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# Regulatory monokines in Multiple Sclerosis and the effects of IL-10 and IFN-β1a on their levels

By

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# ABSTRACT

Multiple Sclerosis (MS) is a chronic, autoimmune disease of the central nervous system. MS is believed to be a Th1 inflammatory disease, speculated to be mediated by CD4+ T lymphocytes. The specific contribution of monokines to disease has not been extensively studied in MS. I thus hypothesize that Th1 regulatory monokine levels are elevated in MS patients, and these increased levels correlate to disease severity. This project deals with the quantitation of regulatory monokine mRNA and protein levels in MS patients and their subsequent comparison to healthy control levels. The influence of cytokine-directed immunotherapy, specifically IFN-β1a and IL-10, on these monokine levels is also addressed.

Highly enriched monocytes were isolated and separately cultured for 24 hours in the presence of RPMI media alone, or supplemented with IFN-β1a or IL-10. Protein and mRNA levels were determined for various monokines by intracellular staining, ELISA, and Riboquant RNase Protection Assay. Increased monokine levels of IL-1, IL-6, IL-12, and TNF-α were found to correlate significantly with increased MS disease severity as compared to healthy control levels. IL-10 and IFN-γ were also detected in MS patient samples, however these levels did not differ significantly from healthy control levels.

IL-10 24-hour exposure was found to significantly reduce all elevated MS patient monokine levels. IFN-β1a showed minimal effects in reducing elevated MS patient monokine levels, and in certain patients boosted their Th1 and proinflammatory monokine levels.

These findings suggest a role for monocytes and monokines in the immunopathogenesis of MS, and also indicate further study into IL-10 as a potential MS therapy may be warranted.

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| | |
|---|---|
| BSA | Bovine serum albumin |
| CD | Cluster of differentiation |
| CNS | Central nervous system |
| CTL | Cytotoxic T lymphocytes |
| ELISA | Enzyme linked immunosorbent assay |
| FCS | Fetal calf serum |
| FITC | Fluorescein isothiocyanate |
| GMCSF | Granulocyte macrophage colony stimulating factor |
| HC | Healthy controls |
| HIV | Human immunodeficiency virus |
| HLA | Human leukocyte antigen |
| IFN | Interferon |
| Ig | Immunoglobulin |
| IL | Interleukin |
| MAP | Myelin associated protein |
| MBP | Myelin basic protein |
| MCP | Monocyte chemotactic protein |
| MCSF | Macrophage colony stimulating factor |
| MFI | Mean fluorescent intensity |
| MHC | Major histocompatibility complex |
| MIP | Macrophage inflammatory protein |
| MOG | Myelin oligodendrocyte glycoprotein |

| | |
|---|---|
| mRNA | Messenger ribonucleic acid |
| MS | Multiple sclerosis |
| NK | Natural killer |
| PAF | Platelet activating factor |
| PBMC | Peripheral blood mononuclear cell |
| PE | Phycoerythrin |
| PFA | Paraformaldehyde |
| PGE2 | Prostaglandin E2 |
| PLP | Proteolipid lipoprotein |
| PMN | Polymorphonuclear neutrophils |
| RNA | Ribonucleic acid |
| RR | Relapsing remitting |
| RR/T | RR undergoing immunosuppressive therapy |
| SP | Secondary progressive |
| TCR | T cell receptor |
| Th | T helper |
| TNF | Tumour necrosis factor |
| TRI | PE linked to CY-5 |
| US-FDA | United States Food and Drug Administration |
| VCAM | Vascular cell adhesion molecule |
| VLA | Very late antigen |

# I. INTRODUCTION

## A. THE IMMUNE SYSTEM

The world surrounding us contains a multitude of organisms capable of infecting our bodies and causing disease. If left to explore our bodies unchecked and continually flourish and multiply, these organisms would lead to our untimely deaths. Our bodies do however, contain a mode of combating any unwanted foreign intruders. This mechanism is known as the immune system.

Two categories of immune responses exist. The first category is a nonspecific mode of protection. It involves clearing microbes through physical and chemical barriers, complement proteins which control inflammation, phagocytic cells such as macrophages, and other leukocytes such as natural killer cells (NK) (1, 2, 3). The second form, the adaptive response, is highly specific for a particular pathogen or antigen. Adaptive immunity is an extremely specialized response housing the ability to remember past encounters with an organism, thus allowing for a faster and more vigorous attack upon repeated exposures (2, 4, 5). The components of adaptive immunity involve T lymphocytes, B lymphocytes and various lymphocytic products, such as cytokines and antibodies. Each antibody is specific for a particular determinant on an antigen, known as an epitope (1, 2, 4, 5). The components and actions of both the innate and specific immune responses can function cooperatively to combat an intruder, thus allowing for an increased protective response (1, 2, 4, 5).

Many different immune cells mediate the innate and adaptive responses. Examples of such important cells include phagocytes, lymphocytes, and monocytes. The

mononuclear phagocyte system consists of cells all of a common lineage and is one of the major cell populations of the immune system. These cells interact in both innate and adaptive immune responses. The function of these cells is to engulf, internalize, and degrade particles (1, 6, 7). These cells, which are all derived from a bone marrow precursor, can be found dispersed throughout the body in areas where they will encounter antigen. These cells are also responsible for the presentation of degraded antigen fragments, in conjunction with an endoplasmic reticulum-originating major histocompatibility complex (MHC), to circulating lymphocytes for their recognition and dual cell activation (5, 7). Examples of such mononuclear phagocytic cells include blood-derived monocytes, tissue-derived macrophages, Kupffer cells in the liver, interdigitating dendritic cells present in the interstitium of most organs, Langerhans cells in the skin, and microglia in the central nervous system. (6, 7, 8, 9).

Another important phagocytic cell is the granulocyte. Peripheral blood contains three types; neutrophils, eosinophils, and basophils. These cells are recognizable due to the presence of multi-lobed morphologically diverse nuclei (9, 10). Of the polymorphonuclear cells (PMN), neutrophils are the most numerous. These immune cells develop from the same early precursor as monocytes and perform similar functions. In response to stimuli, PMN migrate into tissues and subsequently engulf and degrade material. Neutrophils, however, have a very short life span and die following this degradation process (9, 10). Eosinophils are important in allergic reactions and the engulfment of IgE antibody-coated organisms, such as helminthes and their components (1, 5). Basophils are also important granulocytic effector cells involved in immediate IgE-mediated hypersensitivity (1).

Lymphocytes are also extremely important in the innate and adaptive immune responses. Specific antigen recognition and adaptive immune response initiation occur mainly due to the acts of lymphocytic cells. Like phagocytic cells, lymphocytes are derived from bone marrow stem cells (6). Subsequently, however, T lymphocytes develop in the thymus while B lymphocytes mainly develop in the bone marrow (11, 12, 13). B lymphocytes are the only cells capable of producing antibodies. B cells house an immunoglobulin receptor on their surface, which is antigen specific. Interaction of antigen with this membrane-bound antibody surface receptor initiates B lymphocyte activation, resulting in effector cell development and antibody secretion (5, 6).

T cells may be divided, by their T cell receptor (TCR) variable genes, into $\alpha\beta$ and $\gamma\delta$ subtypes (12). $\alpha\beta$ T cells constitute greater than ninety-five percent of the T cells in the blood. Two groups of $\alpha\beta$ T cells exist, those that interact with B cells, which aid in antibody production, and those that interact with mononuclear phagocytes, which aid in pathogen destruction. T cells that assist in the activation and functions of other cells, and are CD4+, are deemed helper T cells (12, 14, 15, 16). The other subset of $\alpha\beta$ T cells that exist, which are responsible for infected host cell destruction, are CD8+ and referred to as cytotoxic T cells (CTL) (12, 17). T cells employ T cell receptors on their surfaces to recognize presented antigen on the surface of cells, but only when it is presented in conjunction with a MHC, as mentioned previously (18, 19, 20). This recognition results in either the release of soluble mediators known as cytokines, which signal to other cells, or direct antigen presenting cell-T cell interaction (5, 6, 20). $\gamma\delta$ T cells are phylogenetically older than $\alpha\beta$ cells and are responsible for more primitive, MHC-

independent, immune responses to gut-derived bacterial antigens and heat shock proteins (21, 22).

Specific immune responses involving T helper lymphocytes can be divided into two types based on the cells and cytokines of the immune response employed. Cell-mediated immunity, also known as a Th1 response, involves T lymphocyte activated phagocytic cells and involves the destruction of microbes or infected cells (16). Th1 responses also involve the production of pleiotropic soluble mediators, known as cytokines, such as IL-2 and IFN-γ (23, 24). Humoral immunity, believed to be associated with the Th2 response, involves antibody production, due to the acts of T and B lymphocytes. Th2 responses also involve the production of cytokines, such as IL-4, IL-5, and IL-13 (1, 14, 23, 24). The various functions of cytokines will be discussed later.

These various features and cellular components of humoral and cell-mediated immunity are absolutely necessary if the immune system is to work effectively at host defense. Specificity, memory, diversity, immune response self-limitation and self / non-self discrimination, are all extremely important fundamental properties of these complex immune system functions (5). Together, the cellular constituents of humoral and cellular immunity, and their interactions, when functioning properly enable our bodies to fight off pathogenic invaders and keep us healthy.

## B. MONOCYTES

A key player residing at the backbone of many immune responses is the monocyte. Monocytes are known to differentiate from pluripotent stem cells. The next step in monocyte differentiation involves the development of CD34 on the cell surface, which is

then followed by an intermediate step known as the monoblast (6, 25). The production of monocytes in the bone marrow is controlled by many different cytokines. Stimulation of monocyte production can occur due to IL-3, and various colony-stimulating factors (GM-CSF and M-CSF). Conversely IFN-α and IFN-β are known to inhibit monocyte production (6, 26). Monocytes are the first cells known to leave the bone marrow and enter the peripheral blood (1, 6, 27).

Monocytes are 10 – 15 μm in diameter and incompletely differentiated. Monocytic cytoplasm is finely granular containing lysosomes, phagocytic vacuoles, and cytoskeletal filaments along with a single bean-shaped nucleus (1, 7). Blood monocytes show much heterogeneity in morphology, metabolic activity, and surface marker expression. Monocytes are known to express the CD14 surface marker. CD14 is a well known endotoxin receptor. Both major and minor CD14 expressing monocyte subpopulations exist which show strong and weak monocyte expression of this molecule respectively (28). As well the surface expression of CD16, also known as the Fcγ receptor type III, varies between monocyte populations (28).

Under normal conditions monocytes remain in the circulation for approximately 5 days. Following this time period if non-activated, they enter tissue sites where they mature into macrophages (6,7). However, if an infection or tissue injury occurs during a monocyte's peripheral blood circulation, travelling monocytes are drawn to migrate to the specific inflamed or injured site (1, 5, 7, 8). Monocyte recruitment is regulated by unique signaling and adhesion molecule combinations (7, 26, 27). Inflammatory mediators, such as cytokines and chemokines, promote and regulate monocyte recruitment from the circulation. During tissue injury, cytokines, such as IL-1, IL-8, TNF-α, and IFN-γ, and

chemoattractants, such as complement protein C5a, leukotriene B, monocyte chemotactic protein-1 (MCP-1), and platelet-activating factor (PAF), modulate the adhesion of monocytes to endothelial cells (3, 27, 29, 30, 31, 32, 33). Once the monocytes arrive at the endothelial barrier to which they were drawn, initial rolling contacts with the endothelium are mediated by selectins. This rolling adhesion is not strong enough to anchor the monocyte to the endothelium. Firm adhesion is thus achieved by the use of other adhesion molecules. CD11a/b, CD18, and very late antigen-4 (VLA-4) on monocytes interact strongly with CD54 and vascular cell adhesion molecule-1 (VCAM-1) adhesion molecules on endothelial cells to mediate the solid adherence (26, 30). Chemokines induce the activation, followed by the subsequent deactivation, of endothelial cell bound VLA adhesion molecules. This process aids in monocyte recruitment by facilitating firm adhesion and thus eventual migration (26, 30, 34). Following firm adhesion monocytes spread over the surface of the endothelial cells and subsequently transmigrate into the subendothelial space, an act known as diapedesis (26). Following this, monocytes traverse the subendothelial matrix and basement membranes and enter extravascular sites (27). Following endothelial transmigration monocytes differentiate into exudate macrophages. In conjunction with T lymphocytes these cells usually comprise the inflammatory exudate (25, 34).

Upon entering tissue sites following blood circulation, monocytes are known to undergo apoptosis (26, 34). The ability to inhibit this process is an important mechanism so as to allow monocyte accumulation in inflammatory lesions. Many different compounds have been found to inhibit this spontaneous apoptotic mechanism, including proinflammatory cytokines such as IL-1 and TNF-$\alpha$ (26, 34). Interestingly, Th2

promoting cytokines, such as IL-4 have been found to induce enhancement of monocytic cell death upon leaving the circulation (26, 35).

Monocytes are important in both innate and adaptive immunity. During an innate immune response, monocytes phagocytose foreign particles, including microbes, complement-coated particles, and injured or dead self- tissues. Phagocytosis is followed by degradation due to lysosomal enzymes. Control of infection spread also occurs due to the monocytic production of enzymes, reactive oxygen species, and nitric oxide, which all have killing capabilities (36, 37, 38). Monocytes also produce cytokines, which are involved in the recruitment of other inflammatory cells and in many of the systemic effects of inflammation, such as fever (26, 27, 37, 39).

Monocytes play extremely important roles in the recognition, activation and effector phases of adaptive immunity. Monocytes act as accessory cells in lymphocyte activation through the processes of antigen presentation and the production of cytokines responsible for T lymphocyte proliferation and differentiation (2, 4, 19, 39, 40). Upon their own activation, monocytes become one of the principal effector cells of cell mediated immunity by their upregulated ability to destroy phagocytosed materials (27, 37). Monocytes also have an intricate role in the humoral response of adaptive immunity. Opsonization, or coating, of foreign pathogens with antibody or complement proteins, leads to an increased rate of phagocytosis since monocytes contain receptors on their surface for these proteins (3, 27, 37). Thus, the ability of monocytes and lymphocytes to activate each other's functions and act collectively to eliminate pathogens remains an extremely important mechanism of specific immunity.

Cytokine release is a common thread of monocytic action in both innate and adaptive immunity. Cytokines are soluble proteins released by cells, which are responsible for such acts as cellular activation, proliferation, and differentiation. Human monocytes can release a large spectrum of cytokines. Examples of the wide range of molecules produced by monocytes include IL-1, IL-6, IL-10, IL-12, TNF-α, and IFN-γ (26, 27, 37, 39). The cytokine profile released by a monocyte depends on the stimuli inciting its release. The function and phenotype of a monocyte depend on the cytokines that act upon it, and the manner in which they do so. Many important cytokines are known to act upon monocytes in an autocrine manner, paracrine manner, or both. Examples of autocrine acting cytokines include IL-1 and various colony stimulating factors. Cytokines that act upon monocytes in a paracrine fashion include IL-2, IL-3, IL-4, IL-7, IL-13, and IL-16. Cytokines that can act upon monocytes in both manners include IL-10, TNF-α, and IFN-γ.

Proinflammatory, IL-1 and TNF-α, and antiinflammatory, IL-4 and IL-10, cytokines both exist, and are known to exert opposing effects on monocyte function (26, 39). Monocytic cytokines are also important in specific Th development. IL-12 and IL-12 induced IFN-γ are responsible for the generation of Th1 responses, while IL-10, in conjunction with IL-4, counteracts their effect, allowing for the downregulation of Th1 and subsequent generation of a Th2 response (14, 16, 20, 37, 39).

Monocytes also release chemoattractant cytokines known as chemokines. Chemokines, in conjunction with other immune cells, are mandatory for the directional migration of leukocytes and also play important roles in hematopoiesis, cell activation, and leukocyte effector functions. Several monocyte chemotactic proteins have been

identified and cloned. Examples of monocyte chemokines include the monocyte chemotactic protein family (MCP-1, -2, -3), RANTES, and MIP-1α/β. These cytokines are both produced by and act as chemoattractants for monocytes, as well as for many other cell types (27, 31, 32, 33, 37, 39).

Monocytes play an important part in both the innate and specific (humoral and cell-mediated) immune responses. Within these responses monocytes are responsible for antigen recognition and clearance, cytokine production for lymphocyte proliferation and differentiation, chemotaxis, and various other inflammatory processes. Thus, monocytes play a central role in the functioning of all major aspects of the immune system.

## C. AUTOIMMUNITY

An individual's immune system can have the capacity to exert an immune response against autologous antigens, or self, during its normal functioning. This process can lead to tissue injury, disease, and even death. Most autoimmune reactions occur due to a breakdown, or deficiency, in the mechanisms normally responsible for self-tolerance (1, 41, 42). Self-tolerance, as mentioned previously, is an important aspect in the specific immune response. Self-tolerance involves the immune system remaining unresponsive to self-antigens during its normal functioning. Self-tolerance occurs by the active deletion or functional inactivation of lymphocyte clones specific for self-antigens during their immune system development (1, 5, 18, 41, 42, 43).

Many factors, which can interact together, contribute to autoimmunity. Autoimmune diseases may be either systemic in nature or organ-specific. The nature of the disease depends on the different antigen types present and the various occurring

immunological abnormalities (41, 42, 43). Immunological abnormalities, such as autoantibody production and self-reactive B and T cells, play major roles in many organ-specific autoimmune diseases, such as Hashimoto's thyroiditis and insulin-dependent diabetes mellitus (11, 15, 18). By acting as highly activated antigen-processing and presenting cells, monocytes and macrophages can contribute to events in many organ-specific autoimmune diseases such as rheumatoid arthritis. Through upregulated cytokine synthesis and secretion, monocytes also play a driving role in many autoimmune diseases (25, 41, 43). Monocyte/ macrophage produced cytokines are abundantly found in specific affected areas in many autoimmune diseases, such as the synovium of the joint in rheumatoid arthritis (25, 43). Systemic autoimmune diseases, such as systemic lupus erythematosus, usually occur due to immunological abnormalities such as deposition of self bound immune complexes (1, 5, 43, 44).

Genetic backgrounds also have the ability to predispose to autoimmune diseases. This predisposition shows a definite bias however, towards organ-specific autoimmune disease. Familial and twin studies have revealed genetic predispositions in many autoimmune diseases, including Hashimoto's thyroiditis, an organ-specific disease (5, 45). Of the genetic factors believed responsible for autoimmunity, disease has often been shown associated with specific MHC genes, also known as HLA haplotypes (5, 46, 47) . In autoimmune diseases with genetic predispositions however, it is usually not solely one genetic factor that is responsible for disease. Genes that predispose an individual to develop an autoimmune disease, as well as others that determine which antigen(s) are involved are both believed responsible in the development of autoimmunity (5, 42, 45, 48).

Gender-related hormonal influences are believed to play a role in many autoimmune diseases. Females are known to acquire various autoimmune diseases with a much higher incidence than do men. Systemic lupus erythematosus, for example, is known to affect females ten times more often than males. The exact physiological reasons for these incidence differences have yet to be elucidated (1, 44, 49).

Environmental factors have also been speculated for many autoimmune diseases. The effect of environment has been studied for different autoimmune diseases, such as Type I diabetes mellitus and systemic sclerosis, where geographical movement affects disease incidence (45, 50).

Infections, both viral and bacterial, are associated with and often precede many autoimmune diseases. Interestingly, the invading microorganism is not usually found in the lesions produced, or even in the individual, upon the development of autoimmunity. The lesions produced during autoimmunity are not due to the infectious organism but due to an altered host response. This host immune response must therefore be dysregulated by the microbe (23, 24, 51, 52). Infections can lead to altered immunity through polyclonal lymphocyte activation, local tissue inflammation leading to enhanced costimulator expression, self-antigen alteration to create partially cross-reactive neo-antigens, and molecular mimicry.

Molecular mimicry involves the production of an immune response against a microorganism antigen epitope which cross-reacts with a self-antigen epitope, thus redirecting the immune response against self upon subsequent recognition by memory T cells (1, 5, 23, 24, 51).

Tissue alterations, due to such things as infection, inflammation, injury, or trauma can lead to an event known as epitope spread. During an immunological attack against a specific epitope, a dispersion of the immune response may occur. This dispersion known as epitope spreading, involves the immune response subsequently directing its attack against other epitopes of the original antigen or against other self antigens. Epitope spread can also occur due to the release of cryptic antigens, which in turn can become targets of the immune system. These events, thus lead to the development of autoimmunity (23, 24, 52).

Autoimmune diseases are responsible for extreme disability, yet the exact aetiology of every disease is not known. Many factors can work together, culminating in loss of self-tolerance, and ultimately leading to an individual's immune system turning against them. Once the exact roles of self-tolerance and the various causes of autoimmunity are elucidated, much work can be done in the development of therapies for these diseases.

## D. MULTIPLE SCLEROSIS

Multiple Sclerosis (MS) is a chronic, inflammatory disease of the central nervous system (CNS) (53, 54). MS is believed to be autoimmune in nature, and is the most common human neurological disease of young adults, affecting women twice as often as men (23, 53, 55).

The central feature of this disease is myelin destruction and oligodendrocyte loss (23). Myelin is composed of a lipid bilayer membrane. Various proteins are anchored within this membrane (23). Myelin is produced by the oligodendrocyte, and is found

encircling axons. This bilayer membrane is critical for neural signaling and transmission (23, 53). Other pathological disease features include axonal loss and central nervous system atrophy. These features accumulate over many years and through continual demyelination and remyelination processes result in the formation of plaques or lesions in the CNS. Manifestations of clinical disease, due to myelin destruction and plaque formation, commonly involve paralysis, sensory disturbances, and visual impairment (23, 53).

Five main types of MS exist. Not every person who acquires MS will develop a progressive form of the disease. Only mild to moderate symptoms occur in 10% of patients. These symptoms do not worsen and do not lead to severe disability. This is known as benign MS disease. Another ten percent of patients with MS undergo a primary progressive disease course. This involves continual deterioration from the first appearance of symptoms without remission periods. Chronic progressive disease is a rare form of MS seen in less than five percent of patients. It involves a primary progressive disease course with the addition of periods of worsened or more severe symptoms. The remaining 75% of patients acquire relapsing-remitting disease. This form of MS and involves one to two flare-ups every few years followed by periods of remission. After years of relapsing disease, around fifty percent of these patients will enter a period of continual deterioration know as secondary progressive disease. (23, 53, 55, 56).

The exact aetiology of MS is unknown. Current evidence classifies MS as a T-cell mediated organ-specific autoimmune disease (54, 57). Susceptibility to MS is considered to be genetically controlled. This belief is due to findings showing MS occurs more commonly in Caucasians and is associated with genes related to immune functioning, such

as MHC alleles including DR2, DR15, and DQ6 (46, 54, 55, 57). Genetic twin studies however, indicate a concordance rate of only 20-30% for monozygotic twins and merely 4% for dizygotic twins and other siblings. Full genomic screening, among various multiplex families, revealed many susceptibility loci on different chromosomal regions, with none of the positively detected loci showing a significantly larger role. Thus, no single major MS susceptibility gene exists. Aetiologically therefore, multiple genes are believed to only exert moderate effects in conjunction with other factors, such as environment (54, 55).

Epidemiological evidence supports an environmental agent, possibly a viral or bacterial infection, in early childhood as an aetiological factor (53, 55). MS is found with greater incidence in areas with increasing latitude. Studies have also shown that migration before puberty from a high MS prevalence area to a low prevalence area results in a reduction in the risk of developing MS (53, 55). This indicates that various unknown environmental factors are operating in the aetiology of MS (53, 55).

Active demyelination in MS is known to be accompanied by inflammation. Damage to oligodendrocytes, axons, and neurons occurs as a result of nonspecific mediators of inflammation, such as complement, cytokine, and chemokine production, drawing immune cells to the central nervous system. Inflammatory infiltrates in MS are found mainly composed of T lymphocytes, activated macrophages and microglial cells, and some B lymphocytes and plasma cells (54, 58). It is generally believed that inflammation, and thus subsequent demyelination, in MS are the result of autoreactive cell- mediated and humoral responses to myelin proteins.

There are at least five mechanisms by which immunological damage to the CNS is believed to occur. These mechanisms are believed associated with bacterial or viral infection. Firstly, damage to the CNS may occur due to a CNS located antimicrobe immune response. In MS this would indicate that the microbe is housed within the myelin coated areas of the brain (59).

It is also speculated that perhaps infection may activate transcriptional machinery leading to the transcription of areas of the genome that are hypermutable, such as *alu* repeat sequences. This leads to the production of neoantigens. These neoantigens are thus recognized by the immune system as foreign and attacked (59).

A third immunological damage mechanism involves a possible antimicrobe response leading to the unregulated dispersion of the immune response. This results in a process known as epitope spread, as mentioned previously. Epitope spreading is believed to play an important role in many ongoing autoimmune diseases. Blocking of this process through costimulatory antagonists or by antigen-specific tolerance induction, has been found to result in the inhibition of clinical MS relapses (59, 60).

Another possible mechanism for MS induction is speculated through the production of molecules known as superantigens. Superantigens are microbial toxins that via engagement of the T cell receptor Vβ chain are capable of stimulating whole populations of T cells. If a superantigen triggered myelin-specific T cell clones, this could lead to eventual CNS damage (53).

Structural homologies may also exist between the invading pathogen and structures in the CNS. Immune system pathogen recognition can then subsequently trigger an immune response against the homologous self antigen. This phenomenon is

known as molecular mimicry. Myelin basic protein (MBP) has been found to share extensive homologies with many viral and bacterial pathogens including the influenza virus, measles, Epstein-Barr virus, herpesviruses, and *Pseudomonas* bacteria (51, 53, 59). Interestingly, it has been found by Hemmer et. al., using a MBP model, that cross-reactivity can occur not only with largely homologous peptides but also between epitopes that share none to only a few critical residues (61). This finding greatly expands the number of potential pathogens with the capability of cross-reacting with self antigens of the central nervous system. Other myelin proteins, speculated to be victims of molecular mimicry, include proteolipid lipoprotein (PLP), myelin oligodendrocyte glycoprotein (MOG), and myelin-associated protein (MAP) (23, 54).

Keeping in mind the speculated mechanisms of MS disease, it is important to determine what cells and molecules are responsible for MS pathogenesis. MS is thought to be a Th1-CD4+ $\alpha\beta$ T-cell mediated disease. Thus, the first step in the pathogenesis of MS is believed to involve the activation of myelin reactive T cells in the periphery. Increased numbers of $\alpha\beta$ T cells to CNS-specific antigens are found more often in MS patients as compared to controls (54). CNS oligodendrocytes, which are responsible for myelin production, do not express MHC II, thus CD4+ T cells cannot have a direct effect on their destruction (23). Once activated, the myelin reactive T cells are believed to expand and rapidly traffic to the CNS.

In MS, it is believed that Th1 responses induce disease activity, and possible disease progression, whereas Th2 responses induce remission. Penetration and migration through the blood brain barrier occurs next, promoted by increased adhesion molecule surface expression and release of proinflammatory Th1 cytokines, such as IFN-$\gamma$, IL-2 and

TNF-α and -β, by activated T cells. Proinflammatory cytokines are believed important in MS inflammation. T cell reactivation occurs upon encountering their specific myelin epitope presented by local antigen presenting cells within the CNS (24, 62, 63). This results in the further production of Th1 cytokines by these cells, including IL-1, IL-6, IFN-γ, and TNF-α, and chemokines, including MIP-1α, MIP-1β, MCP-1, RANTES, and lymphotoxin, which are responsible for the activation and chemoattraction of other immune cells to the central nervous system, ultimately culminating in demyelination (24, 54). Although the exact mechanisms are not known, demyelination is believed to involve T cells. Roles for monocytes, macrophages, and various cytotoxic mediators such as cytokines, complement-cascade-induced membrane attack complexes, demyelinating antibodies, and reactive oxygen species, are also suggested (21, 24, 62).

Although MS is believed primarily associated with CD4+ αβ T cells, other forms of T cells have been found associated with MS lesions. Roles for these T lymphocytes in MS have thus been speculated. Myelin peptides from various human myelin proteins, including MBP, have been found to form stable complexes with MHC class I molecules, inducing a CD8+ cytotoxic T cell response. Thus, CD8+ T cells may be responsible for some forms of disease induction in MS (23, 54). In MS patients, however, fewer circulating CD8+, more specifically CD8+ CD28-suppressor T cells, are found to occur.

Although αβ T cells predominate in MS lesions, significant numbers of γδ T cells also exist. Increased heat shock protein expression is known to occur in MS lesions, and thus, the presence of γδ T cells in CNS lesions may indicate an autoimmune response to these proteins in MS (21, 54).

Although T lymphocytes are believed to be the principal immune cells exerting effects in MS, macrophages and microglia are also known to play a role in MS immunopathogenesis (27). These cells are responsible for antigen presentation, myelinotoxic and neurotoxic factor production, myelin phagocytosis, and even assist in remyelination. Macrophages can induce tissue breakdown by the production of elastase, collegenase, and various lysosomal proteases (27, 54). Macrophage cytokine and chemokine production, including IL-1, IL-6, IL-10, IL-12, and TNF-α are known to drive inflammatory responses (23, 27). These activated phagocytic cells are also known to produce many proteins implicated in myelin damage and oligodendrocyte toxicity including complement components, free oxygen radicals, reactive nitrogen species and tryptophan breakdown products (23, 27). Thus, cells such as monocytes and macrophages deserve much study regarding their roles and pathological effects in MS.

## E. IMMUNOTHERAPY IN MULTIPLE SCLEROSIS

Based on its autoimmune nature, immunopharmacological therapies have been the mainstay of MS therapies. Attempted therapies include agents believed to alter or block various steps in the inflammatory and immunological pathways (56). The initial agents utilized worked through the production of non-specific immunosuppression. Due to their ability to act as physiological inhibitors of inflammation, steroids, such as glucocorticoids, are widely used as immunosuppressive and antiinflammatory agents. Many steps in the inflammatory response including cytokine synthesis, cell surface molecule expression, and leukocyte migration into inflamed areas, are known to be inhibited by glucocorticoids (28, 64). It has been well documented that pregnancy is associated with enhanced humoral

and reduced cellular immunity. Secretion of steroid hormones, such as glucocorticoids, is known to be highly elevated during pregnancy. These steroids are believed responsible for the Th2 anti-inflammatory conditions that occur during pregnancy. Thus, other widely known steroid hormones, which are elevated during pregnancy have also been tested as potential therapies for multiple sclerosis. These steroids include estrone, estradiol, estriol, and progesterone (65, 66).

Many limitations exist with general immunosuppression as a therapy for disease. More specific therapies aimed at inhibiting central nervous system inflammation have recently been developed due to increased immunoregulatory knowledge, such as cytokine interplay. Two separate groups of cytokine directed therapies exist. Those in which the inflammatory or autoreactive T cell response is alterd by cytokine administration or blockage, and therapies in which the direct action of a cytokine itself is blocked (67).

Immune deviation by cytokine-directed therapy has been studied using such cytokines as IL-4, IL-10, and anti-IL-12 (67). IL-4 and anti-IL-12 are critical for the development of Th2 responses and inhibition of Th1 responses respectively (67). IL-10 is a potent anti-inflammatory cytokine that inhibits proinflammatory cytokine and chemokine synthesis (68, 69, 70). IL-10 can regulate humoral immune responses and attenuate cell-mediated immunity by its ability to stimulate proliferation and cytokine synthesis by T cells (69, 71). Immune deviation by cytokine injection or blockade is appealing however, it is conceivable that the deviated response will be more detrimental to the host. This was seen in the initial interferon (IFN) therapies.

Interferons are proteins with anti-viral, anti-proliferative, and immunomodulating properties. IFN-$\alpha$ and IFN-$\beta$ possess antiinflammatory properties which are mediated

through interaction with the type I interferon receptor, while IFN-γ possesses proinflammatory properties and exerts its effects through the type II receptor (48). IFN-γ was initially tested. Administration of this cytokine was found to exacerbate disease in MS patients (48, 67). IFN-β is known to counteract the effects of IFN-γ, therefore study into this cytokine as a potential therapy soon followed.

IFN-β was the first drug to be approved by the US-FDA for the treatment of MS (72). The exact mode of action of IFN-β is unknown, but several hypotheses exist including: downregulation of T cell proliferation and function, or the actions of IFN-γ, TNF-α or other cytokines or inflammatory molecules such as nitric oxide; IL-10 upregulation; induction of T suppressor cell function; alteration of the immune system response to viral infections (56, 67, 73, 74, 75, 76).

Two forms of IFN-β therapy exist. IFN-β1b is a non-glycosylated, human recombinant interferon that is expressed in *E. coli.*, and was approved in 1993 for use in the treatment of MS. IFN-β1a is expressed in chinese hamster ovary cells and was approved for use in 1996 (36, 56, 77). IFN-β1a is a recombinant, fully glycosylated interferon that appears identical to native interferon (36, 56, 77). Studies using both recombinant forms of IFN-β show it effective in reducing relapse rate and severity, and inhibiting the accumulation of inflammatory lesions, and in the case of IFN-β1a reduced the rate of acquisition of disability (78, 79). IFN-β1b is known to produce many side effects including the development of neutralizing antibodies, lymphopenia, anaemia, injection site reactions, and flu-like symptoms. The incidence of side effects with IFN-β1a is quite low. Only flu-like symptoms and mild injection site reactions mainly occur with this therapy (36, 56, 77, 80).

A synthetic amino acid copolymer consisting of alanine, lysine, glutamic acid and tyrosine, is the only other approved therapy for the treatment of MS. This copolymer is similar in charge to, and shares some cross reactivity with, myelin basic protein. In MS patients this molecule has been found to reduce relapse rate and slow the progression of disability. The mechanism of action is believed to involve high affinity binding to various MHC class II molecules. Direct competition with myelin antigens for T cell binding and subsequent activation and induction of specific regulatory T cells is the result of efficient MHC binding by this copolymer (56, 81).

Other pharmacological agents tested in the fight against MS include various immunosuppressive agents such as cyclosporin, cyclophosphamide, and methotrexate. As well, other therapeutic means have been employed which have in trials produced mixed results including, high-dose intravenous immunoglobulin therapy, altered peptide ligand therapy, T cell vaccination therapy, and therapeutic plasma exchange (56, 82, 83, 84, 85).

# II. RATIONALE

MS disease is believed initiated by CD4+ T lymphocytes. Cytokines produced by these activated T cells are important in the regulation of the immune response. In MS, Th1 cytokines are implicated in pathogenesis. Most studies involving Th1 and Th1 regulatory cytokine levels have been performed previously using peripheral blood mononuclear cell (PBMC) or T lymphocyte cultures. Monocytes produce many of the same regulatory cytokines as lymphocytes, as well as many other proinflammatory cytokines. Monokines are known to play important roles in the immunopathogenesis of many diseases. The exact role monokines play however, in MS disease has not been extensively studied. Thus, this project shall elucidate the role of monokines in this inflammatory, autoimmune disease.

Demonstrating a role for monokines in MS raises the question as to what effects immunotherapy will have on these levels. The therapy treatments tested in this project were a widely used interferon therapy, IFN-β1a, and a potential cytokine therapy, IL-10. IFN-β1a was chosen due to its present use as a MS therapy, its extensive study in T lymphocyte and PBMC cultures, and its known effects on immunological and inflammatory processes, such as Th balance and cytokine production (86).

IL-10 exerts many important effects on cells of the immune system. The use of this cytokine as a therapy for MS has not been extensively investigated. IL-10 was included in this project however, due to its profound ability to suppress monocyte activation and monokine secretion (71, 87).

## III. PURPOSE

The purpose of this project was to detect and quantify regulatory cytokine levels, IL-12, IL-10, IL-1, IL-6, and IFN-γ, from whole blood derived monocytes isolated from MS patients, of various disease severity, and healthy controls. Secondly, it was the purpose of this study to determine the effect of IFN-β1a and IL-10 cytokine-directed immunotherapy on the aforementioned monokine levels.

## IV. HYPOTHESES

The working hypotheses of this project are that Th1 and proinflammatory regulatory monokine levels, specifically IL-1, IL-6, IL-12, and IFN-γ, are elevated in MS patients, and that this monokine level upregulation in MS patients may be associated with disease severity. Upon proving, a corollary to this hypothesis is that Th1 inhibitory cytokine directed therapies, IFN-β1a and IL-10, will decrease elevated monokine levels in MS patients to levels similar to healthy controls.

## V. OBJECTIVES

Addressing the following objectives may uncover an important role for monokines in MS. These objectives shall determine whether MS patient monokine levels differ from healthy control levels, vary with disease course, and help determine possible beneficial

effects of therapy on these levels, or trends between disease severity and outcome of therapeutic culture.

1) To quantitate cytokine, IL-12, IL-10, IL-1, IL-6, and IFN-γ, mRNA and protein levels, from whole blood derived monocytes, isolated from healthy controls and MS patients of variable disease course; Relapsing Remitting, Relapsing Remitting undergoing immunosuppressive therapy, and Secondary Progressive.

2) To subsequently compare MS patient monokine levels between categories, and with healthy controls.

1) To determine the effect of cytokine-directed immunotherapy, IFN-β1a and IL-10, on these monokine levels for both MS patients and healthy controls.

# VI. MATERIALS AND METHODS

## A. PATIENT AND CONTROL SAMPLES

MS patients were obtained from the MS Clinic, Ottawa Hospital, General Site. Informed written consent was obtained from all patients and ethical approval for the study was obtained from the Research Ethics Committee, Ottawa Hospital. MS patients were categorized clinically as Relapsing Remitting MS (RR), RR undergoing immunosuppressive steroid therapy (RR/T), or Secondary Progressive MS (SP), by Drs. Mark Freedman and Darius Matusevicius of the MS Clinic. The following patients were entered into the study: 9 RR patients, 6 RR/T patients, and 10 SP patients. One control group consisted of 8 healthy controls (HC).

## B. MONOCYTE ISOLATION AND CULTURE

Patient and control 50 ml blood samples were collected into EDTA Vacutainer tubes (Becton Dickinson, Franklin Lakes, New Jersey). Enriched, non-activated monocytes were isolated from whole blood by the use of Optiprep medium (Nycomed Pharma, Norway, Oslo) density gradients and high speed centrifugation (IEC Centra-7R refrigerated centrifuge, International Equipment Co., Needham Heights, MA), as described previously (88). The cells were enumerated and viability checked by trypan blue staining. A small number of isolated cells ($10^5$) were blocked with heat aggregated gamme-immune (Ottawa Hospital), and BSA-Cy5 (Gibco BRL, Grand Island, NY), for 10 minutes. Following blocking, cells were externally stained with PE-conjugated α-CD4, TRI-conjugated α-CD45, and FITC-conjugated α-CD14 or –CD3 (Sigma, St. Louis, MO),

for 15 minutes. Cells were analyzed following staining on an Epics MCL flow cytometer (Coulter, Hialeah, FL). Cell isolates consisting of <90% monocytes were used for subsequent experiments.

Following isolation monocytes were split and cultured for 24 hours in RPMI media 1640 (Gibco BRL, Grand Island, NY), supplemented with 10 % heat inactivated fetal calf serum (FCS) (Gibco BRL, Grand Island, NY) alone, or the aforementioned media in the presence of IL-10 (1000IU (1ng)/ $10^6$ cells) (Schering-Plough, Madison, NJ) or IFN-β1a (100 IU (.37 ng)/ $10^6$ cells) (MS Clinic, Ottawa General Hospital) without antibiotics at 37° C, in frosted polypropylene culture tubes (Becton Dickinson, Lincoln Park, NJ). Cells were split and separately cultured so that 20 % of cells isolated were used for intracellular staining. The remaining 80 % of cells isolated were used for RNA extraction and the culture supernatant of this cell fraction was used for ELISA experiments.

C. INTRACELLULAR STAINING

Twenty percent of cells isolated ($10^5$-$10^6$cells) were separated out and used for intracellular staining. Monensin (GolgiStop, 4μl/ 6 x $10^6$ cells) (Pharmingen, San Diego, CA) was added in the final 7-8 hours of culture to inhibit golgi transport and induce accumulation of intracellular cytokines in the golgi body. Cells were washed in phosphate buffered saline (PBS) (Sigma, St. Louis, MO), supplemented with 0.5 % FCS and 0.1 % $NaN_3$ (BDH Inc., Toronto, Ont.), fixed and permeabilized for 15 minutes with paraformaldehyde (PFA) (BDH Inc., Toronto, Ont.), and 0.1% saponin (Sigma, St. Louis, MO). Permeabilized cells were washed with PBS supplemented with 0.5 % FCS, 0.1 % $NaN_3$, and 0.1 % saponin, and subsequently incubated with gamme-immune for 10

minutes to block Fc receptor binding. Cells were aliquoted and incubated with PE-conjugated monoclonal antibodies for IL-1β (Biosource, Camarillo, CA), IFN-γ (Caltag, Burlingame, CA), IL-6, IL-10, IL-12, or TNF-α (Pharmingen, San Diego, CA) for 25 minutes. The monoclonal antibody specific for IL-12 recognized both human IL-12p70 and p40 subunits. TNF-α was incorporated into this study because it is known to be expressed by both monocytes and lymphocytes. Due to this constitutive expression TNF-α is the lab standard positive intracellular staining control. All incubations were carried out in the dark at room temperature. Cells were washed with PBS supplemented with 0.5 % FCS, 0.1 % $NaN_3$, and 0.1% saponin, and fixed in a 1% PFA solution. Cells were analyzed on an Epics MCL flow cytometer. Mean Fluorescent Intensity (MFI) for each of the autofluorescent control groups and the specific cytokines being detected were determined. A constant auto (unstained) MFI=0.05 was used to determine cytokine MFIs for each sample tested.

## D. TOTAL RNA EXTRACTION AND RIBOQUANT RNASE PROTECTION ASSAY

Total RNA was isolated from all remaining cultured monocytes using Tri Pure Reagent (Boehringer Mannheim, Indianapolis, IN) guanidine thiocyanate-phenol solution and chloroform extraction. RNA purity was checked by running a small isolate of total RNA on a 1.8% agarose gel and ethidium bromide staining.

Equal quantities (1-5 μg) of total RNA from each obtained sample were used for cytokine mRNA detection using the Riboquant RNase Protection Assay (Pharmingen, San Diego, CA). The reactions were performed per manufacture's instructions. The hCK-2 template (Pharmingen, San Diego, CA) is specific for the simultaneous synthesis of

probes for IL-12p35, IL-12p40, IL-10, IL-1α, IL-1β, IL-1Ra, IL-6, IFN-γ, and 2 housekeeping genes L32 and GAPDH was employed. $\alpha$-P-$^{32}$ labeled antisense RNA probes were transcribed with T7 polymerase, using the linearized, multiple cDNA hCK-2 template set. Following probe synthesis and quantitation in a scintillation counter, RNA was dried and prepared, and RNA-probe hybridization occurred for 12-16 hours at 56° C. Following overnight hybridization RNA samples endured an RNase A and T1 digestion, followed by a Proteinase K digestion and subsequent purification of protected probe-mRNA fragments by phenol:chloroform extraction and ethanol precipitation. Protected fragments were separated via electrophoresis on a 5% denaturing (urea) acrylamide gel with .5X TBE as the running buffer. Gels were run at 50 Watts until the leading edge of the Bromophenol Blue (front dye) runs off the gel. Gels were exposed to a phosphor screen for 4-5 days. Protected mRNA bands were visualized by the use of phosphorimagery (Scannercontrol Software, Storm Phoshorimagery Systems, Molecular Dynamics, CA).

Bands were analyzed and quantitated with the use of ImageQuant Software (Molecular Dynamics, CA). Each protected mRNA band volume was quantitated using the same analysis box area of 3822. Housekeeping genes (L32 and GAPDH) incorporated into the probe template allowed for direct comparison of cytokine mRNA bands between multiple samples and gels. Housekeeping gene band volumes were standardized for each sample tested. All GAPDH (positive control) bands visualized were adjusted by a specific numerical factor so that all samples tested in this study had the same GAPDH housekeeping gene band volume of 322461.6. All other cytokine mRNA bands present in each specific housekeeping gene's lane were adjusted in accordance with their

housekeeping gene's numerical factor. Background (negative control) was determined for each gel and adjustments were made so as to standardize the background level between experiments. Once these levels were adjusted, all band volumes were divided by a predetermined background volume of 10101.93. This produced a final band volume that was used for comparative graphing.

E. ELISA ASSAYS

Culture supernatants were saved and tested for the presence of IL-1β, IL-6, IL-10, IL-12, IFN-γ, and TNF-α. IL-12 ELISA kits were specific for the detection of human and recombinant IL-12 p70 and p40 in the supernatant sample. TNF-α secretion was determined as a positive ELISA control. Cytokine levels were measured using commercial solid phase sandwich ELISAs (Biosource Int., Camarillo, CA) per manufacturer's instructions. The kits employ microtiter strips precoated with antibody specific for the cytokines under study, and a biotinylated secondary noncompetitive mAb. Subsequently, Streptavidin-Horse Radish Peroxidase conjugate solution was added, followed by a specific stabilized chromagen substrate. Following addition of Stop Solution, plates were read at an O.D of 450 nm using an ELISA plate reader (SpectraCount, Canberra Packard, Meriden, CT). Absorbances determined for each provided standard were graphed against the standard concentrations and used to determine the cytokine concentrations in each tested sample.

F. STATISTICAL ANALYSIS

All data obtained were tabulated, graphed and analyzed by one-way ANOVA (PRISM software). If the ANOVA test demonstrated significant levels of difference,

Newman-Keuls multiple comparison post tests were also performed. P values of $\leq 0.05$ were considered significant in all tests. P values of $\leq 0.06$ were considered to be approaching significance in all tests.

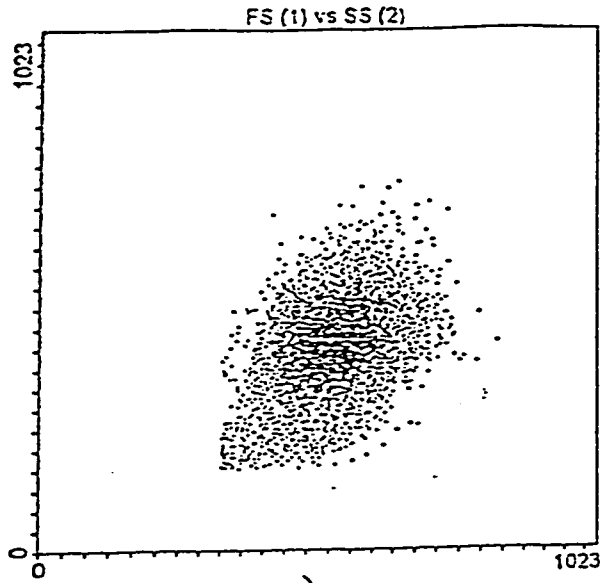
# VII. RESULTS

## A. MONOCYTE PURITY

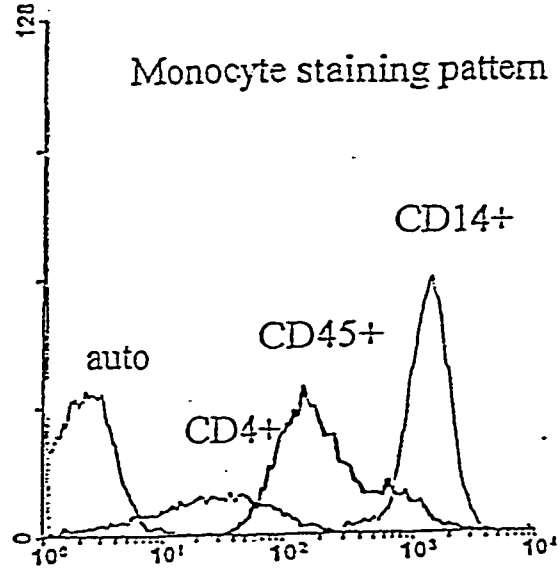
Whole blood samples were collected from each HC and MS patient under study. Monocytes were isolated from each blood sample by our established method (Materials and Methods). One to 15 million monocytes per 50 ml blood sample were routinely obtained. Monocyte purity was determined by flow cytometry. Figure 1 a, shows the Forward Scatter vs. Side Scatter uniform distribution of the monocytes isolated. This population of cells was determined to be CD45, CD4 and CD14 positive (Figure1b). The presence of T lymphocytes was monitored with the use of anti-CD3-FITC staining. As seen in Figure 1c, of the 10 million cells extracted in this monocyte isolation only 5 % (marker M1) stain positively for CD3. The isolated monocyte population was thus deemed 95% pure. Results in this manuscript are reported from monocyte preparations that were at least 90 % pure.

## B. MONOKINE mRNA LEVELS

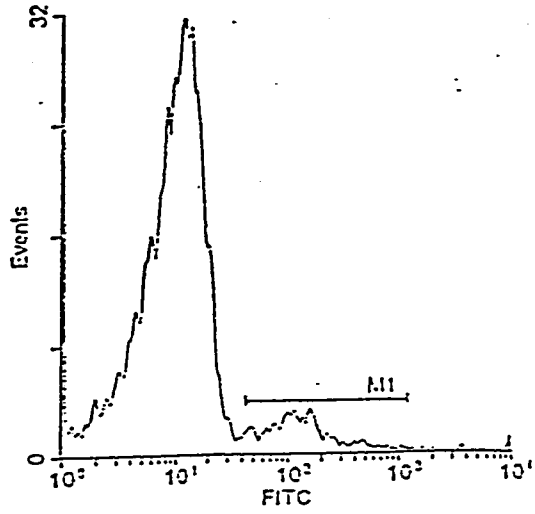
Messenger RNA levels were determined so as to examine the levels of Th1 and proinflammatory monokine transcription occurring in HC and MS patient monocytes. Differences in HC and MS patient monokine transcription levels were also examined. Monokine mRNA levels were assessed by the Riboquant RNase protection assay from total RNA extracted from a large subset (80%) of the monocytes isolated. The Riboquant RNase protection assay was chosen so as to allow the simultaneous detection of multiple monocytic cytokine mRNA species in each sample. Monokine mRNA levels were


FS (1) vs SS (2)
1023
0
0
1023

a)


Monocyte staining pattern
128
CD14+
CD45+
auto
CD4+
0
10⁰
10¹
10²
10³
10⁴

b)


32
Events
M1
0
10⁰
10¹
10²
10³
10⁴
FITC

c)

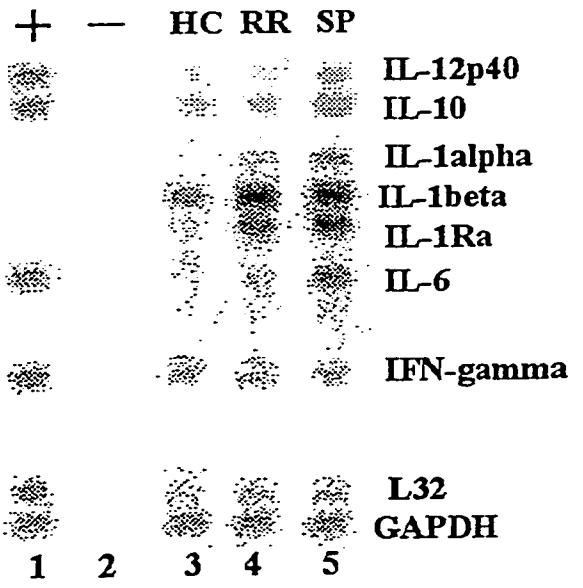
FIGURE # 1: OPTIPREP ISOLATED MONOCYTE PURITY AS DETERMINED BY FLOW CYTOMETRY

Monocyte purity was determined by a) Forward Scatter vs. Side Scatter assessment, b) α-CD4, -CD14, and -CD45 positive extracellular staining, and c) α-CD3 extracellular negative staining indicating 5 % of isolated cells were CD3 positive (M1).

quantitated, as outlined in Materials and Methods, using the GAPDH housekeeping bands as a positive control and a negative background control. Cytokine levels were thus determined for various regulatory monokines to determine what role, if any, they may play in Multiple Sclerosis. Representative cytokine mRNA band intensities, as assessed through ImageQuant software, are shown in Figure 2 for, IL-12p40, IL-10, IL-1α, IL-1β, IL-1Ra, IL-6, and IFN-γ. SP IL-12, IL-10, IL-1, and IL-6 monokine band intensities appear visibly greater than those obtained from HC and RR patients. The intensities of RR patient IL-1 and IL-6 specific bands as well, appear visibly greater than HC bands. L32 and GAPDH housekeeping gene levels are constant for the 3 samples under study.

Cumulative monokine mRNA band volume results, obtained from multiple samples, are shown in Figure 3. As seen in Figures 3 a, b, and e, monocytic IL-12p40, IL-10, and IL-1Ra mRNA levels do not significantly differ between the HC and MS disease categories. SP patients do however, express significantly higher levels of IL-12p40 (Figure 3a) mRNA as compared to RR/T ($p<.05$), and are approaching significance in expressing higher monocytic levels of IL-12p40 mRNA as compared to RR patients ($p<.06$). SP patients also show intradisease differences regarding IL-10 (Figure 3b) and IL-1Ra (Figure 3e) mRNA levels. SP patient monocytes express significantly higher levels of IL-10 and IL-1Ra mRNA as compared to both RR/T ($p<.05$ and $p<.01$ respectively) and RR ($p<.05$ and $p<.01$ respectively) patient levels.

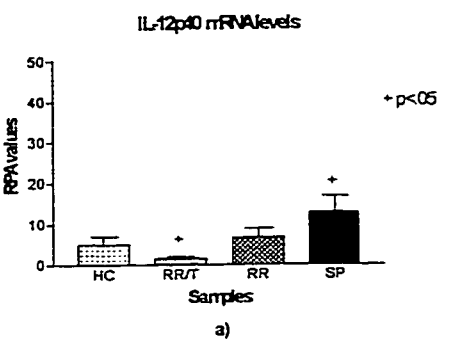
As seen in Figures 3 c, d, and f, SP patients constitutively express significantly higher monocytic levels of IL-1α ($p<.001$), IL-1β ($p<.001$), and IL-6 ($p<.01$) as compared to HC samples. SP patients also express significantly higher levels of these cytokines as


+
—
HC
RR
SP
IL-12p40
IL-10
IL-1alpha
IL-1beta
IL-1Ra
IL-6
IFN-gamma
L32
GAPDH
1
2
3
4
5

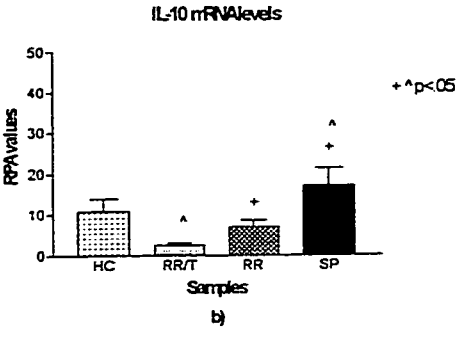
## FIGURE # 2: RIBOQUANT MULTIPROBE RNASE PROTECTION ASSAY COMPARING REPRESENTATIVE MONOKINE mRNA LEVELS

Riboquant RNase Protection assays were performed using the hCK-2 template, specific for IL-12p40, IL-10, IL-1α, IL-1β, IL-1Ra, IL-6, IFN-γ, L32, and GAPDH, and total RNA isolated from all RPMI media/ 10% FCS cultured monocyte samples tested. Cytokine band locations are indicated as determined from positive control.

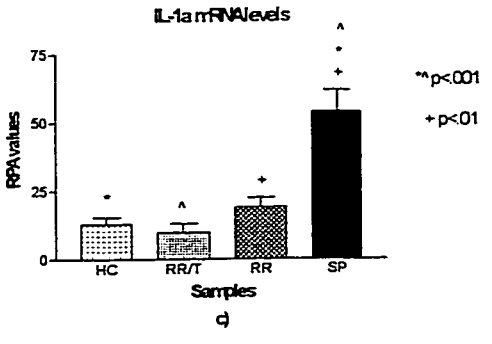
Lane 1= Positive human control RNA (kit provided)
Lane 2= Yeast tRNA negative control (kit provided)
Lane 3= Representative Human Control monocyte RNA
Lane 4= Representative Relapsing Remitting patient monocyte RNA
Lane 5= Representative Secondary Progressive patient monocyte RNA


IL-12p40 mRNA levels
RPA values
50
40
30
20
10
0
HC
RR/T
RR
SP
Samples
+ p<05

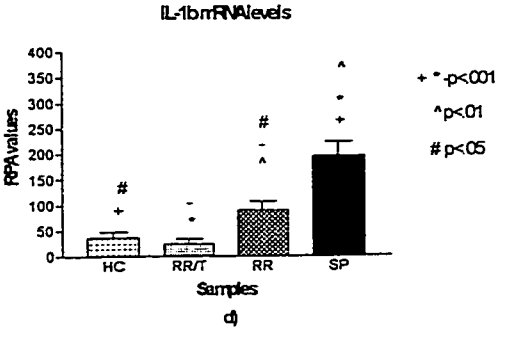
a)


IL-10 mRNA levels
RPA values
50
40
30
20
10
0
HC
RR/T
RR
SP
Samples
+ ^ p<05

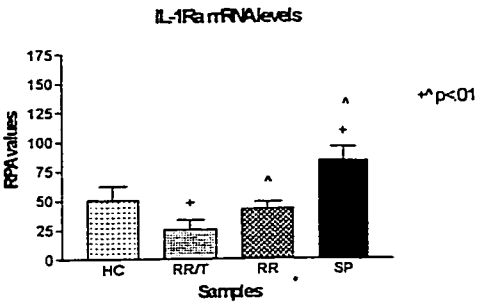
b)


IL-1a mRNA levels
RPA values
75
50
25
0
HC
RR/T
RR
SP
Samples
** p<001
+ p<01

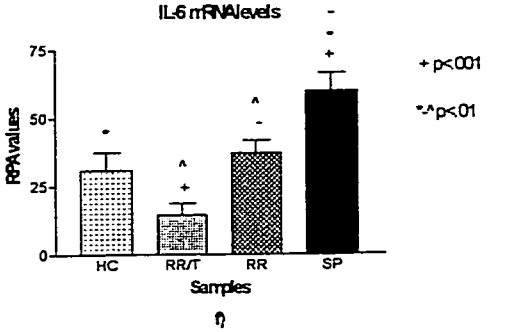
c)


IL-1b mRNA levels
RPA values
400
350
300
250
200
150
100
50
0
HC
RR/T
RR
SP
Samples
+ * - p<001
^ p<01
# p<05

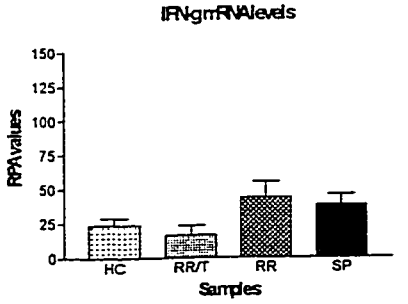
d)


IL-1Ra mRNA levels
RPA values
175
150
125
100
75
50
25
0
HC
RR/T
RR
SP
Samples
+ ^ p<01

e)


IL-6 mRNA levels
RPA values
75
50
25
0
HC
RR/T
RR
SP
Samples
+ p<001
* ^ p<01

f)


IFN-g mRNA levels
RPA values
150
125
100
75
50
25
0
HC
RR/T
RR
SP
Samples

g)

FIGURE # 3: MONOKINE mRNA LEVELS AS DETERMINED BY RIBOQUANT MULTIPROBE RNASE PROTECTION ASSAY

Monocytes were isolated from HC (n=8), RR/T (n=6), RR (n=9), and SP (n=10) blood samples. Monocytes were cultured for 24-hours in RPMI media supplemented with 10% FCS. Riboquant RNase Protection assays were performed using total RNA extracted from HC and MS patient's cultured monocytes. The hCK-2 template was employed allowing for the simultaneous detection of multiple cytokines a) IL-12p40, b) IL-1 0, c) IL-1$\alpha$, d) IL-1$\beta$, e) IL-1Ra, f) IL-6, and g) IFN-$\gamma$ levels. The data for each patient category was tabulated and the mean and standard deviation were calculated. Statistically significant differences ($p < 0.05$) between bars are noted by matching symbols (*+^).

compared to both RR/T ($p<.001$) and RR ($p<.01$) patient levels. RR patients express significantly higher monocytic levels of IL-1β (Figure 3d) as compared to HC ($p<.05$) and RR/T ($p<.001$). HC and RR/T IL-1β levels do not however significantly differ. As seen in Figures 3c and 3f RR patient monocytic IL-1α ($p<.001$) and IL-6 ($p<.01$) mRNA levels are significantly higher than mRNA levels obtained for RR/T patients. Neither RR category however, differs significantly from HC IL-1α or IL-6 levels.

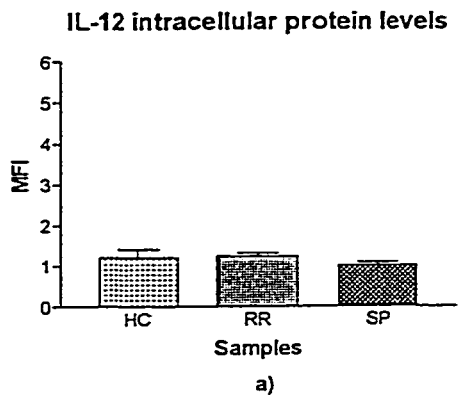
Monocytic IFN-γ mRNA levels, as seen in Figure 3g, do not significantly differ between HC and MS patient categories. As well, IFN-γ mRNA levels do not significantly differ between patient samples within the various MS disease categories.

Monocytic IL-12p35 mRNA levels were undetectable with the use of this technique.

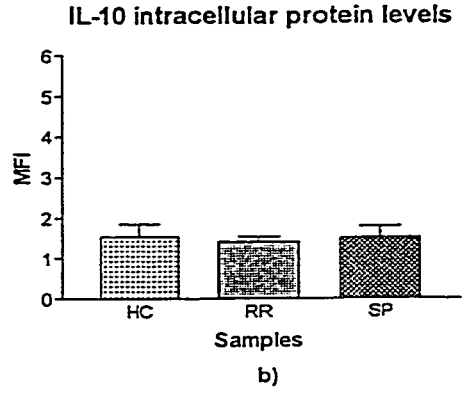
SP patients thus transcribe significantly higher monocytic levels of IL-1α, IL-1β, and IL-6 as compared to HC. RR patients, as well, transcribe significantly higher levels of IL-1β as compared to HC monocyte levels. This indicates that MS patient monocytes transcribe significantly higher levels of proinflammatory cytokines such as IL-1 and IL-6, as well these levels reflect disease severity in that SP patients transcribe greater levels of these monokines than do RR patients.

## C. MONOKINE INTRACELLULAR PROTEIN LEVELS

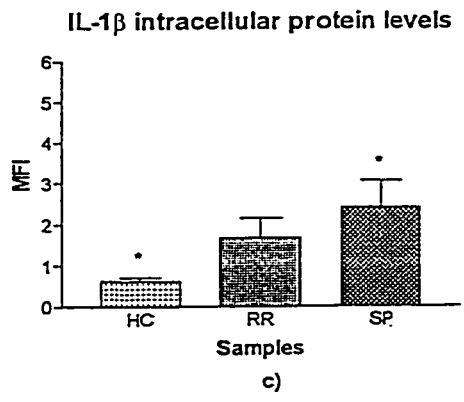
Proinflammatory monokine transcription is thus influenced by MS disease. To determine whether MS disease has a further influence on monokine translation intracellular staining, with the use of monensin golgi inhibitor, was employed. Intracellular cytokine


IL-12 intracellular protein levels
MFI
0
1
2
3
4
5
6
HC
RR
SP
Samples

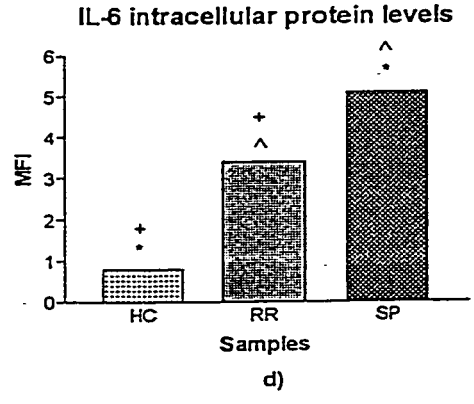
a)


IL-10 intracellular protein levels
MFI
0
1
2
3
4
5
6
HC
RR
SP
Samples

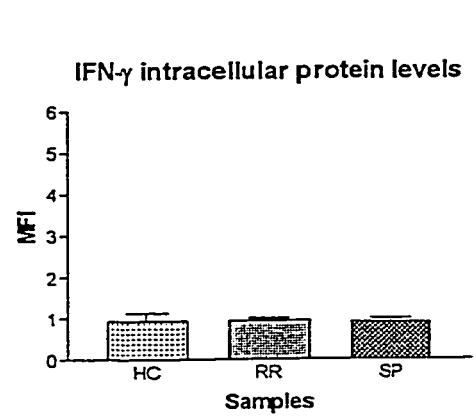
b)


IL-1β intracellular protein levels
MFI
0
1
2
3
4
5
6
HC
RR
SP
Samples

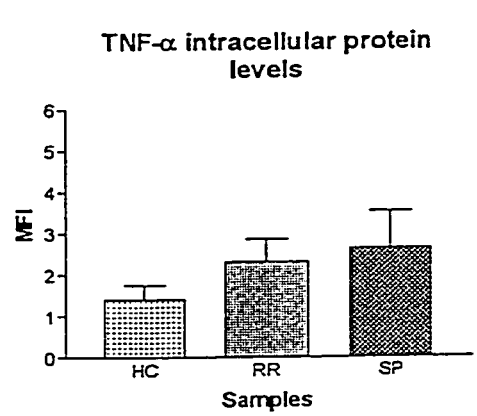
c)


IL-6 intracellular protein levels
MFI
0
1
2
3
4
5
6
HC
RR
SP
Samples

d)


IFN-γ intracellular protein levels
MFI
0
1
2
3
4
5
6
HC
RR
SP
Samples

e)


TNF-α intracellular protein levels
MFI
0
1
2
3
4
5
6
HC
RR
SP
Samples

f)

FIGURE # 4: INTRACELLULAR MONOKINE PROTEIN LEVELS OF ALL DONORS.

Intracellular staining was performed on monocytes cultured for 24 hours in RPMI media supplemented with 10 % FCS. Monensin golgi inhibitor was added to the monocyte samples in the last 10 hours of culture. Cells were blocked with gamme-immune and stained with PE-conjugated antibodies to the various cytokines. The MFI for each cytokine; a) IL-12, b) IL-10, c) IL-1β, d) IL-6, e) IFN-γ, and f) TNF-α, from each HC, RR, and SP category sample (n=4 for each), was tabulated and the mean and standard deviation were calculated. Statistically significant differences ($p > 0.05$) are noted by matching symbols (*^+). *^+ $p < .05$

protein levels were determined for IL-12, IL-10, IL-1β, IL-6, IFN-γ, and TNF-α for a sampling size of n=4. TNF-α was included in the intracellular staining panel as a positive control. TNF-α is a lab standard because it is known to be constitutively expressed by both monocytes and lymphocytes. IL-1α and IL-1Ra levels, although detected by Riboquant RNase protection assay, were not detected by intracellular staining due to antibody unavailability. The levels of autofluorescence for each sample and condition tested were determined and compared. The MFI for intracellularly stained cells is directly proportional to the level of intracellular cytokine in each control and patient monocyte sample. The MFI for all unstained auto samples was 0.05.

Intracellular staining results obtained for all samples tested are shown in Figure 4. As seen in Figures 4 a, b, e, and f, monocytic IL-12, IL-10, IFN-γ, and TNF-α cytokine levels show no significant differences in HC, RR, and SP category intracellular levels.

As seen in Figures 4 c and d, SP patient monocytes have significantly higher levels of IL-1β and IL-6 as compared to HC. RR patient monocytic IL-1β and IL-6 intracellular levels are approaching significance in being greater than HC levels ($p<.06$).

Figure 5 shows histogram overlays depicting monocyte cytokine protein levels as determined by intracellular staining for representative HC, RR, and SP patient samples. The scales in Figure 5 are depicted 10 fold greater than the actual scale used to determine the MFI i.e., the standard auto MFI of 0.5 is shown as 5 in Figure 5. Figures 5 a, b, e, and f show MFI overlays for intracellular cytokine levels of IL-12, IL-10, IFN-γ, and TNF-α respectively, from a representative HC, RR, and SP patient. The histogram overlays show similar MFIs for HC, RR, and SP sample categories, indicating no significant differences in intracellular IL-12, IL-10, IFN-γ, and TNF-α protein levels.

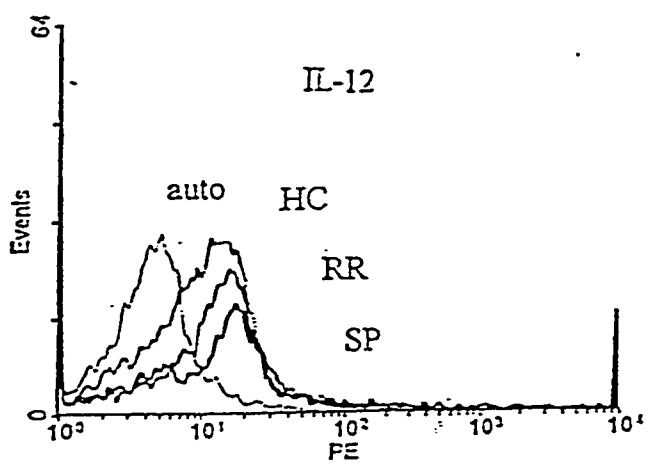
As seen in the histogram overlay in Figure 5c, the SP patient's monocyte sample (MFI=3.93) stains with a higher MFI for IL-1β than the RR sample (MFI=2.15), and both MS samples stain with greater MFIs for IL-1β than the HC sample (MFI=0.65).

Intracellular levels of IL-6, as shown in Figure 5d, follow a similar pattern. The histogram overlay shows the SP patient's monocyte sample (MFI=5.08) stains with a higher MFI for IL-6 than the RR patient (MFI=3.35) and both stain with greater IL-6 MFIs than the HC sample (MFI=0.84).

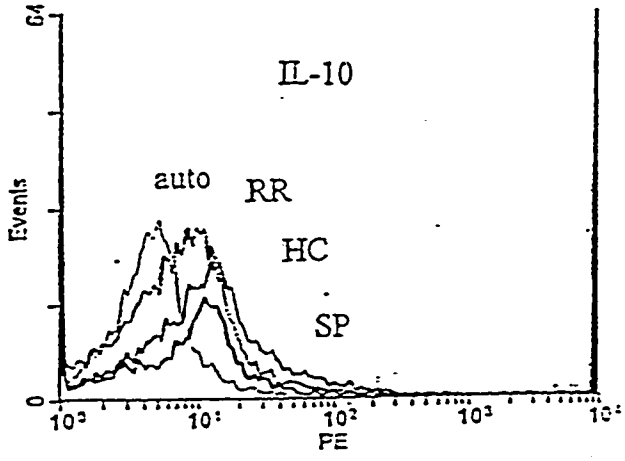
Monokine translation , as determined by intracellular staining, was thus found to reflect transcription patterns in that MS patients translated significantly greater levels of IL-1 and IL-6 as compared to HC. This upregulated translation again reflects disease severity in that SP patients translated significantly greater levels of these proinflammatory regulatory cytokines as compared to the other MS disease categories tested.

## D. EXTRACELLULAR MONOKINE PROTEIN LEVELS

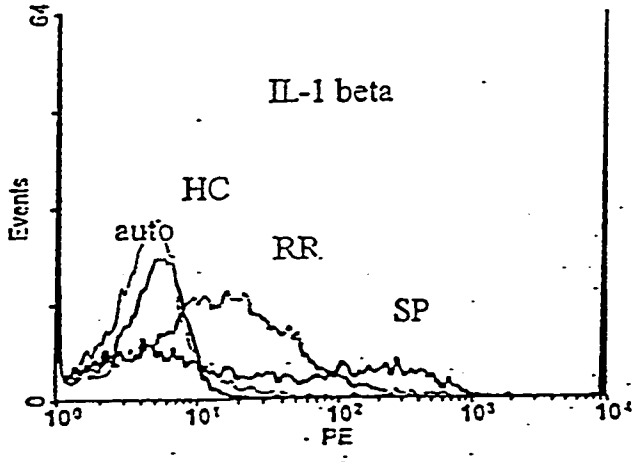
Following 24 hour monocyte culture with RPMI media supplemented with 10 % FCS in polypropylene tubes, supernatants were collected and extracellular cytokine protein levels were tested by noncompetitive sandwich ELISA. Secreted monokine protein levels were assessed and compared to transcribed and translated levels. ELISA determined secreted levels for IL-12, IL-10, IL-1β, IL-6, IFN-γ, and TNF-α are reported in Table 1. TNF-α was included in the study as a positive control. Secreted levels of IL-1α were not determined because this cytokine is known to be membrane bound. IL-1Ra secreted levels were also not determined due to reliable kit unavailability. As seen in


IL-12
auto
HC
RR
SP
Events
PE

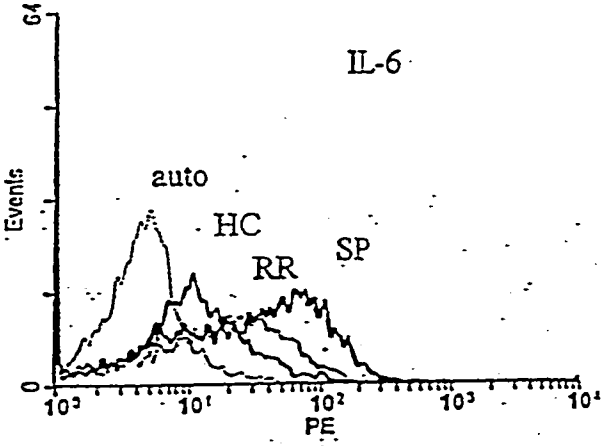
a)


IL-10
auto
RR
HC
SP
Events
PE

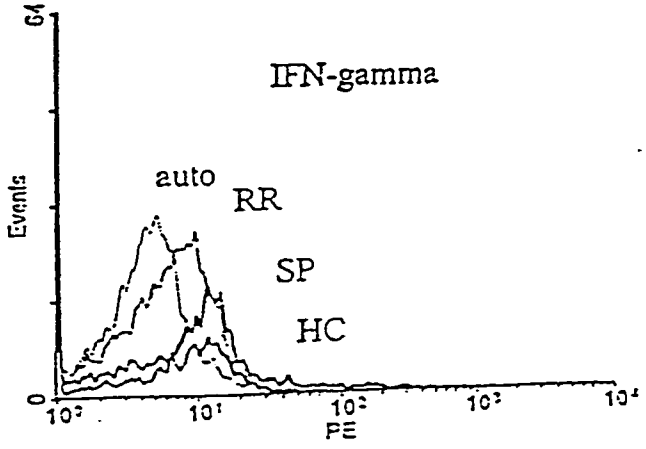
b)


IL-1 beta
HC
auto
RR
SP
Events
PE

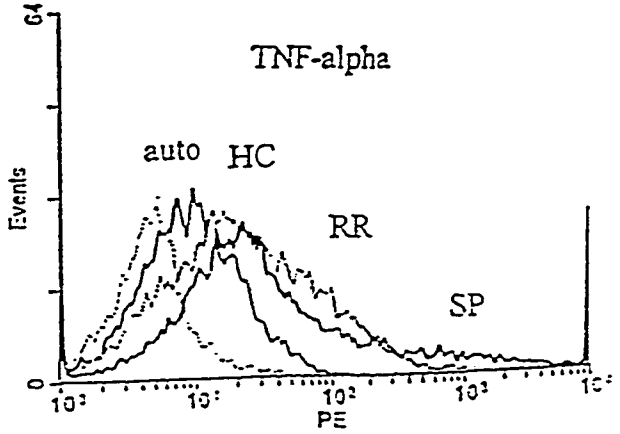
c)


IL-6
auto
HC
RR
SP
Events
PE

d)


IFN-gamma
auto
RR
SP
HC
Events
PE

e)


TNF-alpha
auto
HC
RR
SP
Events
PE

f)

## FIGURE # 5: REPRESENTATIVE HISTOGRAM OVERLAYS SHOWING INTRACELLULAR MONOKINE PROTEIN LEVELS

Intracellular staining of monocytes cultured for 24-hours in RPMI media supplemented with 10% FCS. Monensin golgi inhibitor was added in the last 10 hours of culture. Cells were blocked with gamme-immune and intracellularly stained with PE-conjugated antibodies to the various cytokines. Representative overlayed histograms for SP, RR and HC are shown for a) IL-12, b) IL-10, c) IL-1β, d) IL-6, e) IFN-γ, and f) TNF-α. Each sample has a negative control, shown as auto, with a MFI of 0.05.

column I, SP patient monocytes secreted significantly higher levels of IL-12 as compared to HC ($p<.01$), RR/T ($p<.001$) and RR patient ($p<.001$) monocytes. Levels of IL-10 and IFN-$\gamma$ (columns ii and v) show similar secretion patterns for all sample categories.

Table I also reveals SP patient monocytes secreted significantly higher levels of IL1-$\beta$ (column iii) into the culture media as compared to HC ($p<.05$) and RR/T ($p<.05$) monocytes, and significantly higher levels of IL-6 ($p<.01$) (column iv) as compared to HC monocytes. RR and RR/T monocytes also secreted significantly higher levels of IL-6 into the culture media as compared to HC ($p<.05$).

Interestingly SP, RR, and RR/T patient monocytes all secreted significantly higher levels of TNF-$\alpha$ ($p<.001$, $p<.001$, $p<.05$ respectively) (column vi) as compared to HC samples. SP patient monocytes also secreted significantly higher levels of TNF-$\alpha$ during overnight culture as compared to RR ($p<.05$) patient monocytes, and both SP and RR patient monocytes secreted significantly higher levels as compared to RR/T ($p<.001$ and $p<.01$ respectively) patient monocytes.

Secretion analysis thus reflects transcription and translation data in the revelation that MS patients secrete significantly greater levels of IL-1 and IL-6 as compared to HC. Secretion levels again reflect disease severity in that SP patients secrete significantly higher levels of Th1 regulatory and proinflammatory monokines, including IL-12 and TNF-$\alpha$, as compared to the other MS categories and HC.

TABLE 1: SECRETED MONOKINE LEVELS OF DONORS

| Patient Sample | i) IL-12 pg/ml | ii) IL-10 pg/ml | iii) IL-1β pg/ml | iv) IL-6 pg/ml | v) IFN-γ pg/ml | vi) TNF-α pg/ml |
|---|---|---|---|---|---|---|
| HC 1 | 320 | 18 | 6.5 | 150 | 12 | 1.25 |
| 2 | 650 | 85 | 9.5 | 180 | 150 | 1.4 |
| 3 | 300 | 120 | 14 | 300 | | 2.2 |
| 4 | 300 | 150 | 33 | 380 | | 8.5 |
| | * | | * | *+^ | | *+^ |
| RR/T 1 | 1.4 | 30 | 20 | 2200 | 15 | 9.5 |
| 2 | 250 | 130 | 100 | 2500 | 23 | 75 |
| 3 | 300 | 160 | 400 | 2500 | | 160 |
| | + | | + | ^ | | ^=! |
| RR 1 | 33 | 29 | 40 | 370 | 8 | 150 |
| 2 | 50 | 65 | 800 | 4200 | 15 | 170 |
| 3 | 380 | 140 | 1200 | 4750 | | 180 |
| 4 | 550 | 280 | 1200 | 4800 | | 185 |
| | ^ | | | + | | +#! |
| SP 1 | 1500 | 40 | 600 | 2800 | 14 | 230 |
| 2 | 2100 | 45 | 700 | 3300 | 25 | 240 |
| 3 | 2500 | 95 | 2000 | 4300 | | 240 |
| 4 | 3250 | 400 | 2500 | 5500 | | 240 |
| | *+^ | | *+ | * | | *#= |

## TABLE # 1: SECRETED MONOKINE LEVELS OF DONORS

Noncompetitive sandwich ELISAs were performed using supernatants obtained following monocyte 24-hour culture in RPMI media supplemented with 10% FCS. Secreted monokine protein levels were determined for i) IL-12, ii) IL-10, iii)IL-1β, iv) IL-6, v) IFN-γ and vi) TNF-α, for HC and MS patient samples tested. Statistically significant differences ($p<.05$) between categories are noted by matching symbols (*+^#=!).

IL-12- SP>HC ($p<.01$), SP>RR ($p<.001$), SP>RR/T ($p<.001$)

IL-1β- SP>HC ($p<.05$), SP>RR/T ($p<.05$)

IL-6- SP>HC ($p<.01$), RR>HC ($p<.05$), RR/T>HC ($p<.05$)

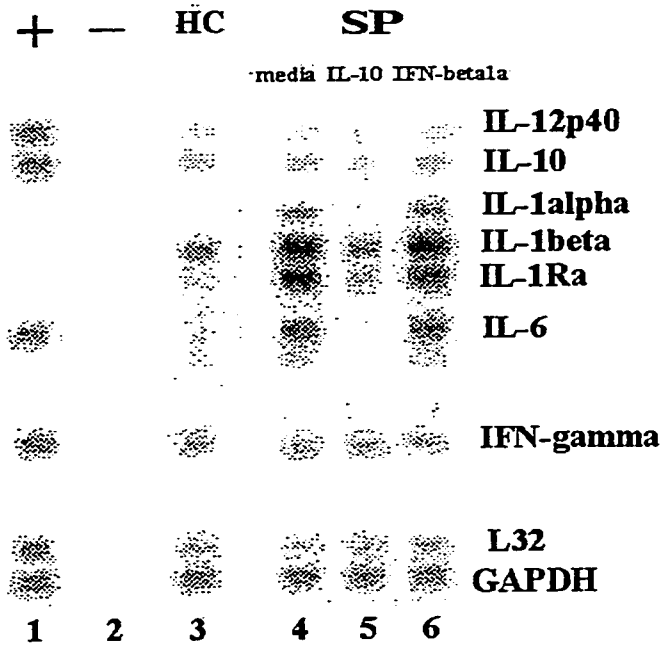
TNF-α- SP>HC ($p<.001$), SP>RR ($p<.05$), SP>RR/T ($p<.001$)
RR>HC ($p<.001$), RR>RR/T ($p<.01$), RR/T>HC ($p<.05$)

E. THE EFFECT OF IL-10 AND IFN-β1a ON MONOKINE mRNA LEVELS

To determine any effect cytokine-directed therapy may have on regulatory monokine mRNA and protein level regulation, monocytes were cultured for 24 hours in the presence of media alone (data shown), or media supplemented with IL-10 or IFN-β1a. As previously described for media-cultured monocytes, total RNA was extracted and sample cytokine mRNA levels were assessed as described in Materials and Methods. Cytokine mRNA levels were thus determined for each control and patient sample obtained, and the effects of IL-10 and IFN-β1a culturing were assessed and shown in Figures 6 and 7.

As shown previously in Figures 2 and 3, SP and RR patients showed significantly higher levels of IL-1β mRNA as compared to HC levels. SP patients alone showed significantly higher levels of IL-1α and IL-6 as compared to HC. SP patients also showed significantly higher levels of IL-10, IL-1α, IL-1β, IL-1Ra and IL-6, and are approaching significance in having higher IL-12p40 levels, as compared to both RR categories under study ($p<.06$). Monocytic IFN-γ levels did not differ between patient samples and control levels. Differences in the effects of IL-10 and IFN-β1a on a SP patient's monokine levels, as determined via the Riboquant RNase protection assay, are shown in Figure 6. IL-10 dramatically reduced these levels as compared to IFN-β1a, which appeared to have minimal effect.

Cumulative graphed results regarding the effects of IL-10 and IFN-β1a are seen in Figure 7. IL-10 significantly reduced SP monokine levels as seen in Figures 7b-f. Levels of IL-10 ($p<.05$), IL-1α ($p<.0001$), IL- 1β ($p<.0005$), IL-1Ra ($p<.0005$), and IL-6 ($p<.0001$) were all significantly reduced in these SP patient samples as compared to levels obtained from media culture alone. As seen in Figures 7 c-g, IL-10 significantly reduced


+
—
HC
SP
media IL-10 IFN-betala
IL-12p40
IL-10
IL-1alpha
IL-1beta
IL-1Ra
IL-6
IFN-gamma
L32
GAPDH
1
2
3
4
5
6

## FIGURE # 6: RIBOQUANT MULTIPROBE RNASE PROTECTION ASSAY SHOWING THE EFFECT OF IL-10 AND IFNβ-1a ON MONOKINE mRNA LEVELS

Monocytes were cultured for 24-hours in the presence of RPMI media supplemented with 10 % FCS alone, or further supplemented with IL-10 or IFN-β1a. Riboquant RNase Protection assays were performed using the hCK-2 template and total RNA isolated from all monocytes samples tested. Cytokine band locations are indicated as determined from positive control.

Lane 1= Positive human control RNA (kit provided)
Lane 2= Yeast tRNA negative control (kit provided)
Lane 3= Representative Human Control monocyte RNA
Lane 4= Representative Secondary Progressive patient media cultured monocyte RNA
Lane 5= Representative Secondary Progressive patient IL-10 cultured monocyte RNA
Lane 6= Representative Secondary Progressive patient IFN-1a cultured monocyte RNA

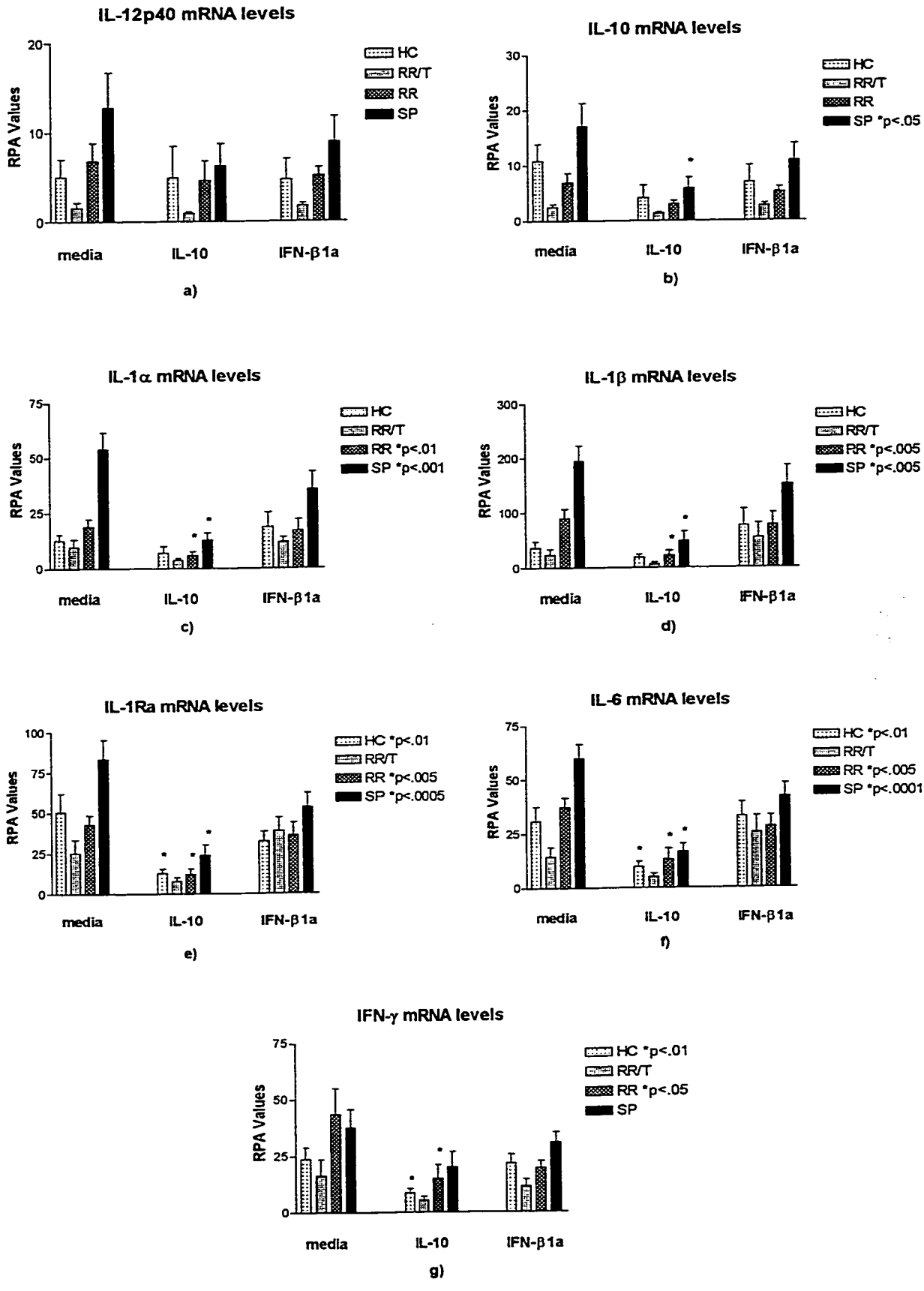

IL-12p40 mRNA levels
RPA Values
20
10
0
HC
RR/T
RR
SP
media
IL-10
IFN-β1a
a)
IL-10 mRNA levels
30
20
10
0
HC
RR/T
RR
SP *p<.05
media
IL-10
IFN-β1a
b)
IL-1α mRNA levels
75
50
25
0
HC
RR/T
RR *p<.01
SP *p<.001
media
IL-10
IFN-β1a
c)
IL-1β mRNA levels
300
200
100
0
HC
RR/T
RR *p<.005
SP *p<.005
media
IL-10
IFN-β1a
d)
IL-1Ra mRNA levels
100
75
50
25
0
HC *p<.01
RR/T
RR *p<.005
SP *p<.0005
media
IL-10
IFN-β1a
e)
IL-6 mRNA levels
75
50
25
0
HC *p<.01
RR/T
RR *p<.005
SP *p<.0001
media
IL-10
IFN-β1a
f)
IFN-γ mRNA levels
75
50
25
0
HC *p<.01
RR/T
RR *p<.05
SP
media
IL-10
IFN-β1a
g)

FIGURE # 7: THE EFFECT OF IL-10 AND IFN-β1a ON MONOKINE mRNA LEVELS

Monocytes were isolated from HC (n=8), RR/T(n=6), RR (n=9), and SP (n=10) blood samples. Monocytes were cultured for 24-hours in RPMI media supplemented with 10 % FCS alone, or further supplemented with IL-10 or IFN-β1a. Riboquant RNase Protection assays were performed using total RNA extracted from MS patient and HC monocytes. Cytokine mRNA levels were tabulated and the mean and standard deviation for each cytokine ; a) IL-12p40, b) IL-10, c) IL-1α, d) IL-1β, e) IL-1Ra, f) IL-6, and g) IFN-γ, were calculated. Statistically significant differences from media levels ($p > 0.05$) are noted by a * symbol.

RR patient monocyte levels of IL-1α ($p<.01$), IL-1β ($p<.005$), IL-1Ra ($p<.005$), IL-6 ($p<.005$), and IFN-γ ($p<.05$) as compared to media levels. The effects of IL-10 are approaching significance in the reduction of RR patient IL-10 levels ($p<.06$) as seen in Figure 7b. IL-10 treatment had no effect on the reduction of any RR/T patient monokine mRNA levels. IL-10 did show however, some reducing effects on HC monokine mRNA levels. As seen in Figures 7 e, f, and g, IL-10 significantly reduced levels of IL-1Ra ($p<.01$), IL-6 ($p<.01$), and IFN-γ ($p<.05$) in HC samples. Therefore, IL-10 had strong transcriptional inhibitory effects on all elevated MS patients mRNA levels, as well as inhibiting certain HC levels.

Surprisingly IFN-β1a had no effect on the reduction of any monokines under study, and only approached significance in the reduction of SP patient IL-1Ra ($p<.06$) and RR IFN-γ ($p=$) levels (Figures 7 e and 7g respectively).

IL-10 and IFN-β1a did not influence the expression of IL-12 p40 levels.

## F. THE EFFECT OF IL-10 AND IFN-β1a ON MONOKINE PROTEIN LEVELS AS DETERMINED BY INTRACELLULAR STAINING

To determine any effect cytokine-directed therapy may have on translated protein levels, intracellular cytokine levels were assessed. The effects of IL-10 and IFN-β1a on IL-12, IL-10, IL-1β, IL-6, IFN-γ, and TNF-α levels were determined. TNF-α was included as a positive control. As shown previously in Figures 4 and 5, SP patient monocytes produced significantly higher levels of IL-1β and IL-6 as compared to HC, while RR patient IL-1β and IL-6 intracellular monokine levels approached significance in being higher than HC levels ($p<.06$). Monocytic IL-10, IL-12, IFN-γ, and TNF-α

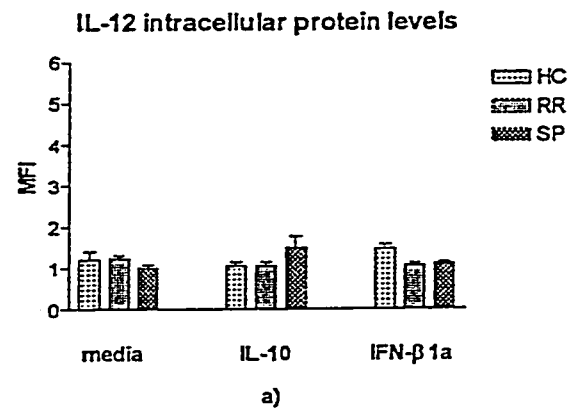
cytokine levels showed no significant differences in the comparison of HC and MS patient (RR and SP) intracellular levels.

The effect of IL-10 and IFN-β1a on intracellular levels of IL-12, IL-10, IL-1β, IL-6, IFN-γ, and TNF-α, for all HC and MS patient samples tested, are shown in Figure 8. As shown in Figures 8a, b, e, and f, intracellular levels of IL-12, IL-10, IFN-γ, and TNF-α monokines remained constant following 24-hour culture, indicating IL-10 and IFN-β1a had minimal effects.

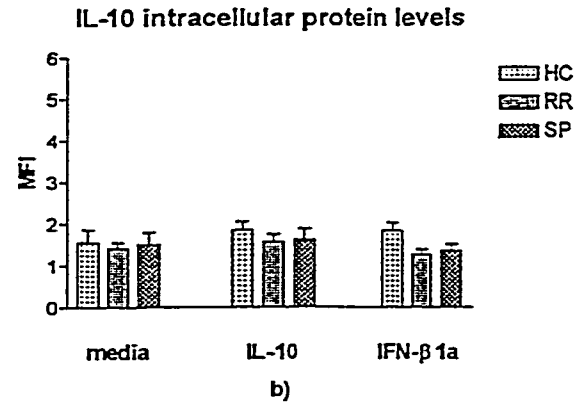
As shown in Figures 8c and 8d RR and SP patient intracellular levels of IL-1β and IL-6 appear visually reduced following IL-10 exposure however, possibly due to very small patient sampling size (n=4), this reduction did not reach significance for IL-1β. IFN-β1a culture appeared to increase intracellular HC IL-1β levels. IFN-β1a culture also appeared to decrease MS patient IL-6 levels to the same extent as IL-10.

To demonstrate the reduction in IL-1β and IL-6 levels caused by IL-10, histograms obtained following intracellular staining from representative HC and MS patient categories are overlayed in Figure 9. These histogram overlays show the actual reduction in monokine MFIs due to the presence of exogenous IL-10 in the culture medium. Reductions shown in Figure 9a are for a SP patient, and indicate IL-1β MFI reductions from MFI=3.1, for media culture alone, to MFI=2.1 following IL-10 culture. IFN-β1a treatment shows little effect with the IL-1β MFI remaining close to media levels (MFI=3).

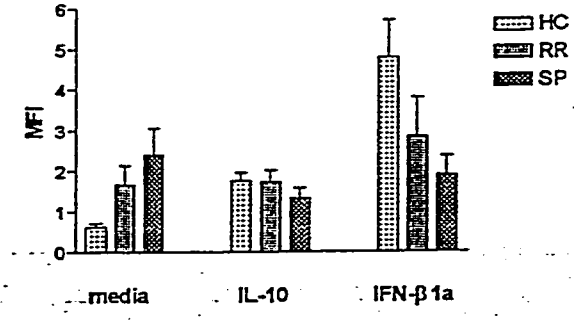
Figure 9b shows the effect of IL-10 on a SP patient's monocytic IL-6 levels. This histogram overlay shows an IL-6 MFI reduction from MFI=5, for media culture alone, to MFI= 1.94 following 24-hour culture in the presence of IL-10. IFN-β1a treatment


IL-12 intracellular protein levels
MFI
HC
RR
SP
media
IL-10
IFN-β1a

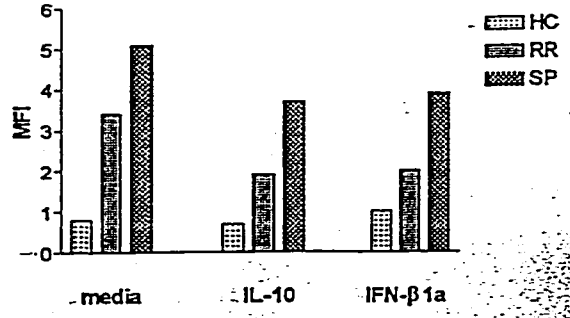
a)


IL-10 intracellular protein levels
MFI
HC
RR
SP
media
IL-10
IFN-β1a

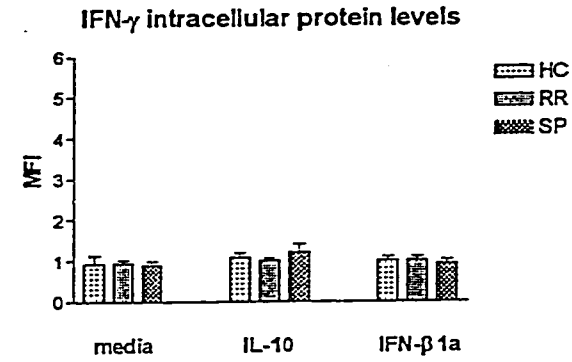
b)


IL-1β intracellular protein levels
MFI
HC
RR
SP
media
IL-10
IFN-β1a

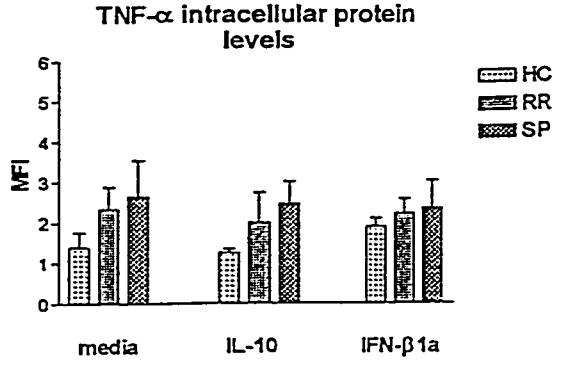
c)


IL-6 intracellular protein levels
MFI
HC
RR
SP
media
IL-10
IFN-β1a

d)


IFN-γ intracellular protein levels
MFI
HC
RR
SP
media
IL-10
IFN-β1a

e)

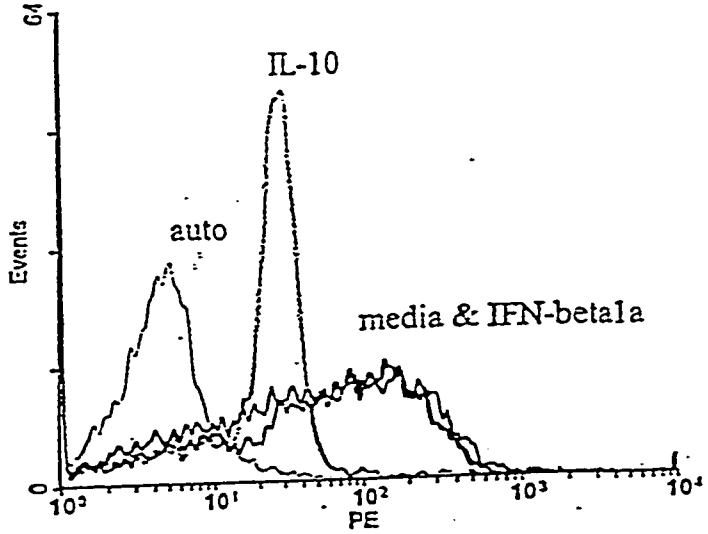

TNF-α intracellular protein levels
MFI
HC
RR
SP
media
IL-10
IFN-β1a

f)

FIGURE # 8: THE EFFECT OF IL-10 AND IFN-β1a ON DONOR INTRACELLULAR MONOKINE PROTEIN LEVELS

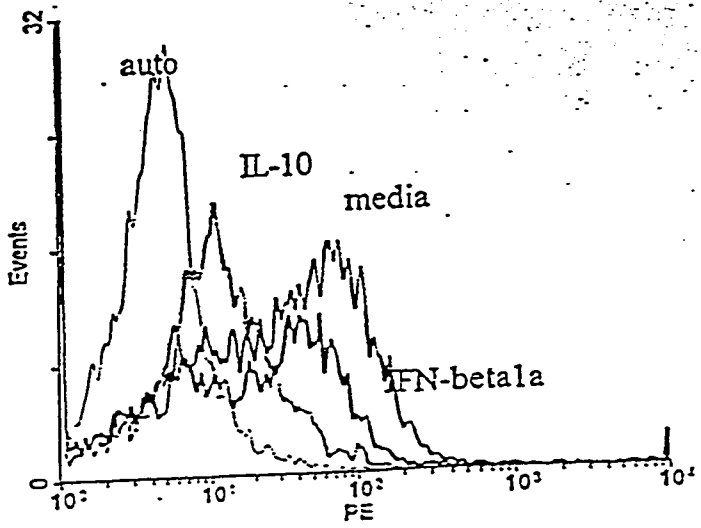
Monocytes were cultured for 24-hours in RPMI media supplemented with 10% FCS alone, or further supplemented with IL-10 or IFN-β1a. Monensin golgi inhibitor was added in the last 7-8 hours of culture. Intracellular Staining was performed on isolated monocytes. Cells were blocked with gamme-immune and stained with PE-conjugated antibodies for a) IL-12, b) IL-10, c) IL-1β, d) IL-6, e) IFN-γ, and f) TNF-α. MFI results from HC, RR, and SP (n=4 for each) samples were tabulated and the mean and standard deviation were calculated. Statistically significant differences ($p<.05$) are noted by matching symbols.

IL-1 beta




a)

IL-6




b)

## FIGURE # 9: THE EFFECT OF IL-10 AND IFN-β1a ON INTRACELLULAR MONOKINE PROTEIN LEVELS

Intracellular Staining was performed on isolated monocytes, following 24-hour cultured in RPMI media supplemented with 10% FCS alone, or further supplemented with IL-10 or IFN-β1a. Monensin golgi inhibitor was added in the last 7-8 hours of culture. Cells were blocked with gamme-immune and stained with PE-conjugated antibodies for IL-1β, IL-6, IL-10, IL-12, IFN-γ, or TNF-α. A representative SP patient's a) IL-1β and b) IL-6 monokine levels are shown. Each sample has a negative control, shown as auto, with a MFI of 0.05.

however, shows an extremely less dramatic IL-6 MFI reduction (MFI=4.1) as compared to the media level.

## G.THE EFFECT OF IL-10 AND IFN-β1a ON MONOKINE PROTEIN LEVELS AS DETERMINED BY ELISA

To better understand and more accurately determine the effects of IL-10 and IFN-β1a on MS patient and HC monokine protein levels, non-competitive sandwich ELISAs were performed. ELISAs were performed to determine the levels of IL-12, IL-10, IL-1β, IL-6, IFN-γ, and TNF-α secreted by MS patient (SP and RR) and HC monocytes during a 24 hour culture period, and to determine the effects of IL-10 and IFN-β1a on secretion patterns.

As shown previously in Table I, SP patient monocytes secreted significantly higher levels of IL-12 and IL-6 as compared to HC, RR, and RR/T, and significantly higher levels of IL-1β as compared to HC. RR and RR/T also secreted significantly higher levels of IL-6 as compared to HC. SP, RR, RR/T patient monocytes were also found to secrete significantly higher levels of TNF-α as compared to HC samples.

As seen in Table II, elevated SP patient media IL-12 levels were found significantly reduced by IL-10 ($p<.05$). IL-10 was also found to significantly reduce levels of IL-1β secreted by SP patient monocytes as compared to media levels ($p<.05$) (Table III). Levels of IL-6 (Table IV) were found significantly reduced by IL-10, as compared to media or IFN-β1a, for SP ($p<.01$ and $p<.05$ respectively), RR ($p<.01$), and RR/T ($p<.001$) patient levels. Levels of TNF-α secreted by all MS patient monocytes

TABLE # II: THE EFFECT OF IL-10 AND IFN-β1A ON MONOCYTIC IL-12 SECRETED LEVELS

| Patient Sample | i) media pg/ml | ii) IL-10 pg/ml | iii) IFN-β1a pg/ml |
|---|---|---|---|
| HC 1 | 320 | 60 | 280 |
| 2 | 650 | 65 | 650 |
| 3 | 300 | 60 | 300 |
| 4 | 300 | 30 | 295 |
| RR/T 1 | 1.4 | 1.5 | 250 |
| 2 | 250 | 11 | 300 |
| 3 | 300 | 125 | 300 |
| RR 1 | 33 | 50 | 75 |
| 2 | 50 | 50 | 380 |
| 3 | 380 | 65 | 650 |
| 4 | 550 | 60 | 1500 |
| SP 1 | 1500 | 50 | 550 |
| 2 | 2100 | 75 | 2250 |
| 3 | 3250 | 85 | 65 |
| 4 | 2500 | 85 | 3250 |
| | * | * | |

## TABLE # II: THE EFFECT OF IL-10 AND IFN-β1a ON MONOCYTIC IL-12 SECRETED LEVELS

A noncompetitive sandwich ELISA was performed using supernatants obtained following monocyte 24-hour culture in RPMI media supplemented with 10% FCS, or further supplemented with IL-10 or IFN-β1a. Secreted IL-12 monokine protein levels were determined for HC and MS patient samples tested. Statistically significant differences between media, IL-10, or IFN-β1a categories are noted by matching symbols (*).

SP- IL-10 < media ($p<.05$)

TABLE # III: THE EFFECT OF IL-10 AND IFN-β1A ON MONOCYTIC IL-1β SECRETED LEVELS

| Patient Sample | i) media pg/ml | ii) IL-10 pg/ml | iii) IFN-β1a pg/ml |
|---|---|---|---|
| HC 1 | 6.5 | 6.5 | 12 |
| 2 | 9.5 | 8.5 | 13 |
| 3 | 14 | 8.5 | 18 |
| 4 | 33 | 19 | 52 |
| RR/T 1 | 20 | 5.5 | 180 |
| 2 | 100 | 10 | 80 |
| 3 | 400 | 10 | 600 |
| RR 1 | 40 | 37 | 65 |
| 2 | 800 | 43 | 300 |
| 3 | 1200 | 20 | 5000 |
| 4 | 1200 | 40 | 25000 |
| SP 1 | 600 | 40 | 25 |
| 2 | 700 | 25 | 140 |
| 3 | 2000 | 35 | 500 |
| 4 | 2500 | 15 | 2500 |
|  | * | * |  |

TABLE # III: THE EFFECT OF IL-10 AND IFN-β1a ON MONOCYTIC IL-1β SECRETED LEVELS

A noncompetitive sandwich ELISA was performed using supernatants obtained following monocyte 24-hour culture in RPMI media supplemented with 10% FCS, or further supplemented with IL-10 or IFN-β1a. Secreted IL-1β monokine protein levels were determined for HC and MS patient samples tested. Statistically significant differences between media, IL-10, or IFN-β1a categories are noted by matching symbols (*).

SP- IL-10 < media ($p<.05$)

TABLE # IV: THE EFFECT OF IL-10 AND IFN-β1A ON MONOCYTIC IL-6 SECRETED LEVELS

| Patient Sample | i) Media pg/ml | ii) IL-10 pg/ml | iii) IFN-β1a pg/ml |
|---|---|---|---|
| HC 1 | 150 | 75 | 290 |
| 2 | 180 | 140 | 350 |
| 3 | 300 | 190 | 370 |
| 4 | 320 | 160 | 1100 |
| RR/T 1 | 2200 | 500 | 3500 |
| 2 | 2500 | 650 | 3800 |
| 3 | 2500 | 270 | 4000 |
| | =@ | =! | !@ |
| RR 1 | 370 | 50 | 4000 |
| 2 | 4200 | 850 | 3750 |
| 3 | 4750 | 140 | 3750 |
| 4 | 4800 | 280 | 4800 |
| | ^ | ^# | # |
| SP 1 | 2800 | 150 | 2250 |
| 2 | 3300 | 130 | 2500 |
| 3 | 4300 | 750 | 550 |
| 4 | 5550 | 350 | 3800 |
| | *> | *+ | +> |

## TABLE # IV: THE EFFECT OF IL-10 AND IFN-β1a ON MONOCYTIC IL-6 SECRETED LEVELS

A noncompetitive sandwich ELISA was performed using supernatants obtained following monocyte 24-hour culture in RPMI media supplemented with 10% FCS, or further supplemented with IL-10 or IFN-β1a. Secreted IL-6 monokine protein levels were determined for HC and MS patient samples tested. Statistically significant differences between media, IL-10, or IFN-β1a categories are noted by matching symbols (*+^=@!>#).

SP- IL-10 < media ($p<.01$), IL-10 < IFN-β1a ($p<.05$)
IFN-β1a < media ($p<.05$)

RR- IL-10 < media ($p<.01$), IL-10 < IFN-β1a ($p<.01$)

RR/T- IL-10 < media ($p<.001$), IL-10 < IFN-β1a ($p<.001$)
IFN-β1a > media ($p<.001$)

TABLE # V: THE EFFECT OF IL-10 AND IFN-β1A ON MONOCYTIC TNF-α SECRETED LEVELS

| Patient Sample | i) media pg/ml | ii) IL-10 pg/ml | iii) IFN-β1a pg/ml |
|---|---|---|---|
| HC 1 | 1.25 | <1 | 28 |
| 2 | 1.4 | 25 | 16 |
| 3 | 2.2 | 9 | 2.1 |
| 4 | 8.5 | 25 | 36 |
| RR/T 1 | 9.5 | 6 | 240 |
| 2 | 75 | 50 | 160 |
| 3 | 160 | <1 | 240 |
| | =@ | =! | !@ |
| RR 1 | 150 | 1.3 | 55 |
| 2 | 170 | 1.6 | 250 |
| 3 | 180 | <1 | 1.2 |
| 4 | 185 | 1.5 | 170 |
| | ^ | ^# | # |
| SP 1 | 230 | 4 | 1.5 |
| 2 | 240 | <1 | 20 |
| 3 | 240 | 32 | 125 |
| 4 | 240 | 4.3 | 230 |
| | * | *+ | + |

## TABLE # V: THE EFFECT OF IL-10 AND IFN-β1a ON MONOCYTIC TNF-α SECRETED LEVELS

A noncompetitive sandwich ELISA was performed using supernatants obtained following monocyte 24-hour culture in RPMI media supplemented with 10% FCS, or further supplemented with IL-10 or IFN-β1a. Secreted TNF-α monokine protein levels were determined for HC and MS patient samples tested. Statistically significant differences between media, IL-10, or IFN-β1a categories are noted by matching symbols (*+^=@!#).

SP- IL-10 < media ($p<.01$), IL-10 < IFN-β1a ($p<.01$)
RR- IL-10 < media ($p<.05$), IL-10 < IFN-β1a ($p<.05$)
RR/T- IL-10 < media ($p<.05$), IL-10 < IFN-β1a ($p<.05$)
IFN-β1a>media ($p<.05$)

were also found significantly reduced following treatment with IL-10, as compared to media cultured and IFN-β1a treated levels (SP $p<.01$, RR and RR/T $p<.05$) (Table V). IFN-β1a treatment decreased SP patient IL-6 ($p<.05$) levels, but showed minimal reducing effects on any RR patient monokine levels. IFN-β1a in fact appears to boost IL-6 and IL-1β levels for some RR patients studied. Interestingly IFN-β1a treatment significantly boosted media levels of IL-6 ($p<.001$) and TNF-α ($p<.05$) found in all RR/T to untreated patient levels. All remaining cytokines tested (IL-10 and IFN-γ) showed similar protein secretion levels, for all control and MS patient samples under study, for all culturing conditions tested.

# VIII. DISCUSSION

The hypotheses addressed in this project were firstly that Th1 regulatory and proinflammatory monokine levels, such as IL-1, IL-6, IL-12, and IFN-γ, are elevated in MS patients in conjunction with disease severity. Secondly it was hypothesized thatTh1 inhibitory cytokine-directed therapies, such as IL-10 and IFN-β1a, would decrease these elevated monokine levels to those of healthy controls. The purpose of this project was thus twofold, firstly to determine what influences MS disease has on specific monokine levels (IL-12, IL-10, IL-1α, IL-1β, IL-1Ra, IL-6, IFN-γ, and TNF-α), and secondly to determine the effects of IFN-β1a and IL-10 on these levels. The data reveal that increased MS disease severity, i.e., SP versus RR disease, produced increased overall monocyte levels of IL-12, IL-1α, IL-1β, IL-6, and TNF-α, and levels of IL-10 and IFN-γ comparable to healthy levels. As well, the data show IL-10 significantly downregulates all monokines under study to healthy levels. IFN-β1a, a well known MS therapy, surprisingly showed minimal downregulatory effects on monocyte cytokine release.

Highly enriched monocytes (Figure 1) were used to test the levels of monocytic cytokines from MS patients (RR/T, RR, SP) and HC. Following 24-hour culture in RPMI media/ 10% FCS alone, or supplemented with IL-10 or IFN-β1a, monokine mRNA and protein levels were determined. Using the Riboquant RNase Protection Assay, levels of IL-12p40, IL-10, IL-1α, IL-1β, IL-1Ra, IL-6, and IFN-γ mRNA present in each monocyte sample isolated, were determined. As seen in Figures 2 and 3, SP patients showed significantly higher levels of IL-1α, IL-1β and IL-6 as compared to HC, RR/T, and RR samples. RR patients showed significantly higher levels of IL-1β and IL-6 as compared to

RR/T patients and showed significantly higher levels of IL-1β as compared to HC. SP patient IL-12p40 levels approached significance in being greater than HC and RR patient levels. Levels of IL-10 and IL-1Ra did not significantly differ as compared to HC levels for any of the MS categories under study. Significant level differences as compared to HC did not occur for these monokines due to the level ranges found in the various HC samples being extremely broad. Although the levels of IL-12p40, IL-10, and IL-1Ra did not significantly differ from HC levels for any of the MS categories, within the disease samples SP patients showed significantly higher levels of these cytokines as compared to both RR categories, thus revealing possible intradisease differences in regulation of transcription of these cytokines. Levels of IFN-γ, an important Th1 cytokine, were surprisingly constant for all samples tested.

So as to examine any differences between levels of monokines transcribed, as shown by the Riboquant data, translated, or secreted, intracellular staining and ELISAs were next performed. Intracellular staining data revealed the cytokines being translated within the media cultured monocytes over an approximate 7-8 hour period (Figures 4 and 5). Staining revealed IL-1β and IL-6 levels generally reflected mRNA data in that SP and RR patient monocytes produced greater levels of these cytokines than did HC. Levels of IL-12, IL-10, IFN-γ, and TNF-α were consistent for all samples tested, thus indicating the various MS patient categories experienced no differences in the translational regulation of these cytokines. TNF-α was included in the staining panel as a positive control. Due to a lack of IL-1α and IL-1Ra antibody availability, these intracellular levels were not determined.

So as to determine whether regulatory differences existed between the levels of monokine proteins translated as compared to secreted, ELISAs were next performed on supernatants obtained following 24-hour media culture (Table 1). TNF-α secretion levels were determined as a positive ELISA control. SP patient monocytes secreted significantly higher levels of IL-12 as compared to monocytes from both RR and the HC categories. SP patient monocytes also secreted significantly higher levels of IL-1β as compared to HC and RR/T. Levels of IL-6 and TNF-α detected from the supernatants of media cultured monocytes were significantly higher for SP and RR patients as compared to HC levels. Other cytokines tested, IL-10 and IFN-γ, showed similar levels secreted between MS patients and healthy controls, for all samples tested. Since IL-1α is a membrane bound protein, secreted levels were not determined. As well, due to the unavailability of a reliable IL-1Ra ELISA kit, secreted levels of this monokine were also not determined in this study.

Differences obtained between RNase protection assay, intracellular staining, and ELISA data could represent time-course differences between mRNA transcription, protein translation and monensin golgi inhibitor addition during the 24-hour culture. The golgi inhibitor, which blocks the movement of cytokines from the golgi body, was added for the last 7-8 hours of culture. The bulk of cytokines produced however, could be transcribed, translated, and secreted during the first 16 hours of culture, thus, they would be detected by RNase Protection Assay and ELISA, but not by intracellular staining, which would only detect cytokine proteins translated during those last 7-8 hours of culture.

So as to determine any effect therapy may have on the aforementioned monokine levels, the same series of experiments examining transcribed, translated, and secreted

monokine levels were performed on monocytes cultured for 24 hours in the presence of IL-10 or IFN-β1a. RNase protection assay analysis (Figures 6 and 7) revealed in this study that IL-10 significantly reduced levels of IL-1α and IL-1β in SP and RR monocytes. IL-10 also significantly reduced its own production in SP monocytes and approached significance in IL-10 reduction in RR patient monocytes. IL-1Ra and IL-6 levels were significantly reduced in HC, RR, and SP patients, while IFN-γ levels were significantly reduced by IL-10 in only the HC and RR patient categories. IL-10 24-hour culture had little effect on IL-12p40 mRNA levels in any sample category tested, although was approaching significance in the reduction of elevated SP IL-12 levels.

IFN-β1a culturing had minimal effects on monokine levels. This cytokine therapy only approached significance in the reduction of SP patient IL-1Ra levels and RR IFN-γ mRNA levels.

Intracellular staining and ELISA experiments were further undertaken to determine the effects of IL-10 and IFN-β1a on cytokine protein translation and secretion. Intracellular staining, (Figures 8 and 9), showed that IFN-β1a had minimal effect on monokine production, while IL-10 appeared, although not significantly, to elicit the same downregulatory effects for IL-1β and IL-6 as seen previously in the mRNA data. Due to possible explanations for these results, such as small sampling size and time course differences in golgi inhibitor addition, monokine release, or therapy action, further research was required. Thus, subsequent to these experiments ELISAs were performed on 24-hour culture supernatants.

ELISA experiments revealed (Tables II, III, IV, and V) that IL-10 addition to the culture environment significantly reduced elevated levels of IL-12, IL-1β, IL-6, and TNF-α

secreted by MS patient monocytes. Importantly in all studies performed, the levels of secreted cytokines reduced following IL-10 exposure did not significantly differ from HC levels.

IFN-β1a again showed minimal effects on downregulating monokine secretion. Only SP patient IL-6 and TNF-α levels were decreased following IFN-β1a culture. Interestingly, IFN-β1a did reveal itself to upregulate IL-6 and TNF-α secretion in all RR patients already undergoing immunosuppressive therapy. Thus, if steroid-IFN-β1a combination therapy was employed in these patients, it could lead to increased disease severity due to boosted secretion of Th1 regulatory and proinflammatory cytokines. This finding indicates an important reason why further study should be performed into the area of combination therapy for MS so as to prevent unnecessary suffering.

It is important to note that all patients found within a specific MS category do not suffer from the same immunopathology, and thus may not show similar monocytic cytokine patterns or respond similarly to treatment. In conjunction with this IFN-β1a was found to boost secretion of IL-12, IL-1β, IL-6, and TNF-α in certain RR patients, while slightly decreasing or not affecting secretion levels in others. This finding may indicate a possible marker for determining whether a RR patient will respond favorably to, or fail, interferon therapy, based on whether the patient's monocytes up or down-regulate the aforementioned monokines release following treatment.

These experiments thus reveal that MS patients transcribe and secrete significantly greater levels of IL-12, a Th1 regulatory cytokine, and IL-1β, IL-6, and TNF-α proinflammatory cytokines as compared to healthy controls. It was also shown that this upregulated production reflects disease severity in that SP patients, which suffer from

more severe and frequent disease episodes secrete significantly greater levels of these cytokines as compared to patients tested from the other relapsing remitting disease categories. This study also reveals that IL-10 significantly reduces the transcription and secretion of these upregulated monokines *in vitro*, to levels similar to healthy controls.

MS pathogenesis can no longer be explained solely by T cell responses. Antigen-presenting-cell functions and inflammatory responses, mediated by monocytes, macrophages and dendritic cells, are believed to be a required and perhaps initial feature of MS demyelination (58). Inflammatory CNS infiltrates, as well as the production of pro- and antiinflammatory cytokines and chemokines, all have proposed roles in MS disease pathogenesis and severity (31, 89). T cells are still largely believed however, as the major effector cells in MS and contribute to inflammation via the production of proinflammatory or regulatory cytokines (90). These experiments collectively reveal MS disease involving normal monocytic IFN-$\gamma$ (Th1), and IL-10 (Th2) levels, and increased monocytic pro-inflammatory and Th1 regulatory cytokine levels, specifically IL-12, IL-1$\beta$, IL-6, and TNF-$\alpha$. Thus, to fully understand the importance and role of these upregulated monocytic cytokines in MS, we must consider their known functions.

IL-12 is composed of two different proteins (p35 and p40), that are encoded by separate genes. The p40 component of this cytokine is believed to be the biologically active protein of IL-12 (91). In response to a variety of stimuli, IL-12 is produced predominantly by macrophages (5, 91). The main effect of this cytokine is T lymphocyte and NK cell function regulation. IL-12 is strongly implicated in the polarization of maturing T cells to the Th1 phenotype (91). A skewed T cell response to the Th1 phenotype is strongly believed implicated in the immunopathogenesis of MS (16). IL-12

levels only appear significantly elevated in SP patients. This finding is approaching significance in terms of mRNA data (Figure 3) and is significant from the ELISA data (Table I). RR and RR/T patients show mRNA and protein IL-12 levels comparable to HC. The upregulated production and/or secretion of IL-12 in SP patients could thus represent an important mode of immune response alteration in these patients by biasing their immune systems for cell-mediated responses. This in turn could lead to the increased disease severity that is seen in this group of MS patients. This alludes to a possible occurrence or trigger, which causes increased monocytic IL-12 production leading to significant Th1 upregulation. This increased IL-12 production thus leads to a more intense cell mediated response, and in turn may be responsible for the clinical entrance into the SP stage from the RR stage.

IL-1β is the pluripotent secreted form of the IL-1 cytokine. IL-1β has been associated with the activation of many immunological and inflammatory responses. In the CNS, overproduction of IL-1β has been considered the core inducer of tissue damage and destruction in many diseases (76, 91). Chronic inflammatory states are known to be induced by IL-1β by its repeated induction of IL-6, TNF-α and its own production (76). CNS dysfunctions, such as HIV encephalitis and Alzheimer's disease, occur due to altered IL-1β expression (76). This cytokine is known to activate T, B and NK cells, upregulate adhesion molecules, alter blood-brain barrier permeability, and induce β-chemokine expression (76, 91). As well, IL-1β is known to induce nitric oxide synthase and peroxynitrite formation, which are responsible for vasodilation and cell damage (36, 76). All of these aforementioned functions are known occurrences in MS pathology (30, 31, 76, 92). Due to its overwhelming immunological effects and induction of inflammatory

processes and CNS dysfunctions, IL-1β has long been considered an important regulatory factor in MS disease (76, 91). The findings of this project fully support that theory and suggest monocytes as the cells responsible for its overproduction and thus, its deleterious effects.

IL-6, which is also produced by T cells, is best known for its induction and control of acute phase protein synthesis and release (93). IL-6 is produced early in inflammation, shortly after IL-1 and TNF-α, and displays several proinflammatory and regulatory properties. Such properties include macrophage maturation, neutrophil maturation and activation, and differentiation of CTL and NK cells (91). IL-6 is also known to cause increased expression of IL-1 and TNF-α (91, 93). Although IL-6 is not regarded as a typical proinflammatory cytokine, since it does possess some antiinflammatory properties, abnormal production of IL-6 has been suggested involved in rheumatoid arthritis and many other autoimmune diseases (91, 93). Thus a role in MS, due to increased monocyte production, is quite feasible since its various roles are known to affect the MS disease course (89, 92, 94).

TNF-α was initially included in the intracellular staining and ELISA experiments as a positive control. TNF-α is a lab standard due to its known constitutive expression by monocytes and T lymphocytes. TNF-α has however, been associated with T lymphocytes in MS pathogenesis since it is known to drive the Th1 response (95, 96). Translated TNF-α protein levels, as determined by intracellular staining, showed no significant differences between HC and MS patient levels (Figure 5), however, ELISA data revealed extremely high quantities of TNF-α being released from MS patient monocytes as compared to HC levels (Table I). This contradiction in data could be due to the time course of TNF-α release in

relation to when the monensin golgi inhibitor was added to the samples, or due to a strict upregulation of TNF-α secretion in MS patients with translation levels being unaffected.

TNF-α is believed to be produced by inflammatory stimuli, and is known to signal cell proliferation and apoptosis, which are important features of the immune system (91). Excessive TNF-α signaling however, leads to tissue injury, severe inflammatory reactions and shock (91). TNF-α has previously been directly implicated in MS immunopathogenesis (94, 97), thus its upregulation by MS patient monocytes makes these cells an important contributor to inflammation, Th1 regulation and MS disease activity.

Of interest are the levels of IL-β, IL-6, and TNF-α produced by RR patients undergoing therapy. IL-1β and IL-6 monokine mRNA levels are decreased in patients undergoing immunosuppressive therapy and are comparable to HC levels. However, as determined by ELISA, levels of IL-1β protein secreted remained comparable to HC levels, while IL-6 and TNF-α protein levels secreted by RR/T patient monocytes remained comparable to SP and RR levels. This indicates a possible mode of action of these immunosuppressive therapeutic agents on the immune system. It indicates that perhaps specific pathways such as IL-1β and IL-6 mRNA transcription and IL-1β translation or secretion, but not IL-6 or TNF-α secretory pathways, are affected by these immunosuppressive agents, thus indicating one possible mode of benefit for MS patients on these therapies.

The findings of this study indeed indicate a role for monokines in MS immunopathogenesis. Normal levels of IL-10 and IFN-γ mRNA and protein were found in all MS patients. TNF-α levels were found greatly elevated in the supernatants of MS patients as compared to HC. IL-12 mRNA levels were approaching significance, while

protein levels were found significantly increased in the supernatants of SP patients as compared to RR and HC samples. As well, significant increases in IL-1β and IL-6 mRNA found in RR and SP MS patient monocytes, as compared to HC, correlates with levels of IL-1β and IL-6 protein detected via intracellular staining and ELISA. This study also reveals that increased disease severity (i.e., SP compared to RR disease) results in higher levels of monocytic IL-12, IL-1β, IL-6, and TNF-α regulatory cytokines.

Altered monokine levels are thus a potential factor in MS immunopathogenesis. The control of these elevated monokine levels could thus possibly lead to decreased symptoms and disease severity. Any therapy resulting in Th1 regulatory or proinflammatory monokine level reduction may lead to possible benefits, and as such would be extremely important in a disease, such as MS, where few beneficial therapies exist. Unfortunately MS therapies have mainly focussed on general, nonspecific suppression of the inflammatory response (67). Immunosuppressive methods, like steroids, have many limitations. Luckily, as knowledge regarding cytokine interrelationships has grown, and MS disease has become better understood, cytokine directed therapies have been developed (67). The possible effects Th1 cytokine directed therapies have on Th1 regulatory and proinflammatory monokine levels in MS are therefore important to investigate.

IFN-β1a has been approved as an MS therapy since 1996. Clinical trials have revealed this therapy to significantly reduce disease activity, exacerbation rate, and reduce lesion load (30, 98). IFN-β1a is believed to act mainly through its ability to attenuate the inflammatory response (80). Unfortunately, the exact mechanisms used by IFN-β1a to exert its beneficial outcomes are unknown (67). Study into the actions of IFN-β1a reveal

its ability to inhibit T cell proliferation, impair receptor upregulation on T cells (i.e., IL-2), and decrease IFN-γ production by T cells (48, 67, 99). The beneficial effects of this therapy on monocyte cytokine release are much less documented, and based on the findings of this study, not as pronounced as for T cells. Interestingly, it was found by Ruuls et. al. (1998), that IL-10 secretion by activated PBMC and IL-10 human serum levels are increased by *in vitro* addition and intramuscular injection of IFN-β respectively (67). It was therefore speculated by Ruul's group that a possible mode of action of IFN-β is IL-10 upregulation and that this therapy's beneficial property is due to the immunosuppressive actions of IL-10 (67). As stated previously, IFN-β1a was found to have no effect on IL-10 production in monocytes, and overall minimal effects on monokine production. Perhaps a longer IFN-β1a treatment duration would have produced IL-10 upregulation, or more dramatic Th1/ proinflammatory monokine downregulation. The regulatory effects of IFN-β1a appear to be related to translation. The Riboquant mRNA data revealed no IFN-β1a effect on monokine levels, however the ELISA data did reveal some small regulatory effects of IFN-β1a on protein production and/or secretion.

In MS patients, the beneficial effects of IFN-β therapy on monocytes possibly occurs earlier in the cell's development. Monocyte production, as stated previously, is known to be inhibited by IFN-β (6, 26). Although no direct inhibitory effects were seen on monokine production, perhaps some of the beneficial effects seen with interferon therapy are due to the direct inhibition of monocyte production, thus reducing the possible number of monocytes capable of upregulating Th1 and proinflammatory cytokine production.

IFN-β1a did, however, produce some interesting results involving RR patient monokines. RR patients undergoing immunosuppressive therapy produced significantly boosted levels of IL-1β, IL-6, and TNF-α following IFN-β1a treatment. Combination therapy has been considered for many diseases including MS. This study indicates that the combination of interferon-β and steroid therapy significantly boosts the proinflammatory response in RR patients. This form of therapy could result in more aggressive myelin attack, and thus more severe disability. In certain RR patients not undergoing therapy, IFN-β1a addition also resulted in augmented cytokine levels. The addition of IFN-β resulted in upregulated protein production and/or secretion of IL-12, IL-1β, IL-6, and TNF-α production in certain RR patients tested. The upregulated production of these Th1 regulatory and proinflammatory cytokines could lead to extremely detrimental effects and the development of more severe disease. This finding indicates a possible marker by which the likelihood of a patient benefiting from, or failing therapy, could be determined. This could prove to be extremely beneficial as a therapy outcome predictor, and as such could save the patient much time, money, and needless pain and disease aggravation, if it could be determined beforehand *in vitro* whether or not the patient would benefit.

IL-10 has long been known as an important regulator of proinflammatory cytokine production (100). The biologic effects of IL-10 have been documented to result in immunosuppression, and thus, IL-10 has been found extremely useful in diseases where overexpression of cytokines play a role in pathogenesis (101). As shown, MS disease significantly correlates with increased monocytic production and secretion of IL-12, IL-1β, IL-6, and TNF-α (Figures 3 and 5, Table I). This study clearly demonstrates the

ability of IL-10 to affect transcription, downregulating mRNA, and thus subsequent protein levels, of the cytokines tested to levels of healthy controls. This differs from the IFN-β1a data, which indicated the effects of IFN-β1a to be related to translation. As well, levels of other monocytic cytokines, previously shown to be significantly elevated in relation to disease severity within MS patients, show a more uniform spread of cytokine release amongst all samples tested following IL-10 culture. Importantly, levels of all cytokines elevated in MS upon IL-10 downregulation, were not found to significantly differ from HC levels.

One concern regarding IL-10 therapy is that the downregulation of monokine production could result in another form of disease. Thus, although the levels of proinflammatory cytokines are decreased to healthier levels, the effect of these new levels *in vivo* needs to be determined. As well, the effects of IL-10 on other cytokine levels and immunological pathways need also be investigated before conclusive evidence can be obtained for IL-10 as a beneficial therapy.

# IX. SUMMARY AND CONCLUSIONS

In summary this study sheds some light on the role of monocytes in MS immunopathogenesis. MS is a known chronic, CNS inflammatory disease. The main effector cell believed responsible for inflammatory and regulatory cytokine production has long been T cells. This study shows that T lymphocytes may no longer be the main driving force behind MS immunological and inflammatory processes. A model of the possible role monocytes play in MS, in relation to the findings of this project, is seen in Figure 10. An unknown MS trigger, i.e., superantigen or molecular mimic, leads to T cell activation. Memory T cells are produced and migrate to the blood-brain barrier. The memory T cells are reactivated upon exposure to myelin antigens in the central nervous system. This leads to T cell cytokine release and subsequent peripheral immune cell activation. Peripheral blood monocyte activation leads to upregulated transcription and translation of Th1 regulatory and proinflammatory cytokines, such as IL-1β, IL-6, and TNF-α. These monokines contribute to increased cellular activation and proliferation, activated monocyte apoptosis inhibition, tissue damage, altered blood-brain barrier permeability and thus, MS disease. The production of greater levels of these monokines, thus results in more severe MS disease activity. Upregulated monocytic IL-12 and further upregulated IL-1β, IL-6, and TNF-α production leads to increased damage and disability and subsequent SP disease. The exact trigger responsible for this upregulated monocytic Th1 regulatory and proinflammatory cytokine transcription and translation is unknown.

Immunosuppressive steroid therapy, as used by RR/T, appears to work in this model by the downregulation of IL-1β and IL-6 transcription and IL-1β translation, while

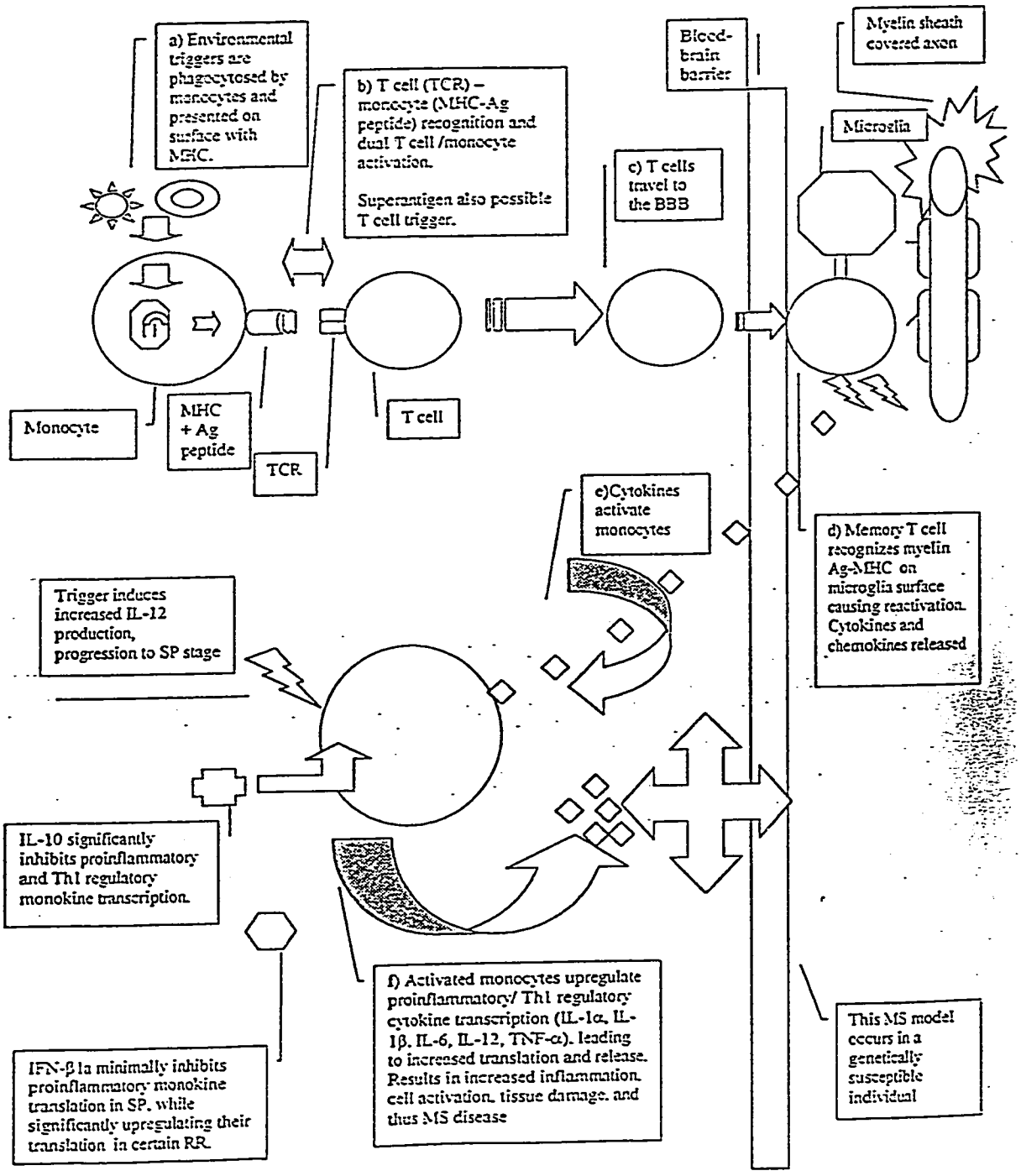

a) Environmental triggers are phagocytosed by monocytes and presented on surface with MHC.
b) T cell (TCR) – monocyte (MHC-Ag peptide) recognition and dual T cell /monocyte activation.
Superantigen also possible T cell trigger.
Blood-brain barrier
Myelin sheath covered axon
Microglia
c) T cells travel to the BBB
Monocyte
MHC + Ag peptide
TCR
T cell
e)Cytokines activate monocytes
d) Memory T cell recognizes myelin Ag-MHC on microglia surface causing reactivation. Cytokines and chemokines released
Trigger induces increased IL-12 production, progression to SP stage
IL-10 significantly inhibits proinflammatory and Th1 regulatory monokine transcription.
f) Activated monocytes upregulate proinflammatory/ Th1 regulatory cytokine transcription (IL-1α, IL-1β, IL-6, IL-12, TNF-α). leading to increased translation and release. Results in increased inflammation. cell activation. tissue damage. and thus MS disease
This MS model occurs in a genetically susceptible individual
IFN-β1a minimally inhibits proinflammatory monokine translation in SP. while significantly upregulating their translation in certain RR.

FIGURE # 10: PROPOSED MODEL FOR THE ROLE OF MONOCYTES IN MS

not affecting IL-6 or TNF-α translation. IL-10 has an important role in the regulation of monocyte cytokine transcription in this monocyte based model of MS. Monocyte cytokines are actively downregulated upon IL-10 treatment. This effect is most importantly seen involving Th1 regulatory and proinflammatory monokine transcription, where IL-10 culture reduces upregulated levels of IL-12, IL-1β, IL-6, and TNF-α, to those of healthy controls. IFN-β1a, a therapy known to benefit some RR MS patients, showed minimal effects on monokine downregulation. Only slight translational inhibitory effects were seen in certain patients. In some RR patients IFN-β1a culture leads to upregulated translation of proinflammatory monokines, thus indicating possible detrimental effects due to this therapy. IFN-β1a cytokine therapy's main beneficial mode of action must thus involve other immune cells or systems, possibly T cells.

In conclusion further study into monocytes and monokine regulation in MS is warranted to firmly cement a role for monocytes in this disease. As more consideration is given to the important role of monocytes in MS immunopathogenesis, more research should also be performed into IL-10 as a potential therapy for MS disease, due to its effective ability to downregulate proinflammatory and Th1 regulatory monokine production.

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