL-10 in microglia-T cell interactions, although some IL-10 production occurs in T cells. and that IFNβ pretreatment of T cells imparts on microglia an increased capacity to produce IL-10.

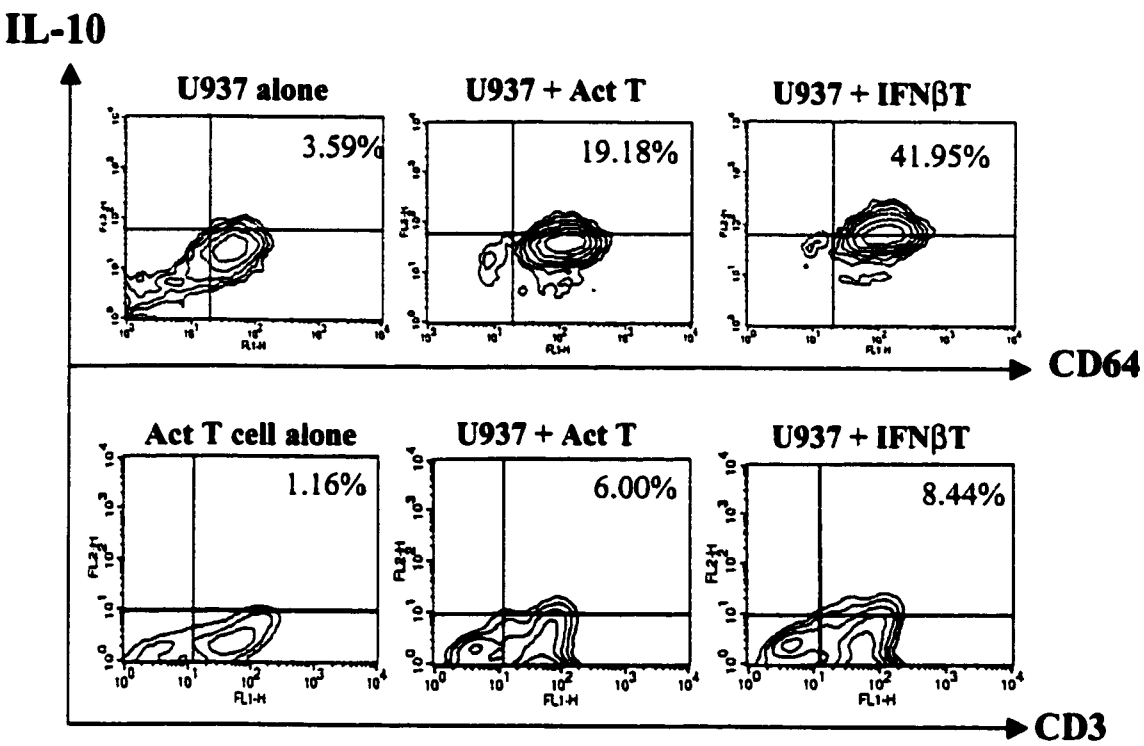


Figure 33. Source of IL-10 in co-cultures of PMA/IFNγ-treated U937 cells and IFNβ-treated T cells. PMA/IFNγ-treated U937 cells (CD64+), used as a model of human adult microglia, or activated T cells in isolation express low levels of intracellular IL-10. When co-cultured, PMA/IFNγ-treated U937 cells and activated T cells express higher levels of intracellular IL-10. The pretreatment of T cells with IFNβ prior to their co-culture with PMA/IFNγ-treated U937 cells further increases levels of IL-10 in both IFNβ-treated T cells and PMA/IFNγ-treated U937 cells, but this increase was more marked in PMA/IFNγ-treated U937 cells.

7.1.2E. *Contact between IFNβ-treated T cells and microglia is necessary for IL-10 production*

The mechanisms that lead to the increase of IL-10 in co-cultures of microglia with IFNβ-treated T cell were evaluated. Two approaches were used to investigate the role of soluble factors produced from IFNβ-treated T cells and microglia in inducing the production of IL-10. First, the conditioned medium of IFNβ-treated T cells culture was added to microglia, and second, IFNβ-treated T cells were placed in a culture insert, where they were in close proximity, but not contacting, microglia. In both cases, minimal

amounts of IL-10 protein are detected by ELISA, suggesting that soluble factors produced from each cell type do not play any significant role in the production of IL-10 generated in co-cultures of IFNβ-treated T cells and microglia (Figure 34A). It is only when the two cell types are allowed to make contact with one another that significant increased levels of IL-10 are produced (Figure 34A). These results suggest that contact is necessary in order to trigger the production of IL-10, and that soluble factors play a minor role in this process.

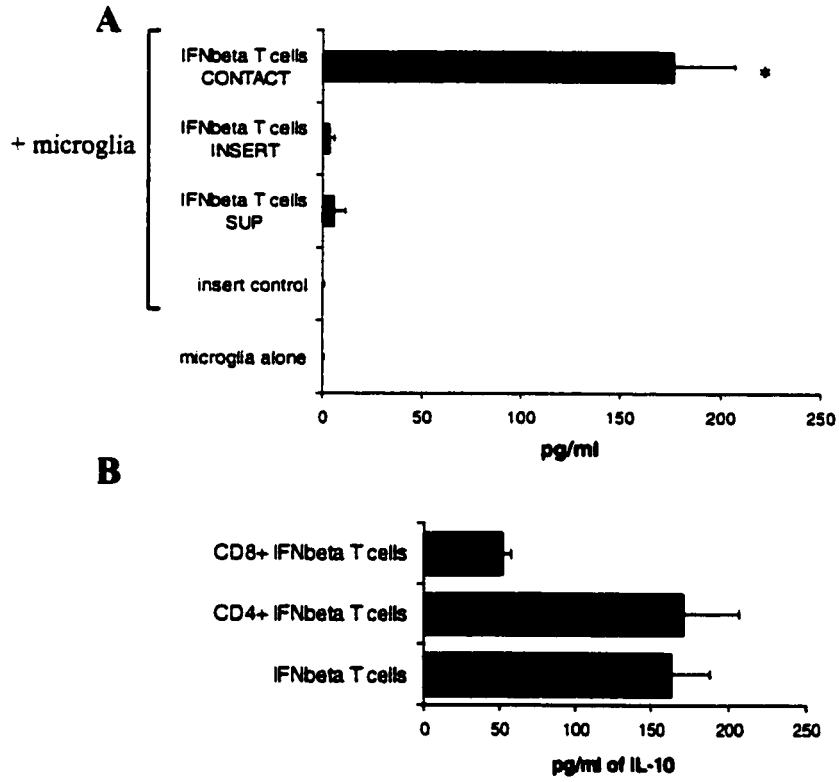


Figure 34. Contact between IFNβ-treated T cells and microglia is necessary for IL-10 production: role of CD4+ T cells. IL-10 is secreted in negligible amount by human adult microglia alone or when microglia are exposed to the culture insert (insert control) (**A**). When the conditioned medium (SUP) from IFNβ-treated T cells is added to the microglia, or when IFNβ-treated T cells are placed in a culture insert, in close proximity but not contacting the microglia, low levels of IL-10 are detected by ELISA. Significant production of IL-10 is obtained only when microglia and IFNβ-treated T cells are in contact. In **B**, purified CD4+ and CD8+ T cell population were obtained for co-culture with microglia. When compared to the total population (IFNbeta T cells), CD4+ IFNβ-treated T cells are mainly responsible for the production of IL-10 resulting from their co-culture with microglia. Values are mean ± SEM of triplicate experiments. * $p<0.05$ compared to activated T cells plus microglia controls.

To address which population of T cell induces the production of IL-10 in co-cultures of microglia with IFNβ-treated T cell, magnetic beads were used to isolate IFNβ-treated CD4+ and CD8+ T cells from the entire IFNβ-treated T cells population. Purified cells were then co-cultured with microglia, and IL-10 protein levels were assayed by ELISA. In co-cultures of IFNβ-treated CD4+ T cells and microglia, IL-10 is produced to a greater extent than in co-cultures involving IFNβ-treated CD8+ T cells (Figure 34B), suggesting that IFNβ-treated CD4+ T cells play a more significant role in this production of IL-10.

7.1.2F. *IFNβ treatment does not affect the CD40, B7, and CD23 pathways*

Because the contact between IFNβ-treated T cells and microglia is necessary to induce the production of IL-10 (Figure 34A), we postulated that IFNβ treatment could modulate the expression of adhesion molecules on the surface of T cells. We have previously reported that CD40, B7 and CD23 molecules, on microglia, and their ligands on activated T cells, namely CD40L, CD28/CTLA-4 and CD11b, respectively, play an important role in the production of IL-10 in microglia-T cell co-cultures (Chapter 4 / Chabot et al, 1999). In order to test whether the pretreatment of activated T cells with IFNβ modulates the surface expression of those ligands, flow cytometry analyses were performed. Figure 35 shows that IFNβ treatment does not affect cell surface expression of all molecules tested, suggesting that it induces IL-10 production in microglia-T cell interaction through a mechanism independent to that which involved CD40, B7 and CD23 (Chapter 4/Chabot et al, 1999). These results are in agreement with those obtained from a study by Mena and Rohowsky-Kochan (1999) which demonstrated that

expression levels of CD80, CD86, CD28, and CTLA-4 seen on CD4+ and CD8+ T cells from patients with MS are not altered by treatment with IFNβ.

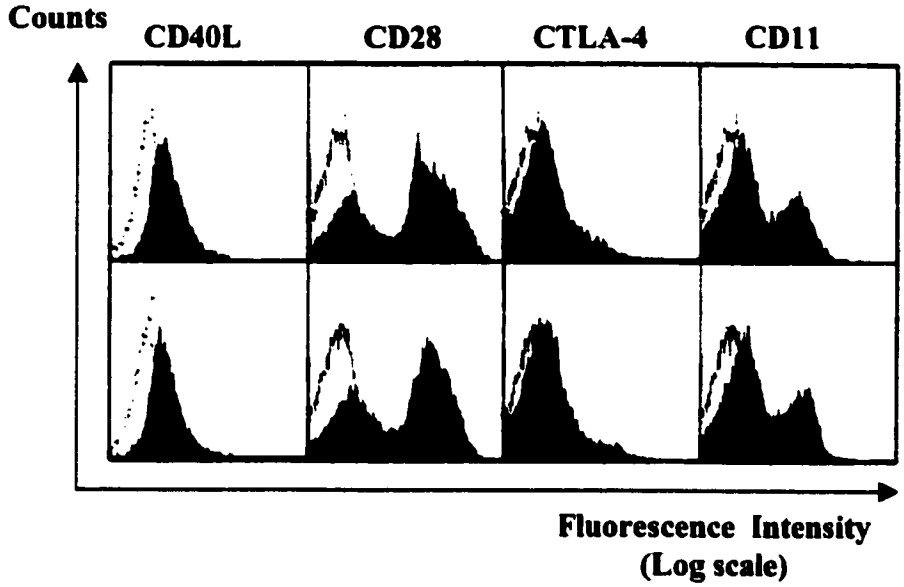


Figure 35. Effect of IFNβ treatment on T cell surface expression of CD40L, CD28, CTLA-4, and CD11b. Flow cytometry analyses show that IFNβ pretreatment of activated T cells does not affect surface expression of CD40L, CD28, CTLA-4 and CD11b. The top panels are of control cultures while the bottom panels are of T cells treated with 1000 IU/ml of IFNβ. These results are representative of 3 separate experiments involving 3 different donors.

7.1.2G. *Effect of IFNβ treatment on cytokine production in other co-culture systems*

I evaluated whether the effect of IFNβ on co-cultures of T cells with adult human microglia can be reproduced in other co-culture systems, specifically those involving co-cultures of activated T cells with PMA/IFNγ-treated U937 cells or fetal human microglia. Figure 36 shows that co-cultures of those microglia cell models with activated T cells result in increased levels of IL-10 and TNF-α. However, when IFNβ-pretreated T cells are used, levels of IL-10 were further increased, while TNF-α levels decreased compared to co-cultures of control T cells. Thus, these results suggest that PMA/IFNγ-treated U937

cells and fetal human microglia respond to IFNβ-pretreated T cells in the same manner as human adult microglia.

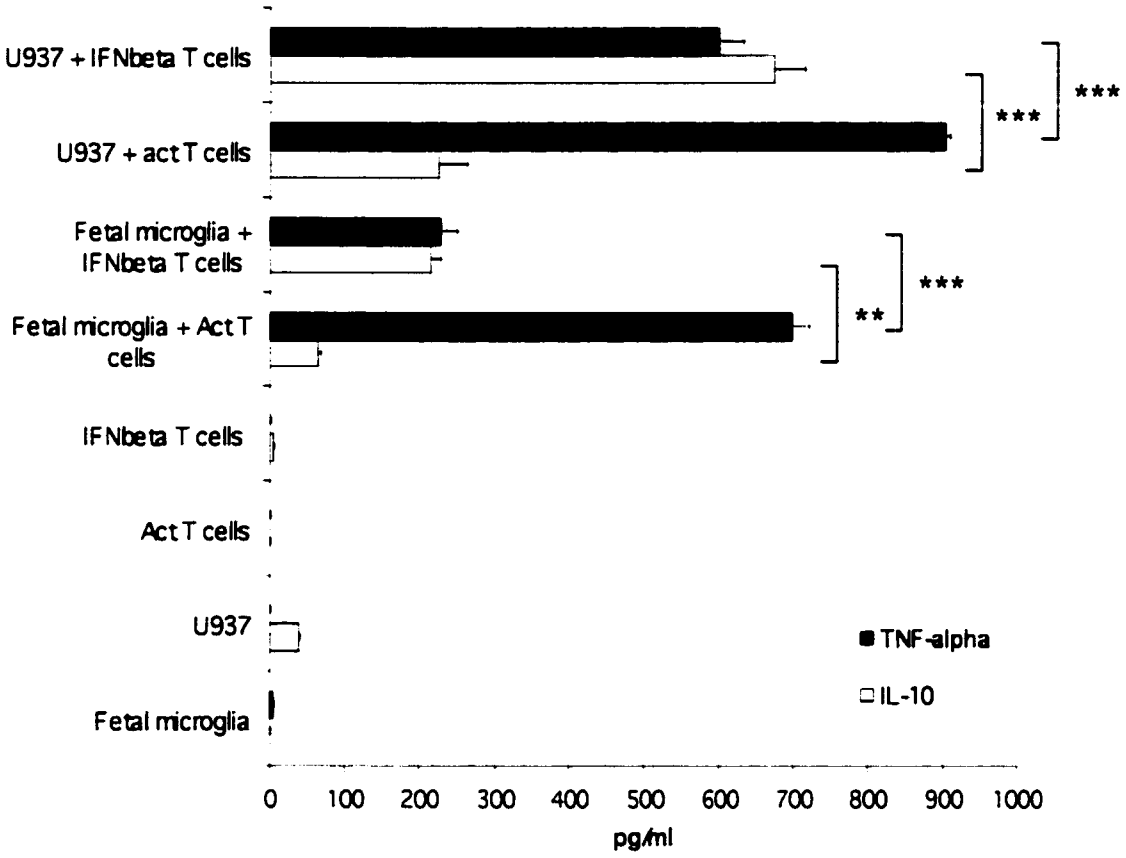


Figure 36. Effect of IFNβ treatment in other microglia-T cell interaction system. PMA/IFNγ-treated U937 cells, fetal human microglia, activated (Act) T cells and IFNbeta T cells produce no or little amount of IL-10 and TNF-α in isolation. Significant levels of TNF-α, and IL-10 are present in the conditioned medium of co-cultures of activated T cells with fetal microglia or PMA/IFNγ-treated U937 cells. IFNβ treatment of T cells prior to their exposure with fetal microglia or PMA/IFNγ-treated U937 cells enhances the production of IL-10, while inhibiting that of TNF-α. Values are mean ± SEM of triplicate experiments. **p<0.01, ***p<0.001.

Finally, the effect of IFNβ on the production of other cytokines generated in co-cultures of PMA/IFNγ-treated U937 cells with activated T cells was investigated. Figure 37 demonstrates that the pretreatment of activated T cells with IFNβ prior to their co-culture with PMA/IFNγ-treated U937 cells decreases in a concentration-dependent manner levels of TNF-α, IL-4, IL-12, and IL-13, and increases that of IL-10. These data suggest that IFNβ preferentially increases the production of IL-10.

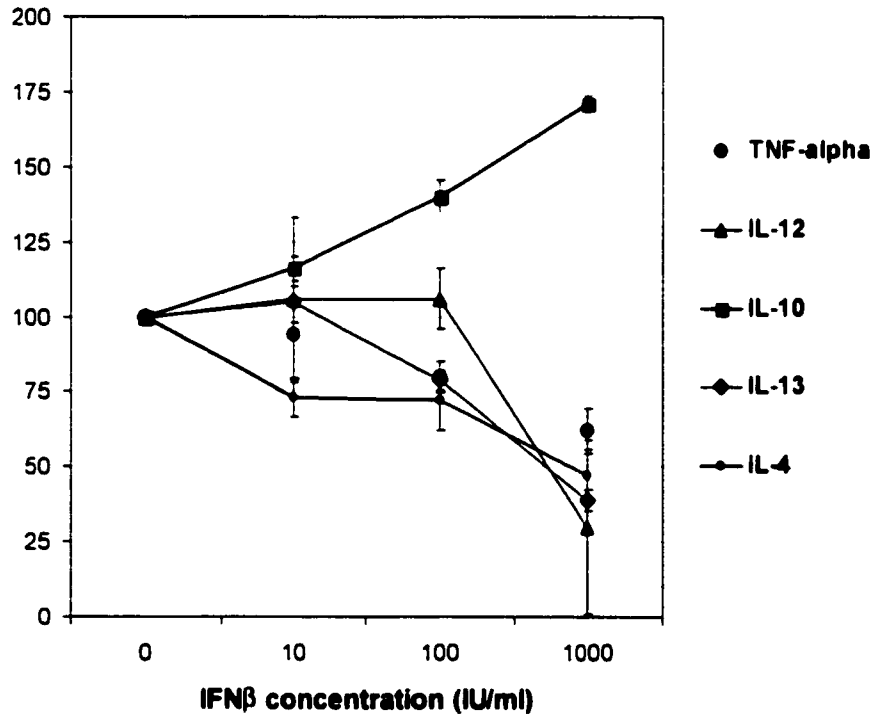


Figure 37. Effects of IFNβ on cytokine production in co-cultures of PMA/IFNγ-treated U937 cells and activated T cells. Pretreatment of T cells with IFNβ prior to their co-culture with PMA/IFNγ-treated U937 cells preferentially increases the production of IL-10 in a concentration-dependent manner. In contrast, protein levels of TNF-α, IL-4, IL-12, and IL-13 are decreased by such a treatment. All results are expressed as percentage of controls, where controls are co-cultures of PMA/IFNγ-treated U937 cells plus T cells without IFNβ treatment. Values are mean ± SEM of triplicate experiments.

7.1.3. *Discussion*

Since the initial reports in 1993 (Paty et al, 1993, IFN MS Study Group, 1993), several studies, using either IFNβ-1b or other forms of human IFNβ, have shown that these interferons decrease the number of relapses and MRI-detected lesions in relapsing-remitting MS patients (Rudick et al, 1996; Fernandez et al, 1995; Jacobs et al, 1996; Lublin et al, 1996). Despite the clinical advances, however, the precise mechanism(s) by which IFNβ-1b is effective in the treatment of MS has remained unclear. Suggested modes of action refer primarily to systemic immune-mediation (IFN MS Study Group, 1993; Panith et al, 1993) and include an effect of IFNβ-1b in decreasing T cell reactivity or its synthesis of IFN-γ (Soilu-Hanninen et al, 1995; Noronha et al, 1993), attenuating antigen presentation to T cells, improving the suppressor function of T lymphocytes (Noronha et al, 1995), or modifying the humoral immune response (O'Gorman et al, 1987). We recently reported that IFNβ-1b decreases the T cell production of the matrix-degrading protease, matrix metalloproteinase-9 (MMP-9), with the resultant reduced capacity of T cells to proteolytically remodel and infiltrate across barriers (Stuve et al, 1996).

Our current results have uncovered another mechanism by which IFNβ-1b may be efficient in MS. In this regard, IFNβ-1b attenuates the ability of T cells to stimulate the production of the oligodendrocyte-toxic cytokine, TNF-α. With respect to whether the mechanism of IFNβ-1b involves the T cell VLA-4 integrin, the results are unclear. Although *cellular* level of the α4 chain of VLA-4 is reduced by IFNβ-1b as determined by Western blot analyses, cell *surface* level of α4 was not affected as shown by flow cytometry. Besides decreasing intracellular α4 level, it is possible that IFNβ-1b might have affected the affinity state of the VLA-4 integrin, which was not reflected by the cell

surface flow cytometry results. For VLA-4 and other integrins, the switch from a low to a high affinity state on the cell membrane, with an associated increase in cellular function, can occur without alterations of the level of that integrin (reviewed in Hynes et al, 1992). That the affinity state, and function, of the VLA-4 integrin could have been affected by IFNβ-1b is supported by the findings of the lack of synergy of IFNβ-1b with the functional α4 integrin blocking antibody in affecting TNF-α production.

A multitude of evidence suggests that IL-10 is a cytokine with important anti-inflammatory properties. IL-10 is considered to be a mediator of macrophage deactivation because of its ability to inhibit the functions of macrophages. For example, IL-10 inhibits the monocytic production of pro-inflammatory cytokines, such as TNF-α, IL-1β, IL-6, and IL-12 (Sawada et al, 1999), and downregulates the expression of cytokine receptors on the surface of macrophages-like cells, including microglia (Sawada et al, 1999). Moreover, there is evidence to suggest that IL-10 suppresses the antigen-presenting capacity of macrophages and other antigen-presenting cells. Indeed, it was shown that IL-10 down-regulates the expression of MHC class II (Koppelman et al, 1997; O'Keefe et al, 1999), and the costimulatory ligands, B7-1 and B7-2, in cells of the monocytic lineages (Ding et al, 1993; Kawamura et al, 1995; Menedez Iglesias et al, 1997). Furthermore, the ability of IL-10 to induce anergy of peripheral T cells has been demonstrated (Akdis et al, 1999; Becker et al, 1994). Experiments performed in IL-10-deficient mice revealed that the absence of IL-10 resulted in the acceleration of experimental autoimmune encephalomyelitis (EAE) following immunization with myelin oligodendrocyte glycoprotein (MOG), suggesting that IL-10 plays a role in the recovery

phase of EAE (Samoilova et al, 1998). IL-10-deficient mice also develop a chronic inflammatory disease of the colon (Kuhn et al, 1993).

Recently, several reports have indicated that IFNβ treatment results in the increase of IL-10 in the plasma and PBMCs of patients with MS (Rep et al, 1999; Ozenci et al, 1999). Furthermore, the elevation of IL-10 has also been noted in the CSF, suggesting that IL-10 increases also within the CNS (Rudick et al, 1998). The results of this chapter demonstrate a mechanism by which IFNβ may cause a local increase of IL-10 in the CNS of MS patients. Specifically, we show that IFNβ increases the production of IL-10 induced from cognate interactions between activated T lymphocytes and human adult microglia, human fetal microglia or PMA/IFNγ-treated U937 cells. The increased production of IL-10 by IFNβ requires cell-cell contact. Finally, we demonstrate that IFNβ preferentially increases IL-10, since it also decreases the production of other cytokines TNF-α, IL-1β, IL-4, IL-12, and IL-13.

It has been suggested by several lines of evidence that microglia and T cells do interact *in vivo*. In the graft-versus-host disease (GvHD) model, activated microglia were shown to cluster around T cell infiltrate, and to be associated with single or clustered microglia (Sedgwick et al, 1998). Microglia isolated from GvHD animals were also shown to proliferate, and to exhibit functions of activated microglia, such as phagocytosis and motility (Sedgwick et al, 1998). These results suggest that microglia activation occurs as a consequence of their interaction with infiltrated T cells. Direct contact between microglia and T cells was further demonstrated by Raivich and co-workers (1998), who showed that CD3+ T cells infiltrate the site of injury 3 days following facial nerve resection, and form direct contact with microglia. Interestingly, the influx of T cells

correlated with an increase in the production of cytokines, such as IL-1β and TNF-α, as detected by RT-PCR. Given those *in vivo* observations, and the proximity of activated T cells and microglia in the perivascular space or CNS parenchyma of MS lesions (Traugott et al, 1983), we believe that the model of microglia-T cell interaction described in this manuscript is relevant to MS pathogenesis.

The ability of IFNβ to induce a cytokine shift has previously been demonstrated in an antigen-dependent system, where an antigen-presenting cell (APC) interact in an MHC-restricted manner with naïve CD4+ T helper cells, resulting in their activation and differentiation into T helper 1 (Th1) or T helper 2 (Th2) cells. Typically, Th1 cells produce IL-2, such as IFNγ and TNF-α, while Th2 cells produce IL-4, IL-10 and IL-13. It was shown that MBP-reactive CD4+ T cells proliferate and produce Th1-type cytokines, such as IFNγ and IL-2, in the presence of MBP and an APC, such as macrophages (Kozovska et al, 1999). When IFNβ was added to this system, a cytokine shift, termed immune deviation, was induced as T cells began to produce Th2-type cytokines, which include IL-10 and IL-4 (Kozovska et al, 1999). In the current study, an antigen-independent system was used. In this case, IFNβ had a preferential increasing effect on IL-10 (Figure 38), and did not strictly induce a Th2-type response since it decreased the production of IL-4 and IL-13. Thus, taken together, it suggests that IFNβ can impact upon interactions that are either dependent or independent on the presence of an antigen by upregulating the production of IL-10.

Results of this chapter demonstrate that the IFNβ-induced production of IL-10 from microglia-T cell interactions depends on a cell-cell contact mechanism, suggesting that IFNβ modulates the expression of molecules present at the membrane of activated T

cells. We have previously demonstrated that CD40, B7 and CD23 and their ligands are involved in IL-10 production from microglia-T cell interaction (Chabot et al, 1999 / Objective 2), however surface expression levels of CD40L, CD28, CTLA-4 and CD11b on activated T cells remained unchanged following treatment with IFNβ. Nonetheless, expression by flow cytometry does not reveal possible alterations of the affinity of molecules for their ligands, so this needs to be further explored. Thus, the nature of the molecules affected by treatment with IFNβ in this study is not known. Binding of IFNβ to its receptor results in the activation of Janus protein tyrosine kinases (Jak), namely Jak1 and Jak2, which is commonly followed by tyrosine phosphorylation of the transcription factor Stat1 and Stat 2 (Grumbach et al, 1999). Thus, IFNβ must affect the expression of an IFN-responsive gene or affect other metabolic pathways through the signalling cascade that it induces. Recently it was shown that gangliosides GD1a and GM3, which are sialic acid-containing glycolipids with immunomodulatory functions, are strong inducers of IL-10 production by human T cells, and that this production of IL-10 was completely blocked by protein tyrosine kinase (PTK) inhibitors (Kanda, 1999). These data raises the possibility that gangliosides are also inducers of IL-10 production by microglia. One may speculate that IFNβ treatment increases the production and surface expression of gangliosides, which interact with their receptors on microglia to induce the production of IL-10. There is also evidence that type III and IV phosphodiesterase play a role in the induction of IL-10 production by microglia (Eigler et al, 1998; Yoshikawa et al, 1999), suggesting that cAMP may also be involved in IL-10 production from microglia-T cell interaction.

In summary, we provide evidence that IFNβ pretreatment of T cells prior to their encounter with microglia preferentially increases the production of IL-10, while inhibiting that of TNF-α, IL-1β, IL-4, IL-12, and IL-13. Thus, this is not a simple Th1 to Th2 deviation since the Th2 cytokines, IL-4, and IL-13, were also inhibited by IFNβ. Given the functions of IL-10, its increased production in the CNS is of great significance as it may result in the deactivation of macrophages/microglia, the inhibition of antigen presentation, and the induction of T cell anergy. Hence, we conclude that through the induction of IL-10 production and the down-regulation of pro-inflammatory cytokines production, IFNβ treatment creates an anti-inflammatory milieu in the CNS, which helps accounts for its efficacy to treat MS.

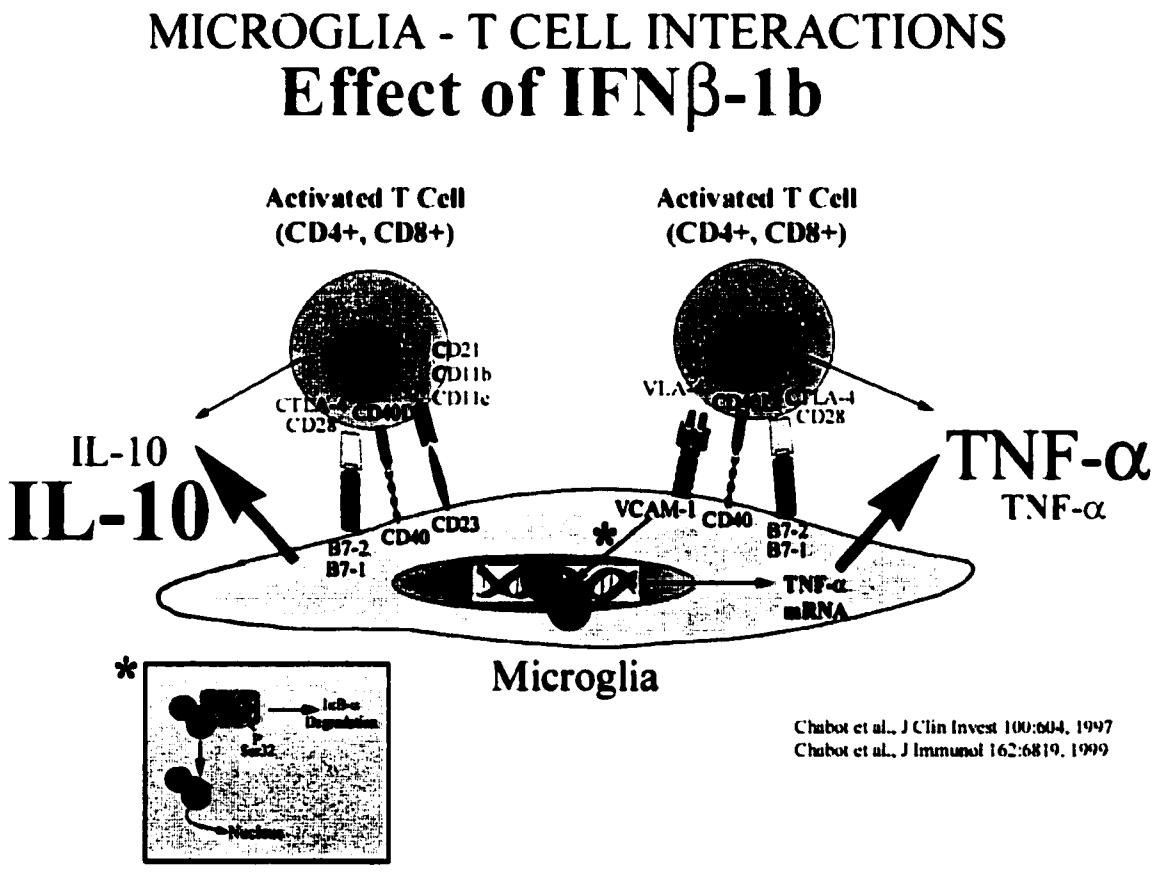


Figure 38: Schematic representation of the effects of IFNβ on cytokine production in microglia-T cell interaction.

7.2 Effects of Glatiramer Acetate

7.2.1. *Introduction*

Gatiramer acetate (Copaxone^{R}) is a synthetic random polymer of 4 amino acids (L-alanine, L-glutamate, L-lysine and L-tyrosine) that has efficacy in the treatment of patients with relapsing-remitting multiple sclerosis (MS) (Bornstein et al., 1987; Johnson et al., 1995, 1998). In an animal model of MS, experimental autoimmune encephalomyelitis (EAE), glatiramer acetate suppresses both the acute and chronic disease induced by several myelin proteins including myelin basic protein (MBP), proteolipid protein and myelin oligodendrocyte glycoprotein (Ben-Nun et al., 1996; Teitelbaum et al., 1996; 1999). The principal mechanism of action of glatiramer acetate in MS is currently thought to involve antigen presentation, by interfering with both the antigen presenting cell and the responding T cell. For the former, glatiramer acetate has been shown to compete with, or displace, myelin peptides from binding to major histocompatibility complex (MHC) molecules (Teitelbaum et al., 1992, 1996; Racke et al., 1992; Ben-Nun et al., 1996; Arnon et al., 1996; Fridkis-Hareli and Strominger, 1996). With respect to the responding T cell, glatiramer acetate has been described to act as an antagonist at the T cell receptor to the immunodominant 82-100 epitope of MBP (Aharoni et al., 1999). Consequences of affecting antigen presentation include the decreased activation of encephalitogenic T lymphocytes that mediate the disease, and the induction of T helper 2 (Th2) suppressor/regulatory cells that participate in the shutdown of the disease (Aharoni et al., 1993, 1997; Teitelbaum et al., 1999).

In this chapter, I sought to elucidate novel effects of glatiramer acetate that may help account for its efficacy in MS. In particular, I have addressed whether glatiramer

acetate affects the production of cytokines in T cell - microglia interaction, an important encounter in the MS brain.

7.2.2. *Results*

7.2.2A. *Glatiramer acetate suppresses cytokine levels in T cell – microglia co-cultures*

PMA/IFNγ-treated U937 cells were first used as a model of microglia (Chapter 5/ Chabot et al, submitted). Anti-CD3 activated T cells or PMA/IFNγ-treated U937 cells in isolation produce undetectable levels of TNF-α; IL-10 was detected in U937 but not T cells (Figure 39). In co-culture for 24h, substantial increase in levels of TNF-α and IL-10 are obtained. 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 production of TNF-α and IL-10 (Figure 39). The effect of glatiramer acetate appears to be principally on T cells since the pretreatment of PMA/IFNγ-treated U937 cells with glatiramer acetate did not influence cytokine production in subsequent T cell – U937 interactions.

Interactions between activated T cell and PMA/IFNγ-treated U937 also lead to the upregulation of two Th2-like anti-inflammatory cytokines, IL-4 and IL-13 (Figure 40), although the amounts produced are about a log fold lower than those of IL-10 or TNF-α. Significantly, the production of IL-4 and IL-13 was reduced by glatiramer actetate in co-cultures of T cells with PMA/IFNγ-treated U937, and this decrease was also observed for the pro-inflammatory cytokine, IL-12 (Figure 40). In summary, glatiramer acetate pretreatment (72h) of anti-CD3 ligated T cells results in the suppression of all inducible cytokines that we have studied in T cell – U937 interactions.

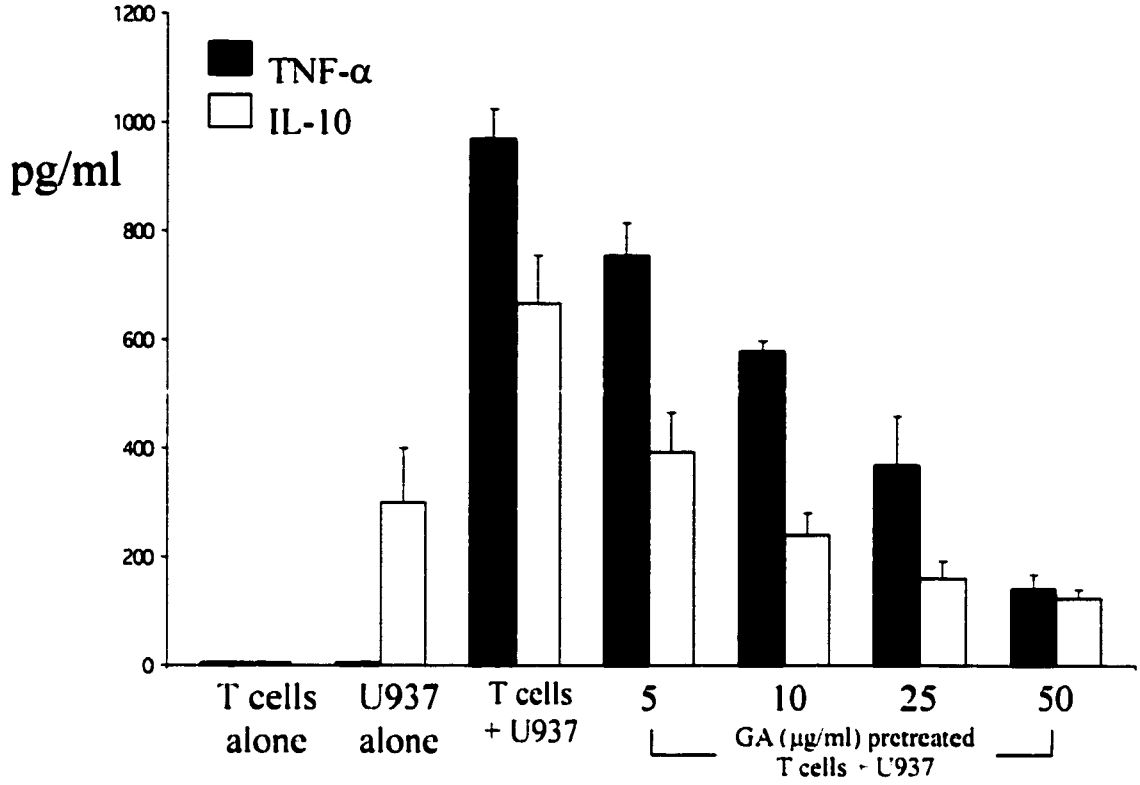


Figure 39: Glatiramer acetate pretreatment of T cells suppresses IL-10 and TNF-α production that is generated in T cell – U937 interactions. T cells in isolation produce undetectable IL-10 or TNF-α; PMA/IFNγ-pretreated U937 cells have detectable IL-10 but negligible TNF-α. In co-culture, both IL-10 and TNF-α levels are significantly elevated and this is reduced dose-dependently by glatiramer acetate pretreatment of T cells. Values are mean ± SEM of triplicate analyses.

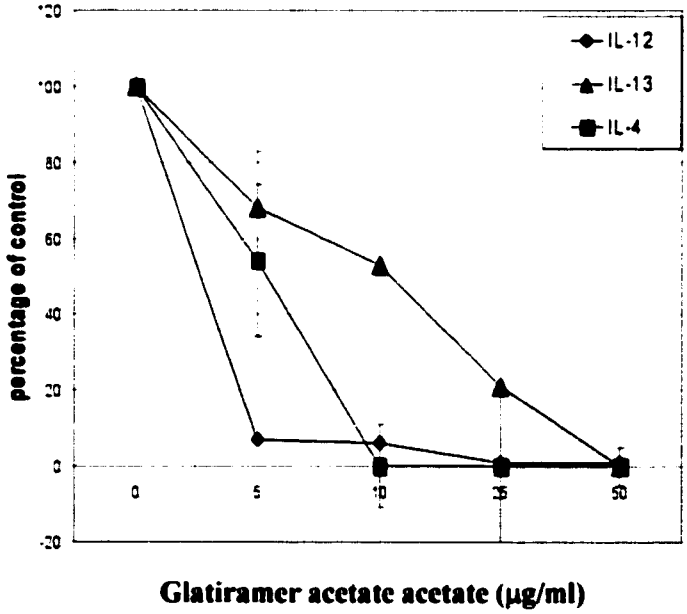


Figure 40: Glatiramer acetate treatment of T cells also lead to the suppression of IL-4, IL-12 and IL-13 in T cell – U937 interactions. Both T cells and PMA/IFNγ-pretreated U937 cells do not secrete detectable amounts of IL-4, IL-12 or IL-13 into their conditioned medium. With co-culture, levels of these cytokines are increased, although the levels are low in comparison to those for IL-10 or TNF-α (Fig. 4); amounts of IL-4, IL-12 and IL-13 in T cell – U937 interactions are, respectively, 13, 12 and 62 pg/ml. These are dose-dependently reduced by glatiramer acetate pretreatment of T cells.

The effect of glatiramer actetate in reducing cytokine production in T cell – U937 co-cultures is not a result of a decrease in the proliferation of T cells, since glatiramer acetate does not decrease the number or size of T cell aggregates that form following anti-CD3 treatment, indicating that it does not affect the proliferation of T cells in any significant manner. Indeed, when the total number of cells was counted after 72h of glatiramer acetate, cell numbers were comparable in control (24 ± 2 x 10^3) versus glatiramer acetate (5, 25 and 50 μg/ml) groups (26 ± 1, 26 ± 2 and 22 ± 1, respectively, x 10^3). Moreover, equal number of T cells was added to microglia or U937 cells in all test situations.

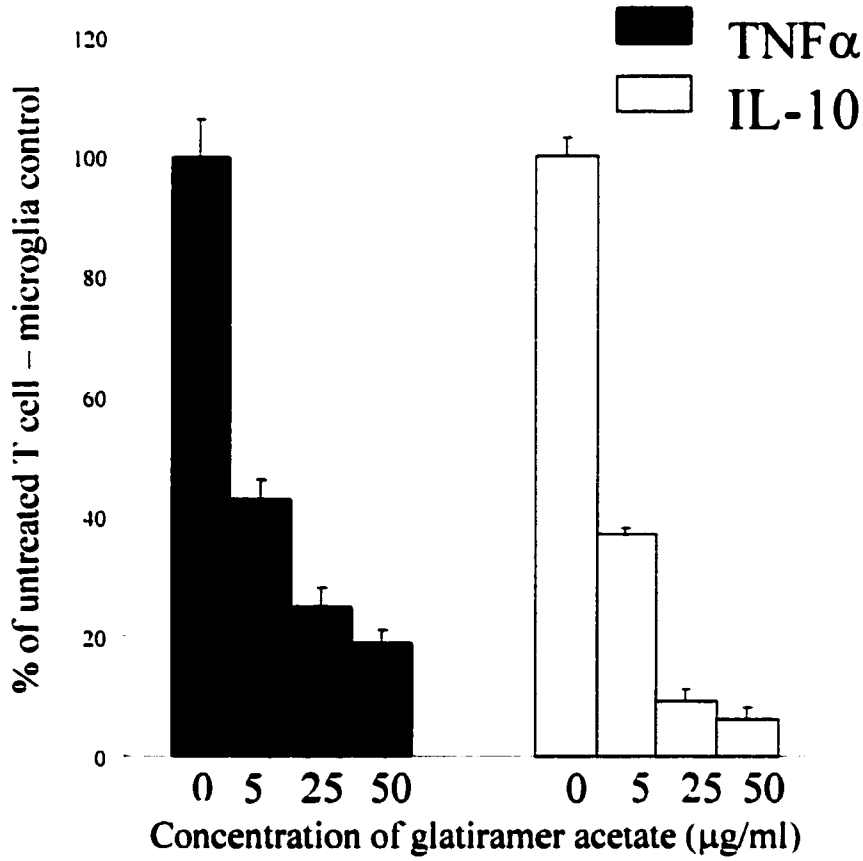


Figure 41: Cytokine production in co-cultures of T cells with fetal human microglia is reduced by glatiramer acetate. Values are mean ± SEM of 3 or 4 analyses and are expressed as % of the mean of control T cell - microglia co-cultures (i.e. 0 μg/ml glatiramer acetate). The amount of TNF-α in control T cell – microglia co-culture was 1068 ± 68 pg/ml while that for IL-10 was 139 ± 4 pg/ml.

With respect to primary microglia, fetal human microglia in isolation do not secrete detectable amounts of IL-10 or TNF-α into the culture medium; thus, it appears that these cells are similar to their adult counterparts (Chapter 3 / Chabot et al., 1997; Chapter 4 / Chabot et al, 1999). When fetal human microglia are co-cultured with activated T cells, significant amounts of IL-10 and TNF-α are induced. With glatiramer acetate pre-treatment of T cells, the resultant IL-10 and TNF-α in T cell – microglia co-cultures was reduced in a concentration-dependent manner (Figure 41).

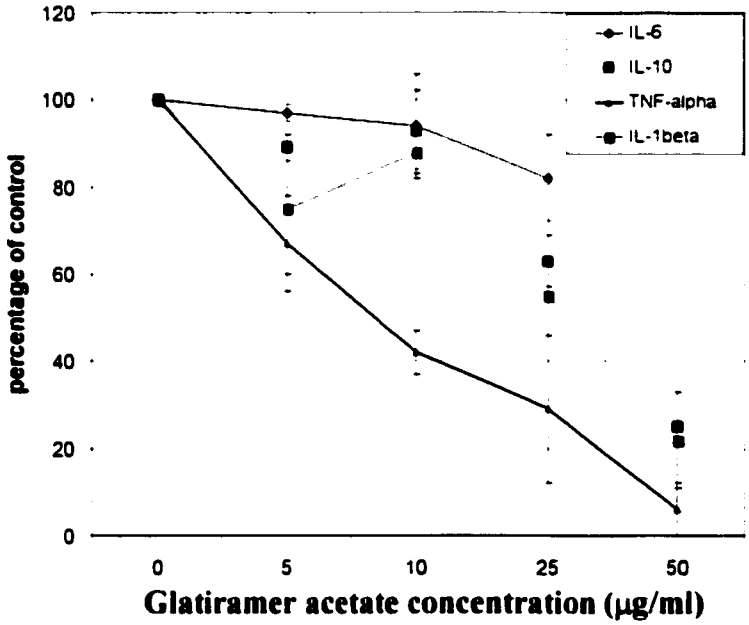


Figure 42: Adult human microglia and T cell co-cultures: Effects of glatiramer acetate on cytokine production. In isolation, neither T cells nor microglia express IL-1β, IL-10 or TNF-α. In co-culture of microglia and activated T cells, the levels of cytokines induced after 24h were 1012 ± 86 pg/ml for TNF-α, 18 ± 2 pg/ml for IL-1β and 46 ± 2 pg/ml for IL-10. However, IL-6 was constitutively expressed in microglia (1010 ± 215 pg/ml) but not T cells. Glatiramer acetate pretreatment of T cells reduced the level of inducible cytokines in co-culture with microglia but did not alter the level of expression of IL-6. Values are mean ± SEM of triplicate cultures.

Since MS affects primarily adults, we evaluated further the effect of glatiramer acetate on T cell – microglia interactions using neural cells from adult humans. The engagement of adult microglia with T lymphocytes led to the upregulation of IL-10 and TNF-α from previously undetectable levels, and this was also the case for IL-1β, another pro-inflammatory cytokine associated with MS (Schrijver et al., 1999). IL-6 was constitutively expressed at high levels by microglia (Figure 42). The pretreatment of T cells with glatiramer acetate resulted in a dose-dependent inhibition of the inducible cytokines (IL-1β, IL-10 and TNF-α), but glatiramer acetate did not affect the expression of the constitutive cytokine, IL-6 (Figure 42).

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 have 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 does not result in increased production of TNF-α. Moreover, it is necessary that T cells are pretreated with glatiramer acetate since its reducing effect on cytokine production does not occur if it is added at the time of co-culture. Indeed, our current results indicate that the pretreatment period of T cells should be at least 24h before these cells are co-cultured with microglia.

7.2.2B. *Effect of GA on morphological changes in microglia-T cell co-cultures*

I have previously shown that when microglia encounter activated T cells, the morphology of microglia transforms from a ramified/bipolar morphology to an ameoboid rounded form (Chapter 4/Chabot et al., 1999). This is reminiscent of an activated

microglia in vivo which transforms progressively from a ramified resting morphology to an ameoboid form (Bo et al., 1994). In Figure 43, we have reproduced our previous data and demonstrate that microglia isolated from adult human biopsies transforms to an ameoboid morphology when co-cultured with activated T cells. 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. This was also the case for fetal human microglia or PMA/IFNγ-treated U937 cells in co-culture with activated T cells (data not shown). 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.

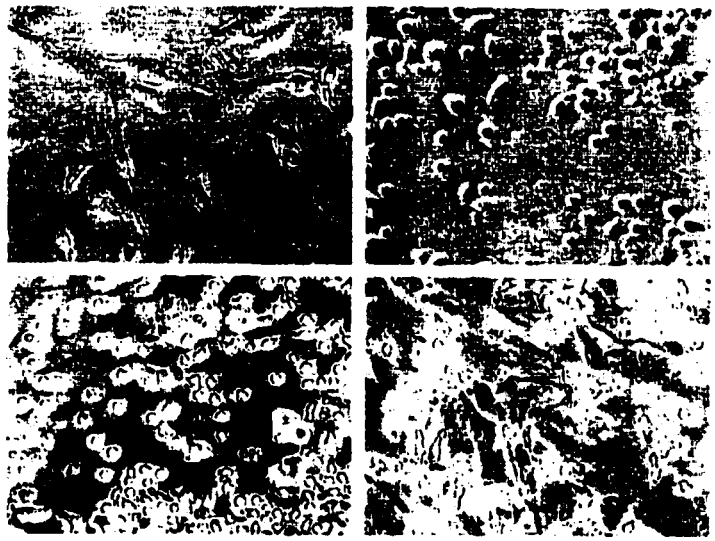


Figure 43: Morphology of microglia in microglia-T cell co-cultures. Adult human microglia are mostly bipolar in morphology in culture (A). T cells are present as single cells or clumps (B). When T cells are co-cultured with microglia in the absence of glatiramer acetate, bipolar microglia become rounded/ameboid 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 magnification, 400x.

7.2.3. *Discussion*

The treatment of patients with MS has improved significantly in the past few years, with drugs such as glatiramer acetate and interferon-β having a favorable impact on the clinical course of the disease. Despite this, the mechanisms of how these drugs work in MS remain incompletely understood (Yong et al., 1998b; Noseworthy, 1999). The experiments presented in this chapter aimed to increase the understanding of how glatiramer actetate may work in MS. Although glatiramer acetate is known to affect antigen presentation (Teitelbaum et al., 1992, 1996; Racke et al., 1992; Ben-Nun et al., 1996; Arnon et al., 1996; Fridkis-Hareli and Strominger, 1996; Aharoni et al., 1999), I sought to uncover other potential mechanisms of its efficacy, particularly those that relate to the CNS.

When T lymphocytes enter the CNS, they encounter neural cells that include microglia. A prevailing view on lymphocyte reactivity within the CNS of MS patients is that T lymphocytes become reactivated when they encounter antigens presented by antigen presentating cells such as microglia. However, as noted earlier, it is believed that the majority of T cells that enters the brain of EAE animals are antigen non-specific (Sedgwick et al., 1987; Cross et al., 1993), raising the possibility that activated T cells may engage CNS constituents such as microglia in a non-antigen dependent manner to evoke cytokine production. Our model of T cell – microglia interaction would support such a postulate, since cytokine production occurs in the absence of any identifiable antigen, and because cytokine production occurs whether or not the microglia and T cells are MHC matched or mismatched (Chapter 3 / Chabot et al, 1997). Thus, we would suggest that the interaction between activated T cells and microglia is a prominent

engagement that generates inflammatory cytokines within the CNS milieu. 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 (Raivich et al., 1998). In a graft versus host disease model, activated microglial cell clusters were invariably intimately associated with T cell infiltrates (Sedgwick et al., 1998). In this light, it is significant that glatiramer acetate treatment of T cells impairs their subsequent ability to interact with microglia, at least with respect to cytokine production. It is noteworthy that while T cell – microglia interactions induce the production of a large spectrum of cytokines, this induction was negated by glatiramer acetate treatment of T cells. Our findings would suggest that even if T cells infiltrated the CNS of MS patients on glatiramer acetate therapy, given the lack of effect of this drug on leukocyte transmigration, the T cells would be unable to promote inflammation within the CNS. The lack of production of TNF-α, a cytokine that is capable of killing oligodendrocytes (Louis et al., 1993; Loughlin et al., 1994; Selmaj et al., 1995; D'Souza et al., 1996), would also lessen the degree of oligodendrocyte loss and demyelination in the brain of patients on glatiramer acetate.

At present, the mechanisms by which glatiramer acetate affect cytokine production in T cell – microglia interactions are being investigated. Some clues may be found in the observations that T cells, rather than microglia, have to be preincubated with glatiramer acetate, and that a period of at least 24h of pretreatment is necessary. Also, only inducible but not constitutively expressed cytokines are affected.

Immune deviation is a concept that has gained attention in recent years. This concept has its origin in the observation that uncommitted T cells can differentiate along

either the Th1 route, with the production of pro-inflammatory cytokines such as IFNγ, IL-12 or TNF-α, or into the Th2 pathway and the production of Th2-like anti-inflammatory cytokines, including IL-4, IL-10, IL-13 or transforming growth factor-βs (TGFβs); cells that produce TGFβs have been referred to as Th3 cells. Susceptibility to EAE in mice or rats correlates with a predominant Th1 response to myelin antigens and resistance to disease induction correlates with a predominant Th2 response (Mustafa et al., 1991; Kuchroo et al., 1994). In concordance with EAE, a Th1 cytokine profile is associated with relapse in MS while Th2 cytokines are thought to predominate during clinical recovery (Brod et al., 1991; Reickmann et al., 1995; Nicoletti et al., 1996; van Boxel-Dezaire et al., 1999; Huang et al., 1999). Drugs such as IFN-β has been reported to induce a Th2 immune deviation in MS (reviewed in Yong et al., 1998b; Kozovska et al., 1999) while glatiramer acetate, at least in EAE, may induce Th2 suppressor/regulatory cells that down-regulate the disease (Aharoni et al., 1993, 1997; Teitelbaum et al., 1999). In MS patients on glatiramer acetate therapy, serum IL-10 levels were elevated, as were transcripts encoding IL-4 and TGFβ in PBMCs; in contrast, level of TNF-α mRNA was reduced (Miller et al., 1998). Thus, the results in the systemic circulation would suggest that glatiramer acetate causes a shift from a Th1 profile to a Th2-like state. It is not clear if such an immune deviation occurs within the CNS of MS patients on glatiramer acetate therapy. Our results, using an antigen independent system, suggests that glatiramer acetate does not have a preferential effect on Th1 or Th2 type cytokines within the CNS, since all inducible cytokines, including TNF-α, IL-4, IL-10, IL-12 and IL-13, are suppressed in the T cell – microglia interactions, creating a non-inflammatory milieu.

In summary, results demonstrate that the pretreatment of T cells with glatiramer

acetate results in a substantial diminution of cytokines that are induced following T cell – microglia interaction (Figure 44). I postulate that cytokine production in the brain of MS patients on glatiramer acetate therapy is significantly attenuated, even when there is lymphocyte influx.

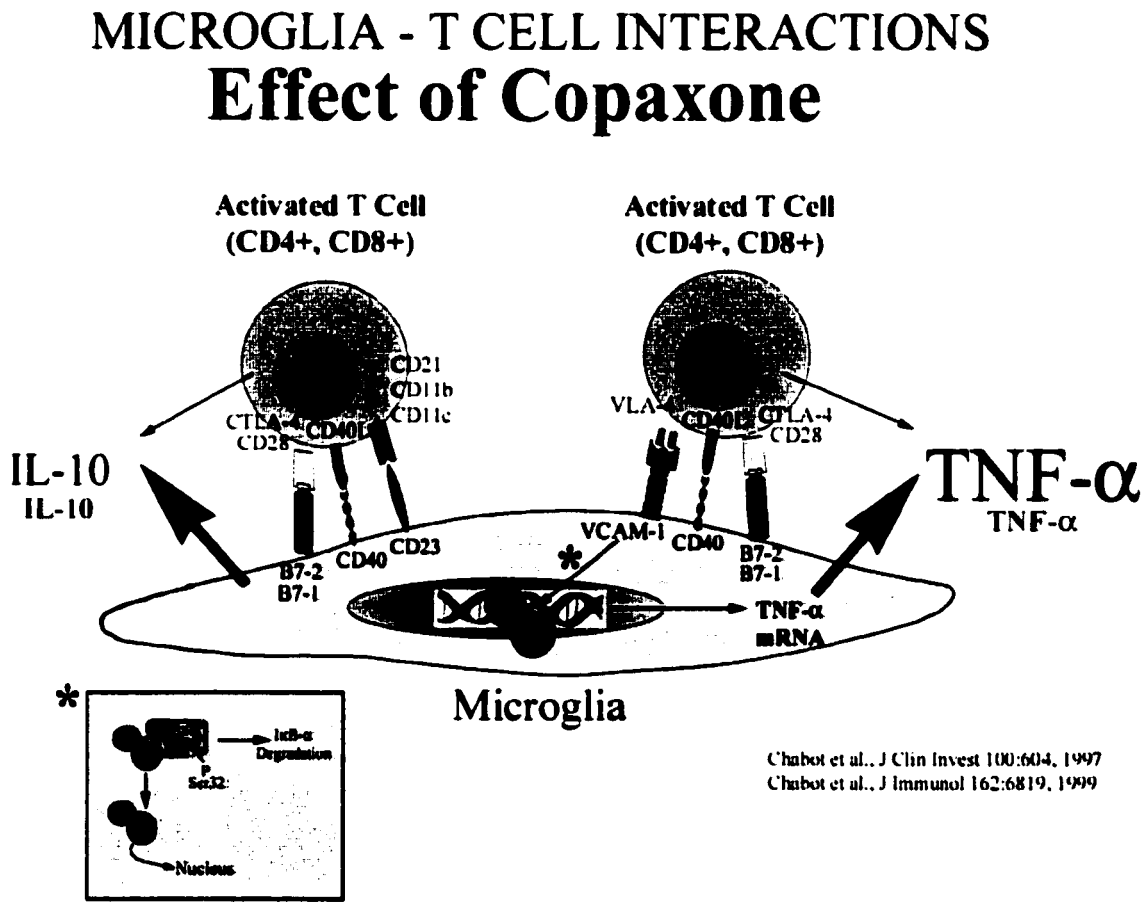


Figure 44: Schematic representation of the effects of GA on cytokine production in microglia-T cell co-cultures

CHAPTER EIGHT

Summary and Conclusions

8.1. Summary of results

Multiple sclerosis is a chronic inflammatory disease of the CNS, which is characterized by oligodendrocyte loss and demyelination, axonal atrophy, and neurological functions. Demyelinating MS lesions, a histopathological hallmark of MS, are dominated by activated T lymphocytes, the majority of which are thought to be antigen non-specific, activated microglia and augmented levels of inflammatory cytokines. Given these characteristics, the general hypothesis of this thesis is that activated antigen non-specific T lymphocytes interact with microglia to produce inflammatory cytokines, which include TNF-α and IL-10, following their infiltration into the CNS, thereby contributing to the amplification of inflammatory responses in MS lesions. In order to test this hypothesis, an *in vitro* model of microglia-T cell interaction was used, where anti-CD3-activated T lymphocytes are co-cultured with human adult microglia, human fetal microglia or PMA/IFNγ-treated U937 cells. Five objectives were designed to test the proposed general hypothesis.

The first objective of this thesis was to determine whether TNF-α is produced from microglia-T cell interactions, and if so, to elucidate the mechanisms involved. TNF-α is a pro-inflammatory cytokine involved in many inflammatory conditions such as Crohn's disease, rheumatoid arthritis, cachexia, AIDS and MS. TNF-α is produced mainly by cells of the macrophage-lineage, which includes microglia in the CNS. Results presented in chapter 3 demonstrate a mechanism through which TNF-α is generated by microglia. In isolation, OKT3-activated T cells or human adult microglia secrete negligible amount of

TNF-α. However, significantly enhanced levels of TNF-α are found in the conditioned medium of microglia-T cells co-cultures. We show that activated human T lymphocytes induce the microglial production of TNF-α, which is attenuated by a functional blocking antibody to CD49d, the alpha chain of the VLA-4 (α4β1) integrin on T cells. Furthermore, cross-linking of VCAM-1, the receptor of VLA-4 present on microglia, results in an increase in TNF-α mRNA levels, further indicating that VLA-4-VCAM-1 interactions between microglia and T cells is one of the mechanisms by which TNF-α production occurs in microglia-T cell interactions.

The second objective of this thesis was to determine whether IL-10 is also produced from microglia-T cell interactions, and to identify the mechanisms involved. Interleukin-10 (IL-10) is a cytokine with important anti-inflammatory properties, and is generated within the CNS during neuro-inflammation. The mechanism for its production is poorly understood. Using our *in vitro* human microglia-T cell co-culture system, the mechanisms of IL-10 production were addressed. We demonstrate that microglia or activated T cells alone secrete negligible amounts of IL-10 but that their co-culture results in significant IL-10 production, which was effected by both cell types. IL-10 generation was found to be cell contact-dependent; and treatment with anti-CD40, CTLA-4-Fc or anti-CD23 decreased IL-10 content in microglia-T cell co-cultures. The combination of anti-CD40 and CTLA-4-Fc reduced IL-10 levels to the negligible amounts seen with T cells or microglia in isolation. By also measuring TNF-α levels, specificity of cytokine regulation was observed, whereby anti-CD40 and CTLA-4-Fc reduced IL-10 and TNF-α levels, and anti-CD23 did not affect TNF-α while attenuating IL-10 generation. Anti-VLA-4, which decreased TNF-α levels, did not affect IL-10.

These results implicate the CD40, B7 and CD23 pathways in IL-10 production following microglia-T cell encounter, and have relevance to the regulation of an anti-inflammatory response within the CNS.

To study at the molecular level the mechanisms involved in cytokine production in microglia-T cell interactions, a large number of cells are necessary. Given the difficulty of obtaining large numbers of microglia, particularly from human sources, it is thus useful to identify human cell lines that mimic the properties of microglia to allow limitless biochemical, molecular and immunological studies to be performed to facilitate our understanding of microglia biology. In the third objective of this thesis, I investigated whether interactions between cells can serve as a model of human adult microglia. Results presented in chapter 5 demonstrate that interactions between PMA/IFNγ-treated U937 and activated T lymphocytes results in the production of cytokines, such as TNF-α, IL-12, IL-10, and IL-4. Furthermore, as shown with human adult microglia, pretreatment of PMA/IFNγ-treated U937 cells with CTLA-4-Ig blocked both TNF-α and IL-10 production induced by cognate interactions with activated T lymphocytes, while anti-CD23 inhibited IL-10 release only. Moreover, the morphological transformation of PMA-treated U937 cells upon co-culture with activated T cells mimics that observed in microglia-T cells co-culture. Given their resemblance to microglia with respect to interaction with activated T cells, I propose that PMA/IFNγ-treated U937 cells can serve as a model for human adult microglia.

In the first objective, evidence suggests that VLA-4-VCAM-1 engagement plays a role in TNF-α production generated from microglia-T cell interactions. Using PMA/IFNγ-treated U937 as a model of microglia, we investigated in objective 4 whether

VCAM-1 serves as a signaling receptor in microglia activation. We have demonstrated that the cross-linking of VCAM-1 on PMA/IFNγ-treated U937 cells leads to the phosphorylation and degradation of the NF-κB inhibitor, IκB-α, followed by the translocation of NF-κB into the nucleus. The translocated nuclear NF-κB binds to its consensus DNA sequence, which results in NF-κB-dependent TNF-α transcription. Taken together, these results demonstrate a novel functional role for VCAM-1 in macrophage activation, where it serves as a signaling receptor that induces the activation of NF-κB, and the transcription of NF-κB-dependent genes, such as TNF-α.

Interferonβ-1b (IFNβ-1b) and glatiramer acetate are two drugs currently used in the treatment of relapsing-remitting MS, although their mechanisms of action remain unclear. The last objective of this thesis investigated whether these drugs affect the production of cytokines generated from microglia-T cell interactions, a mechanism through which these drugs may be effective in MS.

In chapter 7, results demonstrate that IFNβ-1b reduces the production of TNF-α possibly by down-regulating the expression of CD49d, the alpha chain of VLA-4, on the surface of OKT3-activated T lymphocytes. In contrast, pretreatment of T cells with IFNβ-1b prior to their co-culture with human adult microglia, human fetal microglia or PMA/IFNγ-treated U937 cells results in increased production of IL-10. The enhancing effect of IFNβ on IL-10 requires cell-cell contact, but does not seem to depend on pathways that we have implicated in microglia-T cell interactions (chapter 3), involving CD40, CD23 and B7. In addition, IFNβ inhibits the production of other cytokines, including IL-1β, IL-4, IL-12, and IL-13 in co-cultures of T cells with microglia or PMA/IFNγ-treated U937 cells. I propose that the increase of IL-10 in the microglia-T cell

interaction by IFNβ, together with a decrease of other cytokines, lead to a non-inflammatory milieu in the CNS, and that this is a novel mechanism that helps account for the efficacy of IFNβ in MS.

The efficacy of glatiramer acetate in multiple sclerosis (MS) is thought to involve interference with antigen presentation; however, other mechanisms cannot be excluded. In chapter 7, I evaluated whether glatiramer acetate affects microglia-T cell interactions. Significantly, T lymphocytes pretreated with glatiramer acetate had a reduced capacity to induce cytokine production (TNF-α, IL-1β, IL-4, IL-10, IL-12 and IL-13) in their subsequent encounter with human microglia or PMA/IFNγ-treated U937 cells. Morphological transformation of bipolar/ramified microglia into an activated ameboid form was also reduced by glatiramer acetate. These results suggest that glatiramer acetate impairs the ability of T cells to effectively interact with microglia to produce inflammatory cytokines. The net result of a non-inflammatory milieu, in spite of T cell infiltration, may help account for the reduced severity of disease observed in patients on glatiramer acetate treatment.

In summary, I conclude that the interaction of activated Ag-non-specific T cells with microglia may be an important pathogenic event of MS since it induces the production of inflammatory cytokines, and that IFNβ and glatiramer acetate modulates this production of cytokines to create a non-inflammatory milieu in the CNS. This mechanism may help account for their efficacy in MS.

8.2. *What is the role of activated antigen-non-specific T cells in MS?*

The infiltration of activated T lymphocytes into MS lesions is a prominent feature of MS pathology. It is unclear how T cells within the CNS aggravate MS. A prevailing view supposes that T cells within the CNS re-encounter antigen (Ag), whereupon their reactivation and secretion of toxic metabolites occur (Noseworthy, 1999; Conlon et al., 1999). A difficulty with this view is that the majority of T cells (over 95%) that enter the brain of MS patients are thought to be non-reactive to myelin or Ag-non-specific. Indeed, studies in EAE demonstrated that activated T lymphocytes present in demyelinating areas are primarily Ag-non-specific, since only 1.5% of all T cells were shown to be MBP-reactive (Sedgwick et al., 1987; Cross et al., 1993). So far, the role of activated Ag-non-specific T cells in MS has been underestimated. However, given the predominant presence of Ag-non-specific activated T cells in MS lesions, it is likely that Ag-non-specific T cells play an active role in the pathogenesis of MS in engaging CNS constituents in Ag-non-specific dependent manner. Results of this thesis demonstrate that activated Ag-non-specific T cells interact in a contact-dependent manner with microglia to produce inflammatory cytokines, suggesting for the first time that they participate actively in the pathology of MS. By inducing the production of cytokines, such as TNF-α, Ag-non-specific T cells may act as regulators of the inflammatory response observed in MS lesions, and may contribute to the process of demyelination by causing the apoptotic death of oligodendrocytes. In addition, by activating microglia, activated Ag-non-specific T cells contact dependent signals may trigger myelin phagocytosis by microglia, another mechanism by which demyelination is thought to occur.

Based on studies performed in EAE, it is believed that MS is an autoimmune diseases that is mediated by autoreactive CD4+ Th1 cells reactive to myelin protein

encephalitogenic epitopes. However, it has been difficult to firmly demonstrate this hypothesis since T cells reactive to myelin antigens exist not only in MS patients but also in healthy individuals (Pette et al, 1990; Hafler et al, 1987; Olsson et al, 1992). Also, the nature of the myelin antigen(s) responsible for the autoimmune attack in MS remains unknown. It is therefore not clear whether MS is a T-cell mediated disease.

There is evidence to suggest that MS may be a microglia-mediated disease. First, it was shown that acute MS lesions sometimes develop in the absence of T cell infiltrates (Cuzner, 1997), and secondly, microglia have the ability to actively participate in the process of demyelination by phagocytosing myelin (Li et al, 1996; Williams et al, 1994; Trotter et al, 1986; Reichert et alk, 1994). Thus, if microglia activation is an important finding in MS, I propose that microglia-T cell interaction is a pathogenic event as it results in microglia activation.

8.3. *How are microglia activated in MS lesions?*

Microglia are often referred as CNS macrophages. Under normal conditions, microglia are found in their resting state with a ramified morphology. Under neuro-inflammatory conditions occuring during inflammatory diseases such as MS, microglia become activated and function as accessory cells for T cell activation, as pro-inflammatory cells, as effector cells which mediate tissue damage, and as anti-inflammatory cells which promote wound healing. Mechanisms of microglia activation are not very well understood. In addition to the roles of soluble factors, such as IFNγ, TNF-α, LPS, and complement fragments, in promoting the microglia activation, the results of this thesis demonstrate for the first time that contact-dependent signaling

occuring in microglia-T cell interactions is another important mechanism of microglia activation. Moreover, the data shows that the T-cell-contact-dependent signals are as potent as LPS to activate microglia and induce their production of inflammatory cytokines. Given that the migration of activated T cells to the CNS is a consistent and predominant pathological feature of MS, and that infiltrated T cells are located in close proximity to the microglia in MS lesions, one may propose that T cell contact-dependent signaling is an important mechanism of microglia activation in MS.

8.4. *How are cytokines produced in MS lesions?*

Cytokines are important regulators of inflammation, including neuro-inflammation, and are often considered as potential targets for the development of therapeutic strategies that are aimed to treat inflammatory diseases, such as MS. Thus, an obvious step towards the achievement of this goal is to understand the mechanisms involved in cytokine production. To date, mechanisms of cytokine production remain unclear, but appear to depend on soluble factors and cell-cell interactions. The results of this thesis further demonstrate cell-cell interaction is a mechanism of cytokine production in the CNS, which may involve microglia and activated T cells. Therefore, as a treatment of MS, it may be of interest to design therapies that can block microglia-T cell interactions to prevent the production of cytokines in the CNS and subsequent CNS inflammation.

8.5. *Microglia-T cell interaction in other CNS diseases*

Microglia activation appears to play a central role in the pathogenesis of CNS pathologies other than MS where CNS infiltration of T cells is also known to occur.

These include cerebral ischemia, traumatic brain injury, experimental globoid cell dystrophy, and cerebral malaria. Since our system did not involve any specific antigen, it is possible that the results of the present thesis also apply to the pathogenesis of these CNS pathologies. This possibility remains to be explored.

8.6. *Future Directions*

To test the hypothesis proposed in this study, an in vitro experimental model of microglia-T cell interactions was used. Results obtained from this model suggest that microglia-T cell interaction is a pathogenic event of MS because it results in the production of inflammatory cytokines. In order to confirm this hypothesis, it would be important to study microglia-T cell interactions *in vivo* using EAE, as an animal model of MS. To mimic our microglia-T cell system, T cells may be isolated from lymph nodes of EAE animals and activated *in vitro* with anti-CD3 in the presence of allogeneic macrophages following the same protocol of activation used for the studies presented in this thesis. Then, an adoptive transfer of anti-CD3 activated T cells to a recipient syngeneic animal could be performed and the disease could be evaluated using behavioral, histological and molecular tools. Adoptive transfer of T cells activated by other means, such as PMA, and PHA would also be useful. Finally, it would be instructive to evaluate whether T cells aggregate around microglia at any point during the EAE process, and whether treatment of IFNβ and GA affects this aggregation.

Microglia-T cell interactions can result in either T cell activation and T cell death (Sedgwick et al, 1998; Ford et al, 1996). In order to gain some insights into microglia-T

cell interactions *in vivo*, it would be interesting to determine the fate of microglia and T cells following their interaction *in vitro*.

The difference between Ag-specific and Ag-non-specific system are not very well determined. Thus, to better understand these differences, experiments performed in this thesis could be repeated using an Ag-specific system in order to determine whether cytokines production are dependent on mechanisms similar to those described using an Ag-non-specific system.

Results show in chapter 7 that IL-1β, IL-4, IL-12 and IL-13 are also produced from microglia-T cell interactions, however, the mechanisms underlying their production have not been addressed in this thesis. There is evidence that cytokines affects neuronal response. For instance, when produced in the CNS, IL-1β can mediate the activation of the hypothalamic-pituitary adrenal axis by inducing the release of corticotropin releasing factor from hypothalamic cells (Tsagarakis et al, 1989). In long-term potentiation (LTP) studies, it was also shown that IL-1β inhibits LTP in area CA1 and CA3 of the rat and mouse hippocampus, respectively, by either blocking of calcium channel currents through an effect on PKC activation (Bellinger et al, 1993; Katsuki et al, 1990; Cunnigham et al, 1996; Plata-Salaman and ffrench-Mullen, 1994; Araujo and Cotman, 1995), or by prolonging GABAergic-mediated synaptic inhibition (Zeise et al, 1992). Given the modulatory functions of cytokines on neuronal response, and because cytokine dysregulation is a feature of various CNS inflammatory diseases, including MS, it would be of great importance to study mechanisms by which cytokines are produced from microglia-T cell interactions.

In chapter 3, I demonstrated that TNF-α is produced from microglia-T cell interactions in both allogeneic and syngeneic systems. I have shown that other cytokines (i.e. IL-1β, IL-4, IL-10, IL-12 and IL-13) are produced in an allogeneic system, but it is still unknown whether this production of cytokine also occurs in a syngeneic system. It is also worth noting that results obtained using an allogeneic system have relevance to the pathogenecity of graft-versus-host disease.

Results presented in chapter 3 show that the blockade of α4-dependent binding inhibits TNF-α production in microglia-T cell interaction. Since α4 can form a heterodimer with either β1 or β7, it would of interest to determine the role of β subunits in cytokine production from microglia-T cell interaction.

Since inflammatory cell types other than activated T cells are known to express VLA-4, a molecule involved in TNF-α production from microglia-T cell interactions, one may not exclude the possibility that cognate interactions of microglia with other VLA-4-expressing cells, such as neutrophils, may also result in cytokine production. This theory should be further explored as it may play an important role in the pathogenecity of CNS neutrophil-dependent diseases, such as stroke.

The population of activated T cells used in the experimental model described in this thesis consist mainly of CD3+ T cells, but other cell types were also present in low numbers, such as NK cells and B lymphocytes. Thus, using purified populations, it would be interesting to determine whether NK cells or B lymphocytes interact with microglia to produce cytokines, and to determine the mechanisms involved. Moreover, because the CD3+ T cell population may consist of both αβ and γδ T cells, it would be interesting to

determine the role of these T cells subpopulation in inducing the production of cytokines when interacting with microglia.

ELISA was used as a mean to quantify cytokines produced from microglia-T cell interactions. Although this approach indicates the concentration of cytokines produced, it does not represent the biological activity of those cytokines which may vary according to their post-translational modifications (van Damme et al, 1999). Therefore, it would be important to perform biological assays as a measure of cytokine activities.

I have demonstrated that IFNβ and GA inhibits TNF-α production in microglia-T cell interactions. Since TNF-α can cause the apoptotic death of oligodendrocytes (D'Souza et al, 1995; Ladiwala, 1998), one may investigate whether these drugs inhibit TNF-α killing in a system where oligodendrocytes are exposed to microglia-T cell co-cultures, where the later may be placed in a cell culture insert.

Finally, it remains unclear how IFNβ and GA affect cytokine production in microglia-T cell interactions. Thus, in order to gain further insight into the modes of actions of these drugs, future experiments will be necessary to determine the mechanisms involved. The relevance of such experiments is of significance as it may lead to the development of improved therapies for MS.

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