Mast cell interaction with myelin and oligodendrocytes a new process in the pathogenesis of multiple sclerosis
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MULTIPLE SCLEROSIS

Multiple sclerosis (abbreviated MS, formerly known as disseminated sclerosis or encephalomyelitis disseminata) is autoimmune, chronic, inflammatory, demyelinating disease that affects the central nervous system (CNS). MS was first described in 1868 by Jean-Martin Charcot. It is characterized by myelin loss, gliosis, and varying degree of axonal and oligodendrocytes pathology. More specifically, MS destroys oligodendrocytes which are the cells responsible for creating and maintaining a fatty layer, known as the myelin sheath, which helps the neurons carry electrical signals. MS results in a thinning or complete loss of myelin and, less frequently, the cutting (transection) of the neuron's extensions or axons. When the myelin is lost, the neurons can no longer effectively conduct their electrical signals.

Multiple sclerosis (MS) can range from relatively benign to somewhat disabling to devastating, as communication between the brain and other parts of the body is disrupted. Most people experience their first symptoms of MS between the ages of 20 and 40; the initial symptom of MS is often blurred or double vision, red-green color distortion, or even blindness in one eye. Most MS patients experience muscle weakness in their extremities and difficulty with coordination and balance. These symptoms may be severe enough to impair walking or even standing. Approximately half of all people with MS experience cognitive impairments such as difficulties with concentration, attention, memory, and poor judgment, but such symptoms are usually mild and are frequently overlooked. Depression is another common feature of MS. Generally, MS is not lethal, but imposes devastating neurological and psychiatric limits on patients. (Frohman E. M., et al., 2006).

There are two main views about disease onset in MS: central versus peripheral. Some neuropathologists believe that MS begins within the CNS and then propagates to the peripheral nervous system. Barnett and Prineas reported clinical and pathological findings in patients with relapsing-remitting multiple sclerosis (RRMS). They observed extensive oligodendrocyte apoptosis. Microglia were activated in the myelinated tissue which contained few or no lymphocytes or myelin-associated phagocytes (Barnett, M. H., and Prineas, J. W. 2004).

On the other hand, the majority of neuroscientists believe that MS is triggered only after an individual’s exposure to certain environmental factors like viral agents (Steinman, L., and Zamvil, S. S. 2006). This initial viral exposure/infection activates CD4+ T cells against CNS
tissue antigens. These imprinted cells eventually gain access to the CNS microenvironment, which in turn perpetuates ongoing cycles of neuroinflammation and neurodegeneration (Lovett-Racke, A.E., and Racke, M. K. 2006).

Strictly immunological explanation of MS is that the inflammatory process is triggered by the T cells. T cells gain entry into the brain via the blood-brain barrier (a capillary system that should prevent massive entrance of T-cells into the nervous system). The blood brain barrier is normally not freely permeable to these types of cells, unless triggered by either infection or a virus, where the integrity of the tight junctions forming the blood-brain barrier is decreased. When the blood brain barrier regains its integrity (usually after infection or virus has cleared) the T cells are trapped inside the brain. These lymphocytes recognize myelin as foreign and attack it as if it were an invading virus. That triggers inflammatory processes, stimulating other immune cells and soluble factors like cytokines and antibodies. Consequently leaks form in the blood-brain barrier, which in turn, cause a number of other damaging effects such as swelling, activation of macrophages, more production and release cytokine and other destructive proteins (such as matrix metalloproteinases).

It is known that a repair process, called remyelination, takes place in early phases of the disease, but the oligodendrocytes that originally formed a myelin sheath cannot completely rebuild a destroyed myelin sheath. The newly-formed myelin sheaths are thinner and often not as effective as the original ones. Repeated attacks lead to successively fewer effective remyelinations, until a scar-like plaque is built up around the damaged axons, according to four different damage patterns. (Lucchinetti, C., et al., 2000). The central nervous system should be able to recruit oligodendrocyte stem cells capable of turning into mature myelinating oligodendrocytes, but it is suspected that something inhibits stem cells in affected areas.

The axons themselves can also be damaged by the attacks. (Pascual, AM., et al 2007). Often, the brain is able to compensate for some of this damage, due to an ability called neuroplasticity. MS symptoms develop as the cumulative result of multiple lesions in the brain and spinal cord. This is why symptoms can vary greatly between different individuals, depending on where their lesions occur.

MS is an inflammatory disease, where the main pathogenic role is attributed to the immune system. MS is considered an autoimmune disease. However, unlike classic autoimmune disease, where the antigen is known and can be identified via blood tests, a strong antigen in MS has not been identified to date. Several weak myelin-related antigens are known,
but none of them has proven to be the driving force of the immune response. Fig 1 summerizes the main features of MS.

Figure 1. Multiple Sclerosis is a chronic inflammatory disease of the central nervous system (CNS), the brain and the spinal cord. It is a malfunction of the immune system which leads to attacks against, and causes destruction of the myelin sheath (a fatty covering that insulates
nerve cell fibres in the brain and spinal cord. The loss of myelin insulation degrades the nerve transmission ability. Thus a multitude of various neurological disabilities can be observed in patients affected by this disease depending on which nerves are damaged.

Incidence and Prevalence of multiple sclerosis

The geographic distribution of multiple sclerosis is best defined from prevalence studies, of which there are now over 300. These works indicate that the worldwide distribution may be divided into three zones of high, medium, and low frequency. High frequency areas, with prevalence rates of 30 and above per 100,000 population, now comprise almost all of Europe into former USSR, Cyprus, Israel, Canada and all the coterminous United States, as well as New Zealand and south-eastern Australia. They also seem to include the easternmost part of Russia. These high regions are bounded by areas of medium frequency with prevalence rates of 5-29 and now mostly 15-25 per 100,000, which then include most of Australia, the southern Mediterranean basin, probably Russia from the Urals into Siberia as well as the Ukraine, South Africa, and perhaps much of the Caribbean region and South America. All other known areas of Asia and Africa and possibly Venezuela and Colombia are low, with the prevalence rates under 5 per 100,000 population. A number of nationwide surveys in Europe give evidence for geographic clustering of the disease, which is stable over time, but with diffusion over time. This last is also found in the US. With important exceptions, there is a north-to-south gradient in the northern hemisphere and a south-to-north gradient in the southern hemisphere, with MS being much less common in people living near the equator (Epidemiology and multiple sclerosis Neuroepidemiology Section, Neurology Service, Veterans Affairs Medical Centre, Washington, DC; and Department of Neurology, Georgetown University School of Medicine, Washington DC)
Heterogeneity of Multiple sclerosis:

Multiple sclerosis is classified according to frequency and severity of neurological symptoms, the ability of the CNS to recover, and the accumulation of damage.

**Primary Progressive MS (PPMS)** causes steady progression of symptoms with few periods of remission.

**Relapsing-Remitting MS (RRMS)** causes worsening of symptoms (exacerbations) that occur with increasing frequency, along with periods of reduced symptoms (remission). It is the most common form of MS, affects females twice as often as males (Keegan, B. M., and Noseworthy, J. H. 2002).

**Secondary Progressive MS (SPMS)** is initially similar to relapsing-remitting MS and eventually progresses to MS with no remission.

**Relapsing-Progressive MS (RPMS)** causes accumulative damage during exacerbations and remissions.

Neuropathological studies have shown that each of these different forms of MS show different patterns of morphological and histological alternatons in CNS (Lucchinetti, C., et al., 2000).
MS AS AN INFLAMMATORY DISEASE OF THE CNS

On the basis of cellular infiltrates detected in brain and cerebrospinal fluid (CSF) from patients and data from rodent models such as EAE, MS is largely considered to be a CD4+ T helper 1 (Th1)-mediated inflammatory disease (LaSalle, J. M., et al., 1991), although CD4+ TH17 effector T cells are postulated to have more important roles (Bettelli, E., et al., 2006; Iwakura, Y., and Ishigame, H., 2006; Veldhoen, M., and Stockinger. B. 2006). In the EAE model, it has been shown that the injection of myelin components into susceptible animals leads to a CD4+ -mediated autoimmune disease that shares similarities with MS (Martin, et al., 1992). EAE can also be induced by the adoptive transfer of encephalitogenic CD4+ T cells into a naive animal (Pettinelli, C. B., and McFarlin, D. E. 1981; Zamvil, S. S., and Steinman, L. 1990) As will be discussed later, CD8+ T cells and other leucocytes play important pathogenic roles.

In MS and EAE CNS vessels, proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) induce the expression of endothelial cell surface adhesion molecules such as VCAM-1 and LFA-1. Together with chemokine attractants, of which potential candidates are CCL19 and CCL21 (Alt, C., et al., 2002), this facilitates leukocyte tethering and rolling along the endothelial cell surface. Via α4β1 integrin receptors (VLA-4) on their surface, T cells bind to VCAM-1 on the endothelial surface, allowing them to attach to and finally transmigrate through the endothelial cell layer (Bar-Or, A. 2005; Engelhardt, B. 2006). Once through the endothelial cell layer, the migrating leucocytes traverse the two underlying basement membrane barriers by binding specifically to laminin subtypes which constitute the basement membranes (Sixt, M. et al., 2001). By producing metalloproteases such as the matrix metalloproteinases (MMPs) to specifically cleave components of the basement membrane barriers (Agrawal, S. et al., 2006), they enter the CNS parenchyma. Immuno-histochemical analysis of both MS and EAE tissues have found that leucocytes accumulate in the Virchow-Robin space to form perivascular cuffs (Prineas, J. 1975; Van Horssen, J. et al., 2005) before they enter the CNS parenchyma to result in a massive local inflammation wherein they act to degrade myelin protein components and eventually destroy the underlying axons, resulting in the formation of lesions or plaques within the CNS.

On entry to the CNS compartment, activated Th1 cells must be restimulated, as otherwise they die or leave the CNS. Restimulation, perhaps via an autoantigen or a microbial antigen presented on microglia or resident dendritic cells, causes clonal expansion of the Th1 cells. Th1 cells release an array of proinflammatory cytokines, including interleukin-1 (IL-1), interferon-γ
(IFN-γ), and TNF-α leading to monocytes and microglia activation. Activated astrocytes, monocytes and macrophages can also release a variety of neurotoxic elements, including nitric oxide, oxygen free-radicals, and MMPs, all of which contribute to the destruction of the myelin sheath and axons (Dhib-Jalbut, S. 2002). MRI (Magnetic resonance imaging) scans utilizing gadolinium are used to identify and quantify such lesions which are indicative of blood-brain barrier breach and disease extent.

GENETIC AND ENVIRONMENTAL ASPECTS OF MS AND THEIR IMPACT ON IMMUNE FUNCTIONS

A) GENETIC INFLUENCES IN MS

Although the causes of MS are unknown, at present it is thought to be contributed by the influences of both genes and the environment. The disease is reported to predominantly affect northern Europeans, and there is a higher age-adjusted risk for siblings (3%; full siblings, the risk for half siblings is less), parents (2%), and children (2%) than for second- and third-degree relatives (Compston, A., and Coles, A. 2002). Concordance in monozygotic twins is around 35%, and is higher in children with both parents affected (20%) as opposed to offsprings of single-affected couples (2%).

MS doesn’t result from gene mutations or aberrations; rather, it seems to be contributed by the effect of normal polymorphisms in number of genes. These polymorphisms act independently or through epistasis, and each can exert a small contributory effect on some as yet undefined structure or physiological function (Compston, A., and Coles, A. 2002; Motsinger, A. A. et al., 2007; Willer, C. J. et al 2006). Extensive search and results from population studies have yielded few secure candidate regions for susceptibility genes. An association between a few class II MHC alleles and the gene for TNF-α are encoded within the same linkage group. Although no major susceptibility gene has yet been identified, several promising chromosome linkages are for the time being linked with MS at 1p, 6p, 10p, 17q, and 19q.13 (Brassat, D. et al., 2006).
B) ENVIRONMENTAL EFFECT IN MS

An environmental influence is suggested by migration studies. In this regard, regions to which northern European descendants migrated have high prevalence rates of MS. If one migrated from an area of high prevalence of MS to an area of low prevalence before age 15-16, the lower risk of the new area was acquired; migration after 15-16 did not change the risk (Kurtzke, J. F., 1983). Various factors have been taken into consideration to explain this MS prevalence pattern. Sunlight exposure depending on the latitude is thought to be one causative factor. UV radiation may exert its effects either by influencing immunoregulatory cells or by the biosynthesis of vitamin D (Hayes, C. E., 2000), which has many immunoregulatory properties (Van Etten, E., and Mathieu, C. 2005). As well, melatonin secretion depends on sunlight exposure, and the lack of sunlight could induce an excess of melatonin which enhances Th1 response (Kidd, P., 2003; Ponsonby, A. L., 2005).

C) INFECTIOUS AGENTS AS TRIGGERS OF MS

Two main mechanisms have been proposed to explain how infections could induce MS. The first is molecular mimicry, wherein autoreactive T cells are activated by cross-reactivity between self-antigens and foreign agents. For example, during thymic selection, T cells may recognize self-antigens along with foreign antigens resulting in a positive selection of this population of T cells and their export to the periphery. Cross-reactivity of these “potentially” self-reactive T-cells with foreign antigens can lead to their activation during infection and migration across the blood-brain barrier; if these cells recognize antigens expressed in the brain (such MBP), tissue damage and potentially an autoimmune disease like MS may result (Wucherpfennig, K. W. et al., 1997).

A second mechanism to explain how infections could induce MS is that of bystander activation, which assumes that autoreactive cells become activated due to nonspecific inflammatory events that occur during viral or bacterial infections. This mechanism is classified into two categories, the first category encompasses T cell receptor independent bystander cell activation of T cells via inflammatory cytokines, superantigens, and activation of Toll-like receptors, and the second encompasses the role of APCs which are activated directly by host antigens and the adjuvant effect of infectious agents (Murali-Krishna, K. et al., 1998; Tough, D.
F., and Sprent, J. 1996; Waldner, H. et al., 2004). Pender (2003) suggested a role for autoreactive B cells in MS, where autoreactive B cells infected by a virus (such EBV) begin to proliferate and transform into B cells resistant to apoptosis, thereby triggering costimulation of autoreactive T cells or preventing these cells from undergoing activation-induced cell death. The result is the persistence and accumulation of autoreactive T cells in the inflamed area.

CNS-SPECIFIC CELLULAR CONTRIBUTION IN MS

**Cerebral Endothelial Cells** create the anatomic and physiological barrier of the BBB and play an essential role in forming the demyelinating lesions of MS. Under normal circumstances, CNS endothelial cells prevent transendothelial escape of various blood-brain molecules and limit the migration of leucocytes into the CNS. Endothelial barrier and the supporting astrocytes keep the CNS out of reach of the peripheral immune system, homeostatically maintaining a stable environment for neurons to function.

Passage of the activated leukocytes through the junctions between adjacent endothelial cells involves interaction among passing cells and junctional components. Firm adhesion of the activated leukocyte precedes transendoctalial migration. Resting cerebral endothelium differs significantly from activated cells and has low selectin levels, but constitutively expresses ICAM-1 and ICAM-2. Studies on animals with EAE demonstrated that the expression of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) are dramatically upregulated by inflamed cerebral endothelium (Baron, J. L. et al., 1993; Cross, A. H. et al., 1990; Steffen, B. J. et al., 1994). The activated leucocytes within the perivascular cuffs of MS lesions express ligands for ICAM-1 and VCAM-1 (e.g. LFA-1 and \( \alpha 4\beta 1 \) integrins), suggesting these are involved in the efflux of these cells (Engelhardt, B., et al., 1998). Upregulated VCAM-1 and ICAM-1 expression play a significant role in the pathogenesis of EAE and MS since studies have demonstrated adhesion of encephalitogenic CD4+ T cells and monocytes to mouse and human cerebral endothelial cells via \( \alpha 4\beta 1 \) integrin-VCAM-1 and LFA (leukocyte function antigen)-1-ICAM-1-mediated interactions (Floris, S. et al 2002; Greenwood, J. et al., 1995; Laschinger, M., and Engelhardt, B., 2000; Seguin, R. et al 2003).

During EAE pathogenesis, two members of \( \beta 2 \) integrin family LFA-1 and Mac-1 and their ligands ICAM-1 and ICAM-2 have also been implicated in transendothelial migration of T cells (Laschinger, M. et al., 2002). The role of \( \beta 2 \) integrins in the extravasation of encephalitogenic T cells into CNS is also supported by the fact that ICAM-1 and ICAM-2
endothelial knockouts exhibit significantly decreased T cell migration but it is not clear which of the different structural motifs in ICAM-1 are involved in the adhesion and migration of autoreactive CD4+ T cells in MS (Greenwood, J., et al., 2003).

**IMMUNE COMPONENTS OF MS**

The role of CD4 T cells in the pathogenesis of MS:

Much of the current understanding of the potential mechanistics and role of CD4+T cells in MS comes from the animal models simulating features of MS. Experimental autoimmune encephalomyelitis (EAE) is an inflammatory central nervous system (CNS) demyelinating disease, and may be induced in several animal types via immunization with myelin proteins or peptides. Attempts to isolate T-cell clones from the brains of MS patients failed to show either MBP or PLP reactivity (Hafler, D. A et al., 1987). EAE reproduces many of the clinical and immunologic aspects of MS, and has widely used to study the mechanisms of CD4+ T cell priming and response to myelin components (Bettelli, E. et al., 1998: Chitnis, T. et al., 2001) as well as to test potential therapies for MS (Aharoni, R., et al., 1999; Yednock, T. A. et al., 1992).

T cells in MS lesions:

MS lesions are characterized by perivascular infiltrates of CD4+ and CD8+ T cells and macrophages (Prines, J. W., and Wright, R. G. 1978; Traugott, U. et al., 1983). T lymphocytes play a central role in the pathogenesis of MS (Zhang, J. et al., 1992). Both CD4+ and CD8+ T cells have been demonstrated in MS lesions, with CD4+ T cells predominating in acute lesions and CD8+ T cells being observed more frequently in chronic lesions (Raine, C. S. 1994). Activated myelin –reactive CD+T cells are present in the blood and cerebrospinal fluid (CSF) of MS patients; in contrast, only nonactivated myelin reactive T cells are present in the blood of controls (Zhang, J. et al., 1994).

Interaction of CD40 on APC with CD154 on T cells induces APC (antigen presenting cell) production of IL-12, a major factor in Th1 cell differentiation (Kelsall, B. L., et al., 1996). Expression of both CD40 and CD154 were increased in lesions from postmortem MS brains compared with controls, with CD40 found predominantly on macrophages and microglia, while CD154 colocalized with the CD4 T cell marker(Gerritse, K., et al., 1996). Expression of CD154
was found to be higher in peripheral blood monocytes isolated from SPMS compared with RRMS or healthy controls (Filion, L. G., et al., 2003; Jensen, J., et al., 2001), and was reduced by INF-β treatment (Teleshova, N., et al., 2000).

Cytokines:

In the context of MS, Th1 cytokines (IL-2, TNF-α, INF-γ) are thought to mediate disease, while Th2 (IL-4, IL-5, IL-10, and IL-13) cytokines are believed to play a protective role. But this paradigm is not absolute.

Expression of TNF-α was localized to macrophages, microglia, and astrocytes (Cannella, B., and Raine, C. S. 1995; Hofman, F. M., et al 1989; Selmaj, K., et al 1991) in chronic-active lesions. A separate study found that IL-2 was expressed predominantly in association with perivascular inflammatory cells examining acute MS lesions (Hofman F. M., et al 1986). Studies using RNA microarrays in MS brains at autopsy found increased transcripts of genes encoding for IL-6, IL-17, and IFN-γ (Lock, C., et al 2002), indicating a potential role for both Th1 and Th17 cells in proinflammatory responses in MS. TCR-mediated IFN-γ and IL-10 secretion are increased in relapsing-remitting (RR) and secondary progressive (SP) patients, but not in primary progressive disease, suggesting a disregulation of this signaling pathway in certain MS subtypes (Balashov, K. E. et al., 2000). Administration of IFN-γ to MS patients precipitated clinical attacks, confirming the role of INF-γ as a proinflammatory cytokine in MS (Panitch, H. S. et al., 1987).

Studies of the prototypic Th2 cytokine IL-4 in MS are limited, IL-4 was expressed in high levels in both acute- and chronic-active MS lesions (Cannella, B., and Raine, C. S. 1995). High frequencies of T-cell clones reactive to MBP- and PLP-expressing IL-4 were found in MS patients compared with untreated patients (Chou, Y. K. et al., 2002).

T-cell migration in MS:

T-cell migration into the CNS in MS is believed to follow the sequence of capture, rolling, activation, adhesion strengthening, and finally transmigration through the BBB. Two processes appear to be important in T-cell migration into the CNS:

1) migration of T cells from the blood into the CSF with interactions with dendritic elements on the luminal surface of the choroid plexus resulting in immunosurveillance
2) T-cell migration through inflamed endothelial BBB and interactions with perivascular APCs within the Virchow-Robbins space (Engelhardt, B., and Ransohoff, R. M. 2005).

Evidence exists for both of these processes in MS, however the relative contribution of each is unclear, and may depend on the stage and subtype of disease.

Adhesion molecule ICAM-1 is expressed on the inflamed BBB in MS, while LFA-1 is expressed on infiltrating T cells, suggesting an important role for this pathway in T-cell migration in MS (Bo, L. et al., 1996). Inhibition of interactions between integrin molecule α4β1 present on the surface of T cells with VCAM-1 present on endothelial cells of the BBB was shown to suppress the development of EAE (Yednock, T. A. et al., 1992).

CXCR3 is postulated to be the major chemokine involved in the trafficking of T cells from the blood to the CSF for immunosurveillance in both normal and MS patients (Kivisakk, P. et al., 2002).

T-cell interaction with axons and neurons:

Much attention has focused on the presence of axonal damage in MS plaques as a substrate for chronic progressive disease (Trapp, B. D., 1998, 1999). Although mediators of axonal damage include cytokines, complement, antibody, and nitric oxide, T cells may play a significant role both in neurodegeneration and in neuroprotection. The presence of CD8+ T cells, but not CD4+ T cells, in the MS lesions correlates with axonal damage (Bitsch, A., et al., 2000). Class I expression in the CNS, particularly on neurons, may enhance a cytotoxic CD8+ T-cell response. T cell-mediated neurotoxicity was dependent on IFN-γ-induced expression of MHC class I (Medana, I., et al., 2001).

*In vitro* coculture of human fetal neurons with OKT3-activated CD4+ or CD8+ T cells has been found to produce apoptosis of neurons (Giuliani, K., et al., 2003). This process required physical contact of the cells, as demonstrated by transwell experiments, and was not dependent on MHC I. Protection could be conferred by blocking CD40 on both T cells and neurons, and FasL on neurons. Neuronal production of TGF-β has been shown to play a significant role in the induction of CD4+CD25+Foxp3+ regulatory T cells in murine EAE model (Liu, Y. et al., 2006). Myelin reactive T cells may be neuroprotective under certain conditions including trauma. Local production of neurotrophic factors by inflammatory cells may be neuroprotective.
REGULATORY MECHANISMS IN MS

The activation of myelin reactive T cells is also seen in other conditions such as spinal cord injury (Jones, T. B., 2005). Why do these conditions not give rise to MS? Here, it is important to note that while we have discussed mechanisms by which T cells become activated, there are also other mechanisms that prevent the excessive activation of T cells. These regulatory mechanisms include CD4+CD25+Foxp3+ regulatory T (Treg) cells which are TGF-β dependent (Fehervari, Z., and Sakaguchi, S., 2004). Bettelli (2006) showed that a balance exists in the generation of pathogenic T cells (Th17) which induce autoimmunity and regulatory (Foxp3) T cells that inhibit autoimmune tissue injury, suggesting an important role for Tregs in the determination of the fate of the autoimmune disease.

Tregs are T cells with an immunosuppressive function. Several different types of Tregs, including naturally arising CD4+CD25+Foxp3+ Tregs, IL-10-secreting Tr1 cells, TGF-β-secreting Th3 cells. Qa-1-restricted CD8+ T cells, CD8+CD28- T cells, CD8+CD122+ T cells, γδ T cells, and NKT cells have been reported to date (Baecher-Allan, C., and Hafer, D. A. 2006 ; Lu, L., et al., 2006; Roncarolo, M. G., et al., 2006; Sakaguchi, S. 2006; Shevach, E. M., et al., 2006). Some Tregs populations are naturally generated as functionally distinct populations, while others are adaptively induced from naive T cells as a consequence of antigen exposure (Sakaguchi, S. 2006).

Tregs can actively suppress an immune response and are thereby thought to be capable of inhibiting ongoing pathogenic autoimmunity, allowing self-tolerance to be restored in patients suffering from autoimmune disease (Tang, Q., and Bluestone, J. A. 2006). Tregs can act through dendritic cells to limit autoreactive T-cell activation, thereby preventing T-cell differentiation and activation (Baecher-Allan, C., and Hafer, D. A. 2006).

However, this protective mechanism appears insufficient in autoimmune patients, most likely due to a shortage of circulating Tregs and/or the development and accumulation of Treg-resistant pathogenic T cells over the disease course (Baecher-Allan, C., and Hafer, D. A. 2006 ; Tang, Q., and Bluestone, J. A. 2006).

Viglietta (2004) was the first to show an altered function of CD4+ CD25hi Tregs in patients with MS; they reported a significant decrease in the effector function of CD4+ CD25hi T cells from peripheral blood of patients with MS as compared with healthy donors. They also demonstrated using in vitro cloning experiments that the cloning frequency of CD4+ CD25hi Tregs was significantly reduced in MS patients as compared with normal controls, suggesting
important function and possible future therapeutic roles for Tregs in MS (Hafler, D. A., et al., 2005).

MS THERAPIES AND THEIR EFFECT ON IMMUNE ABNORMALITIES IN MS

a) Natalizumab

Monoclonal antibodies against α4 integrin abrogate the development of EAE in mice. This integrin is important in the trafficking of T cells into the CNS by interacting with its ligand on endothelial cells, vascular cell adhesion molecule-1 (Tubridy, N., et al., 1999; Yednock, T. A., 1992). Natalizumab, one such monoclonal antibody, has been administrated intravenously in the treatment of MS (blockage of integrin α4β1). Administration in phase II and phase III trials in MS have produced significant benefits with results showing both a marked reduction in the formation of new lesions and a significant reduction in the risk of exacerbations within 6 months after the initiation of therapy (Miller, D. K., et al., 2003; Polman, C. H., et al., 2006). In the CSF of MS patients treated with Natalizumab, there is a prolonged reduction in the number of T lymphocytes (Stuve, O., et al., 2006), further supporting the mechanism of Natalizumab in reducing T-cell trafficking in humans.

Unfortunately, the success of Natalizumab had been curtailed when three MS patients administered the drug developed progressive multifocal leukoencephalopathy (PML) (Kleinschmidt-DeMasters, B. K., and Tyler, K. L. 2005; Langer-Gould, A., et al., 2005); two of these died from the PML. Despite the PML setbacks, it is widely believed that targeting leukocyte trafficking using Natalizumab may still be a promising mode of treatment in MS therapy (Li, Y. Y., et al., 2006).

b) Interferon β
IFN-β acts partly by reducing the levels and activity of proinflammatory cytokines, and inducing the expression of anti-inflammatory cytokines (Yong, V. W., et al., 2002). In addition, *in vitro* studies show that IFN-β effectively decreased T-cell migration through reduction MMP-9 levels (Stuve, O. et al., 1996); as T-cell infiltration damages the blood-brain barrier, the decrease of T-cell migration by IFN-β can help account for the clinical finding that IFN-β rapidly reduces gadolinium-enhancing MRI activity and inflammation (Yong, V. W., et al., 2002).

Other *in vitro* studies have shown that IFN-β decreased blood-brain barrier cerebral endothelial cell permeability. *In vivo*, the serum concentrations of the soluble forms of ICAM-1 and VCAM-1 are elevated after 3 months of IFN-β treatment in MS patients compared to the untreated situation (Kraus, J., et al., 2004a); the soluble forms of these adhesion molecules are thought to bind integrins on leukocytes before the latter have access to their ligands on endothelial cells.

Clinical studies have supported the possibility that IFN-β regulates MMP levels in patients with MS. Boz, C., *et al.* (2006) obtained blood and CSF samples from 14 patients with relapsing-remitting MS before and 6 months after IFN-β therapy. They report that after IFN-β treatment, serum MMP-9 as well as MMP-9:TIMP-1 ratio were significantly decreased from pretreatment levels.

IFN-β treatment was associated with a significant suppression of MMP-9 and MMP-7 transcript levels in peripheral blood leukocytes from MS subjects. (Galboiz, Y., *et al.*, 2002). Possible mechanisms of IFN-β in MS include the decrease of MMP levels or activity, and/or the increase in levels of physiological inhibitors of MMPs (TIMP-1) such that the transmigratory capacity of leukocytes into the CNS is reduced.

c) Glatiramer acetate

Glatiramer acetate is a random mixture of four amino acids (Ala, Lys, Glu, and Tyr) which suppresses EAE (Arnon, R., *et al.*, 1996) and has long-term efficacy and tolerability in treatment of relapsing-remitting MS (Ford, C. C., *et al.*, 2006). Different immunomechanisms (Farina, C., *et al.*, 2005; Yong, V. W., *et al.*, 2002) have been suggested to contribute to the beneficial effects of GA, including its ability to affect the antigen-binding groove of MHC class II molecules, thereby potentially competing with other epitopes for antigen binding. Other activity of GA include the generation of Th2 cytokine reactivity with antiinflammatory effects.
(Aharoni, R., et al., 2000; Neuhaus, O., et al., 2000), the increase in activity of CD4+CD25 regulatory cells, the modulation of macrophage activity (Weber, M. R., et al., 2004), and increase in CD8+ cells with suppressor activity (Karandikar, N. J., et al., 2002). Finally, the GA-reactive Th2 cells may also enter into the CNS to confer neuroprotection (Yong, V. W., et al., 2002).

d) Minocycline

The semisynthetic tetracycline derivate, minocycline, has multiple actions including that of the inhibition of MMP activity (Yong, V. W., et al., 2004). In mice afflicted with EAE, minocycline has been described to reduce the expression and activity of MMP-9 in T cells and the administration of minocycline to EAE-afflicted animals has been shown to alleviate disease severity and neuropathology (Brundula. V., et al., 2002). Results from animal studies have encouraged a clinical trial with the use of minocycline in 10 patients with relapsing-remitting MS. With gadolinium (gad)-enhancing MRI activity as a marker of clinical activity, patients beginning the trial showed reduced activity within the first 2 months of treatment with minocycline (Metz, L. M., et al., 2004). Serum samples from the patients in the minocycline treatment is reduced compared to pretreatment values and is maintained for up to 18 months of therapy, the latest time point examined in this immune study (Zabad, R. K., et al., 2007).

Besides the effect of minocycline at the level of the blood-brain barrier, other mechanisms that may contribute to its efficacy include the inhibition of microglia and T-cell activity, the reduction of neural cell apoptosis, the inhibition of glutamate cytotoxicity, and others (Yong, V. W., et al., 2004).

The CD8 T cell in Multiple Sclerosis: suppressor cell or mediator of Neuropathology?

For decades, CD4 T cells have been considered the predominant mediator of neuropathology in MS. This perception was largely due to the similarity between MS and CD4 T-cell-driven experimental allergic encephalomyelitis (EAE), the most commonly studied murine model of MS. In certain EAE models, CD8 T cells are considered suppressor of pathology (Jiang, H., et al., 1992). In 1992, it was determined that CD8 T cells protect against EAE relapses (Jiang, et al, 1992). This initial observation was later confirmed through adoptive
transfer of CD8 T cells isolated from EAE recovered mice into MBP-immunized recipients. These early experiments demonstrated the capacity of CD8 T cells to inhibit the MBP-specific CD4 T cell response. In other EAE models, neuropathology can be exacerbated by adoptive transfer of CD8 T cells. EAE can be induced by adoptively transferring CD8 T cells from an animal vaccinated with CNS protein into a naive recipient animal. CD8 T-cell epitopes in this model include MBP, MOG, and an assortment of other proteins endogenously expressed by oligodendrocytes (Crawford, M. P., et al., 2004; Tsukida, T., et al., 1994).

There is also emerging role for CD8 T cells in human MS. The role of CD8 T-cell suppressors in MS, is more difficult to define. It appears that CD8 T cells could have two potential mechanisms by which they promote suppression of inflammation in MS:

a) Direct mechanism- suppression through cell-mediated cytotoxicity (CD8 T cells mediate suppression through direct recognition of class I molecules on CD4 T cells, this results in death of the CD4 T cells)

b) Indirect mechanism- suppression by affecting antigen presenting cell (CD8 T cells mediate suppression through interaction with APCs, triggering a licensing effect that mediates CD4 T cell unresponsiveness).

Neuropathologic studies demonstrate that CD8 T cells are the most numerous inflammatory infiltrate in MS lesions at all stages of lesions development (Babble, H., et al., 2000; Boos, J., et al., 1993). All CNS cells, including oligodendrocytes, microglia, astrocytes, and neurons, express class I MHC molecules under inflammatory conditions (Hoftberger, R., et al., 2004; Neumann, H., et al., 1995). These cell types are therefore potential targets for CD8 T-cell-mediated cytotoxicity through effector molecules.

Determining the protective and pathogenic attributes of the CD8 T cells that infiltrate MS lesions is further complicated by the heterogeneity of the neuropathology of MS patients.

CHEMOKINES IN MS

Normal physiological trafficking and positioning of immune cells is controlled by lymphoid chemokines during hematopoiesis and immunosurveillance. However, during inflammation, lymphoid cells express inflammatory chemokines to recruit inflammatory cells into these sites (Moser, B., and Loetscher, P. 2001). Together with their receptors, chemokines play important roles in MS and EAE at various levels. On EAE induction, CCL2 and CXCL10
levels are upregulated in the liver and various secondary lymphatic organs (Glabinski, A. R., et al., 1995) prior to appearance of clinical symptoms, suggesting a chemokine-dependent systemic immune activation. A study has suggested that chemokine signals (CXCL12) from glial cells from within the CNS are responsible for the migration of activated T cells from secondary lymphatic organs to the CNS (Flugel, A., et al., 2001); however, how these chemokines exit the blood-brain barrier is still unknown.

In MS, chemokine levels are altered in the blood and CXCR3 and CCR5 expression are reported to be increased in circulating T cells (Calabresi, P. A., et al., 1999; Strunk, T., et al., 2000); these T cells are shown to secrete large amounts of proinflammatory cytokines. In the CSF, levels of CCL5 (RANTES) and CXCL10 (IP-10) are elevated, and CCL2 (MCP-1) levels are significantly reduced. These changes in chemokine levels correlate with the presence of CNS inflammation and gadolinium-enhanced lesions (detected by MRI), although the source of these chemokines in the CSF is yet to be identified. Similar to blood, CCR5 and CXCR3 are also detected in the CSF, while CXCL10 mediates their retention in the inflamed CNS. At the level of postcapillary venules within the CNS, chemokines have been implicated in leukocyte extravasation process. A gradient of CCL19 and CCL21 across the blood vessel endothelium not only acts as homing signals for CCR7-expressing T cells but also strengthens the adhesion of extravasating T lymphocytes to endothelial cells (Alt, C., et al., 2002; Opdenakker, G., et al., 2003) by a proposed increase in the avidity of \( \alpha 4 \beta 1 \)-VCAM-1-mediated interactions (Alt, C., et al., 2002). Once in the CNS parenchyma, T cells interact with astrocytes to produce CCL2, which attracts peripheral/perivascular macrophages (expressing CCR2) to enter the CNS parenchyma. Subsequently, macrophage chemokine (CCL3, CCR5, CXCR4) augment leukocyte recruitment into CNS (Babcock, A., and Owens, T. 2003; Opdenakker, G., et al., 2003)

**ROLE FOR METALLOPROTEINASES IN MS**

A role for various MMPs has been investigated in MS brain, CSF, and serum and also in rodent EAE, particularly in the degenerative processes of the disease. Studies in rat adoptive transfer model of EAE have shown strong upregulation of mRNA levels for MMP-9 and MMP-7, peaking at maximal disease severity, but unaltered levels for MMP-2, MMP-3, MMP-11 and MMP-13 (Clemens, J. M., et al., 1997; Kieseier, B. C., et al., 1998).
Studies on MMP substrates have resulted in the identification of molecules relevant to inflammatory disease: for example, MMP-9 cleaves MBP (Gijbels, K., et al., 1993; Proost, P., et al., 1993) and the CNS proteoglycan, neural glia antigen 2 (NG2) (Larsen, P. H., et al., 2003); several chemokines are cleaved by MMP-9 (McQuibban, G. A., et al., 2002; Overall, C. M., et al., 2002) and TNF-α is cleaved by ADAM17. MMPs have also been shown to regulate inflammatory response by influencing chemotactic gradients and inducing cytokine production (D’haese, A., et al., 2000; Opdenakker, G., et al., 2001; Van den Steen, P. E., et al., 2000).

Synthetic inhibitors of metalloproteases have been shown to attenuate EAE disease course: minocycline, probably because of its Zn$^{2+}$-chelating properties, reduces production of MMP-9 and is efficacious against both mild and severe EAE in mice (Brundula, V., et al., 2002).

Young MMP-9 null mice are less susceptible to EAE induction than wild-type mice (Dubois, B., et al., 1999); however, this difference is no longer apparent in older mice. MMP-2 null mice have been reported to be more susceptible to EAE induction than wild-type controls, due to a compensatory upregulation of MMP-9 activity (Esparza, J., et al., 2004).

MMPs, implicated in the degradation of BM components as well as myelin components in MS, are detectable in the spinal fluid of MS patients, and MMP-9 immunoreactivity is present in endothelial cells, pericytes, macrophages, and astrocytes of MS lesions (Bar-Or, A., et al., 2003). Myelin specific T-cell clones derived from MS patients also produce MMP-9 on activation with antigen, and the presence of MMP-9 in the perivascular infiltrate demonstrated by immunohistochemistry is thought to be associated with disruption of the underlying basement membranes and is critical in the opening of blood-brain barrier.

CONTRIBUTION OF COMPONENTS OF INNATE IMMUNITY IN MS

Various components of the innate immune system have been shown to play roles in MS and EAE. The first example is the family of Toll-like receptors (TLR) on cells such microglia/macrophages and dendritic cells, which in normal situations function as sentinels by recognizing preprogrammed pathogen-associated molecular patterns to generate proinflammatory signals which then initiate an adoptive immune response. In MS, stimulation of TLR may break tolerance and render lymph node-specific APCs reactive against self-antigen to which they were previously unresponsive, thereby creating self-reactive effector T cells (Prinz, M., et al., 2006).
Mast cells are another example of innate immune response playing a role in MS. Normally, mast cells are activated during allergic reactions when surface IgE receptors cross-link and respond by releasing several molecules (including histamine and tryptase). Mast cells have been detected in low numbers in normal CNS tissue; however, their function there has not yet been elucidated. In MS, mast cells are thought to be attracted to areas of lesions via chemokines such as RANTES and by self-antigens (possibly remnant epitopes of destroyed myelin) where they release histamine and tryptase which activate metalloprotease pathways, potentially contributing to the opening of the BBB and the facilitation of leukocytes influx into CNS (Sospedra, M., and Martin, R., 2005; Ziemssen, T., and Ziemssen, F. 2005). Both neutrophils and macrophages are capable of generating enzymes such as inducible nitric oxide synthase (iNOS) which catalyzes the production of nitric oxide (NO), a short lived, highly bioactive-free radical, produced in high amounts in normal bacterial infections. High iNOS levels have been found to be associated with MS lesions (Bo, L., et al., 1994) and NO is thought to augment microglial cytotoxic action and bring about the destruction of myelin and oligodendrocytes in MS.

Other components, normally involved in innate immunity, have also been implicated in MS pathogenesis. For example, complement binding to myelin has been reported as a source of demyelination, and astrocytes may be a major CNS source for complements (Vanguri, P., and Shin, M. L., 1986) Also complement activation has been shown to result in oligodendrocyte lysis and macrophage chemoattraction in MS (Johns, Y. G., and Bernard, C. C. 1997) (Piddlesden, S. J., and Morgan, B. P. 1993). Both NKT and γ/δ T cells have also been reported to participate in MS pathology (Gausling, R., et al., 2001; Triebel, F., and Hercend, T. 1989).
MAST CELLS

All mast cells are derived from progenitors present in the bone marrow. Normally, mature mast cells are not found in circulation. Progenitors migrate to the peripheral tissues as immature cells and undergo differentiation in situ. Mature mast cells are found throughout the body, predominantly located near blood vessels and nerves and beneath epithelia. They are also present in lymphoid organs. Human mast cells can be round, oval, or even spindle-shaped. The nuclei are typically round. The cytoplasm contains membrane-bound granules and lipid bodies. The granules contain acidic proteoglycans, which bind basic dyes.

An important advance in the understanding of mast cell biology is the appreciation that in rodents, mature mast cells may assume one of two phenotypes. Mast cells found in the mucosa of the gastrointestinal tract have chondroitin sulphate as their major granule proteoglycan. Such “mucosal” mast cells contain little histamine. The second phenotype has been found in the lung and in serosa of body cavities. These “connective tissue” mast cells contain heparin as their major granule proteoglycan and produce large quantities of histamine. Mast cells can also be cultured from rodent bone marrow in the presence of IL-3. Such cultured mast cells resemble mucosal mast cells based on granule content of chondroitin sulphate and low histamine. Moreover, the presence of mucosal mast cells in vivo appears to depend upon T cells, the presumed source of IL-3, since they are absent in athymic mice. Bone marrow-derived mucosal mast cells can be changed to a connective tissue mast cell phenotype by co-culture with fibroblasts, or incubation with the ligand for c-kit, sometimes called stem cell factor (Galli, S. J. et al. 1994). Repopulation experiments in mast cell-deficient mice further suggest that the mucosal and connective tissue phenotypes are not fixed and that bidirectional changes may be possible in suitable microenvironment. The precise nature of the mast cell and the mediators it can produce vary with its anatomic location and is probably regulated by local cytokines.
In humans, the factors that regulate mast cell growth and development are less well defined. There appears to be a similar pattern of T cell-independent connective tissue mast cells (that depend on c-kit ligand) and T cell-dependent mucosal mast cells. Major differences between types of human mast cells reside in the composition of serine proteases found in the granule (tryptase or chymase in substrate specificity) and in the ultrastructural morphology of the granules. Nevertheless, it does appear that in humans as well as in mice the pattern of mediators produced by mast cells vary with anatomic location. (Galli, S. J. 1993).

Activation of mast cells

The event that initiates immediate hypersensitivity is the binding of antigen to IgE on the mast cell. Mast cells are activated by cross-linking of FcεRI molecules, which is thought to occur by binding of multivalent antigens to the attached IgE molecules. Experimentally, antigen binding can be mimicked by polyvalent anti-IgE or by anti-FcεRI antibodies. Such antibodies can activate mast cells from atopic as well as non atopic individuals, whereas allergens activate mast cells only in atopic persons. The reason for this is that in an individual allergic to a particular antigen, a significant proportion of the IgE bound to mast cells is specific for that antigen. Administration of the antigen will cross-link sufficient IgE molecules to trigger mast cell activation. In contrast, in non atopic individuals, the mast cell-associated IgE is specific for many different antigens (all of which may have induced low levels of IgE production). Therefore, no single antigen will cross-link enough of the IgE molecules to cause significant mast cell activation. Anti-IgE antibodies, on the other hand, can cross-link these IgE molecules regardless of antigen specificity and lead to comparable triggering of mast cells from both atopic and non-atopic individuals (Romagnani, S. 1990).

Activation of mast cells results in three types of biologic responses:

a) Mast cells undergo regulated secretion in which the pre-formed contents of their granules are released by exocytosis

b) Mast cells enzymatically synthesize lipid mediators derived from precursors stored in cell membranes and, in some cases, in the lipid bodies.

c) Mast cells initiate transcription, translation, and secretion of cytokines.

The mechanism of granule exocytosis are partly understood, largely from studies of rodent mast cell and basophil leukaemia cell lines. The cross-linking of FcεRI results in
recruitment and activation of Syk and Fyn protein tyrosine kinases in a process involving the ITAMs of the FcR \( \gamma \) chain. These enzymes, in turn, phosphorylate and activate other proteins, including \( \gamma \) isoforms of a phosphatidylinositol-specific phospholipase C (PI-PLC\( \gamma \)) that catalyze phosphatidylinositol bisphosphate breakdown to inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG). IP3 causes elevation of cytoplasmic calcium, which, in combination with DAG, activates protein kinase C. The activated protein kinase C phosphorylates myosin light chains, leading to disassembly of actin-myosin complexes beneath the plasma membrane, thereby allowing granules to come in contact with the plasma membrane. Fusion of the granule membrane with plasma membrane is mediated by interaction between proteins associated with the plasma membrane; these events are regulated by the GTP-bound (activated) form of the Ras-related proteins Rab 3b or d, which are present in mast cells.

Cross-linking of Fc\( \varepsilon \)RI also activates the enzyme adenylyl cyclase through a heterotrimeric GTP-binding protein. This, in turn, elevates cyclic adenosine monophosphate levels (cAMP), and cAMP activates protein kinase A. Protein kinase A inhibits degranulation, suggesting that this pathway is a negative feedback loop.

The synthesis of lipid mediators is controlled by activation of the enzyme cytosolic phospholipase A2 (cPLA2). This enzyme is activated by two signals: elevated cytoplasmic calcium, released by IP3, and phosphorylation catalyzed by a mitogen-activated protein (MAP) kinase. MAP kinases are activated as a consequence of a kinase cascade initiated through the receptor ITAMs, probably utilizing the same pathways as in T cells.

Cytokine transcription in mast cells is also presumed to be similar to the events that occur in T cells. Syk appears to activate various adaptor molecules, leading to nuclear translocation of NFAT and NFkB, as well as activation of AP-1 by stress-activated protein kinases, such as cJun N-terminal kinase. Overall, this response leads to transcription of several cytokines (IL-3, IL-4, IL-5, IL-6, and tumor necrosis factor, among others), but in contrast to T cells, not IL-2.

Mast cells can be activated by mechanisms other than cross-linking of Fc\( \varepsilon \)RI. For example, certain types of mast cells may respond to mononuclear phagocyte-derived chemokines produced as part of innate immunity, to T cell-derived chemokines produced as part of specific cell-mediated immunity, and to complement-derived anaphylatoxins such as C5a produced during humoral immune responses. The cell-derived chemokines probably constitute the so-called “histamine releasimg factors” observed in delayed type hypersensitivity reactions. Mast cells can be activated by neutrophil granule contents or by neurotransmitters
such as norepinephrine and substance P. These latter agents are potentially important as links between the nervous system and the immune system. The nervous system is known to affect the expression of immediate hypersensitivity reactions. The “flare” produced at the edge of the wheal in elicited immediate hypersensitivity reactions is in part mediated by the nervous system, as shown by the observation that the flare is markedly diminished in skin sites lacking innervation.

Figure 3. Mast cells are activated primarily through the IgE receptor (FceRI) by allergens to cause rapid release of granules (stained blue) that contain histamine, potent proteases, and proteoglycans such as heparin. Other responses include rapid production of arachidonic acid-derived prostaglandins and leukotrienes. At later stages, numerous inflammatory cytokines and chemokines are produced as a result of gene transcription. These responses are markedly augmented by pathogenic ligands of Toll-like receptors (TLR) and the growth factor, Kit ligand also known as stem cell factor.

MAST CELL DERIVED MEDIATORS

Mast cells are heterogeneous and not all mast cells will release the same mediators or the same combinations of mediators. Nevertheless, the same general classes of mediators appear to be made by most mast cells. These may be divided into pre-formed mediators, which include biogenic amines, cytokines and granule macromolecules, and newly synthesized mediators,
which include lipid-derived mediators and again cytokines. (Swartz, L. B., and Austen, K. F. 1984).

These mediators are active in either (or, in some cases, both)

a) **recruiting** all the types of white blood cell to the site

- monocytes that become macrophages when they leave the blood and enter the tissue
- neutrophils
- antigen-presenting dendritic cells
- all kinds of lymphocytes:
  - B cells and T cells, leading to an adaptive immune response;
  - NK cells (an effector cell in innate immunity).
- eosinophils

b) **activating** many of these recruited cells to produce their own mediators of inflammation

**Biogenic amines:**

The granules of mast cells contain non-lipid, low molecular weight vasoactive mediators. In humans, prototypic mediator of this class is histamine, but in certain rodents serotonin may be of equal or greater import. Histamine acts by binding to target cell receptors, and different cell types express distinct classes of receptors (H1, H2, H3, H4) that can be distinguished by pharmacologic inhibitors. Upon binding to cellular receptors, histamine initiates intracellular events, such as phosphatidylinositol bisphosphate breakdown to IP3 and DAG, which cause different changes in different cell types. In vascular endothelial cells, binding of histamine leads to endothelial cell contraction and leakage of plasma into the tissues. Histamine also causes endothelial cells to synthesize vascular smooth muscle cell relaxants, such as prostacyclin (PGI2) and nitric oxide, which cause vasodilation. These actions of histamine produce the wheal and flare response of immediate hypersensitivity. H1 histamine receptor antagonists can inhibit the wheal and flare response to intradermal allergen or anti-IgE antibody.

Histamine also causes constriction of intestinal and bronchial smooth muscle. Thus, histamine may contribute to the increased peristalsis or bronchospasm associated with ingested allergens or asthma, respectively. However, in these instances, especially in asthma antihistamines are not effective at blocking the reaction. Moreover, bronchoconstriction in asthma is more prolonged than the effect of histamine, which is rapidly removed from the
extracellular milieu by amine-specific system. Other mast cell-derived mediators are clearly important in some forms of immediate hypersensitivity.

**Granule proteins and Proteoglycans:**

In addition to vasoactive amines, mast cell granules contain several enzymes, such as serine proteases and aryl sulfatase, as well as proteoglycans such as heparin and chondroitin sulphate. The enzymes may cause tissue injury when released upon mast cell degranulation. One function of the negatively charged proteoglycans may be to bind and store the positively charged biogenic amines.

**Lipid mediators:**

Three classes of lipid mediators are synthesized by activated mast cells. In general, these reactions are all initiated by the actions of cPLA2, which releases substrates from precursor phospholipids stored in membranes or in the lipid bodies. The substrates are then converted by enzyme cascades into the ultimate mediators.

The first mast cell lipid mediator to be described was prostaglandin D\(_2\) (PGD\(_2\)). Released PGD\(_2\) binds to receptors on smooth muscle cells and acts as a vasodilator and as a bronchoconstrictor. PGD\(_2\) is synthesized from arachidonic acid derived from phospholipid by the sequential actions of enzymes. PGD\(_2\) synthesis can be prevented by inhibitors of cyclooxygenase, such as aspirin and nonsteroid anti-inflammatory agents.

The second class of mast cell arachidonic acid-derived mediators is the leukotrienes. Mast cells convert arachidonic acid, by the action of 5-lipoxygenase and other enzymes, into leukotriene C\(_4\) (LTC\(_4\)) which can be subsequently degraded into LTD\(_4\) and LTE\(_4\). Mast cell-derived leukotrienes bind to specific receptors on smooth muscle cells, different from the receptors for PGD, and cause prolonged bronchoconstriction. When injected into skin, these leukotrienes produce a characteristic long-lived wheal and flare reaction. Collectively, LTC\(_4\), LTD\(_4\) and LTE\(_4\), constitute what was once called “slow-reacting substance of anaphylaxis” (SRS-A) and are now thought to be major mediators of asthmatic bronchoconstriction.

The third lipid mediator produced by mast cells is called plateled-activating (PAF) for its original bioassay as an inducer of rabbit platelet aggregation. In mast cells PAF is synthesized by acylation of lysoglyceryl ether phosphorylcholine, which is derived from a membrane
phospholipids by cPLA$_2$-mediated release of a fatty acid from the sn2 position. PAF has direct bronchoconstricting actions. It also causes retraction of endothelial cells and can relax vascular smooth muscle. However, PAF is very hydrophobic and is rapidly destroyed by a plasma enzyme called PAF acetyl hydrolase, limiting its biologic actions.

Figure 4. The breakdown of tolerance and/or immunoregulatory mechanisms leads to autoimmune activation and recognition in the tissues. These responses, which are 'adaptative' in their anti-self specificity, generate primary 'innate' inputs into mast cells, such as immune complex binding to FcRs, and C3a and C5a anaphylatoxins of the complement pathway binding to specific receptors. The molecular route for direct 'bystander' activation of mast cells by T cells remains conjectural. The mast cell, owing to the abundance and diversity of secondary mediators in its granules, responds by activating a host of pathways, thus amplifying the local response. Vascular permeability is increased, allowing influx of additional molecules (antibody, complement). The adhesiveness of the vascular endothelium
is increased, facilitating the homing of leukocytes (and in particular neutrophils) provoked by chemokine and TNF-α release. These leukocytes are also activated by the same cytokines. Mast cell mediators may be also involved in remodelling connective tissue, or in biasing secondary T-cell responses. Mast cell activation may also signal to local neuronal constituents by the release of NGF, serotonin or dopamine. Thus, the mast cell takes in what may be a low pro-inflammatory input and amplifies it to bring about a much wider response.

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Cytokines:

Mast cells are significant sources of cytokines, including TNF, IL-1, IL-4, IL-5, IL-6, and various colony-stimulating factors (CSFs) such as IL-3 and granulocyte-monocyte colony-stimulating factor (GM-CSF). Large amounts of TNF-α are quickly released by stimulated mast cells. All the cells involved in inflammation have receptors for TNF-α, and are activated by it to synthesize more on their own. This positive feedback quickly amplifies the response. IL-1 has both paracrine effects on cells in the vicinity (e.g., causing them to produce tissue factor and thus triggering the blood clotting cascade, stimulating the synthesis and secretion of a variety of other interleukins, helping to activate T cells and thus initiate an adaptive immune response) and endocrine (hormonal) effects as it is carried in the blood throughout the body, decreasing blood pressure and inducing fever. IL-1 causes fever by stimulating the release of prostaglandins, which act on the temperature control centre of the hypothalamus. (Paul, W. E., et al. 1993).
Figure 5. Mast cells produce cytokines and chemokines involved in regulating T cell differentiation and effector function leads to an appreciation of a wider role for these cells in adaptive immune responses.
THE ROLE OF MAST CELLS IN MULTIPLE SCLEROSIS

Multiple sclerosis (MS) is a chronic inflammatory disease which affects central nervous system (CNS) white matter. Inflammatory cells are perivascular and predominant cells in the active lesions are lymphocytes and macrophages. In MS, the immune system attacks myelin which is stripped off the nerve fibres, either partially or completely, leaving scars known as lesions or plaques. Animal model of MS is experimental autoimmune encephalomyelitis (EAE). It shares many of clinical features with MS. EAE studies have demonstrated that other cells, not only T and B lymphocytes and macrophages are involved in innate and/or adaptive immune responses like dendritic cells, natural killer cells and mast cells. (El Behi, M., et al., 2004). Mast cells are present in the CNS where they are found predominantly in the leptomeninges, thalamus, hypothalamus and intraparenchymal vessels (Brown, M., et al., 2002) They are located perivascularly, often in close association to neurons, and are critical for allergic and neuroinflammatory reactions. Mast cells in the meninges are found in apposition to neurons and can be activated by neurotransmitters. (Dimitriadou, V., et al., 1987). The mast cells within and around plaques of demyelination were described first time by Neumann et al., (1890). In the last few years many authors have reported different evidence about the role of mast cells in MS.
Ibrahim et al confirm that MCs are present within and around MS plaques, especially the ‘chronic active’ lesions (Ibrahim, M. Z., et al., 1996). The fact that MCs are more common in the ‘chronic’ cases suggests that MCs appear as a consequence of inflammation and not vice versa (Princeas, J.W, et al., 1978). They could conceivably play their classical roles and furthered the inflammatory state contributing to expansion of established plaques (Ibrahim et al.). More recently Secor et al. demonstrated in vivo role for mast cells during the acute phase of myelin oligodendrocyte glycoprotein (MOG)-induced EAE. Mast cell-deficient mice W/W\(^{V}\) exhibit significantly reduced incidence and disease severity when compared with wild type controls. The selective reconstitution of mast cells in W/W\(^{V}\) mice restores severe disease confirming their role in EAE (Secor, V.H, et al., 2000). In EAE, susceptibility to disease induction is correlated with the variability and distribution of brain MCs. For example, the CNS of SJL/J mice, susceptible to demyelinating disease, contains three times more MCs than the CNS of C3H mice which are less sensitive to the induction of demyelinating disease. (Johnson, D., et al., 1991). It is very likely that MCs can play their role through the secretion of their granule content. In cerebrospinal fluid of MS patients are present the products of MC secretion such as histamine and tryptase (Rozniecki, J. J., et al., 1995), (Tuomisto, L., et al., 1983). Many could be mechanisms through which MC may affect MS progression/triggering. The key event of MS development is breakdown of blood-brain barrier (BBB), and one of the roles of MC mediators could be modulation of BBB permeability and as a consequence extravasation of inflammatory cells (Abbot, N. J., et al., 2000). Acute stress by immobilization was shown to lead to mast cell-dependent increase in BBB permeability. (Esposito, P., et al., 2001). Zhuang et al. have shown local alternations of BBB permeability after injection of the MC secretagogue 48/80 (Zhuang, X., et al., 1996). Mast cells are also a rich source of most known cytokines including tumor necrosis factor TNF-\(\alpha\). Soluble TNF receptor and adhesion molecules were detected in cerebrospinal fluid (CSF) of MS patients and may correspond to BBB impairment. (Tsucada, N., et al., 1993). Mast-cell derived proteases are capable of degrading myelin, and myelin components can directly stimulate mast cells degranulation in vitro. (Johnson, D., et al., 1988). Inhibitors of mast cell degranulation and mast cell mediator activity, including theophylline, proxicromil, and cyproheptadine, can decrease or suppress EAE or MS. (Pedotti, R., et al., 2001). Gene microarray analysis revealed that transcripts encoding tryptase and receptors for IgE, IgG, and histamine, which are all expressed by mast cells, are upregulated in CNS plaques from patients with chronic MS. (Lock, C., et al., 2002).
Theoharides et al. have reported that 17β-estradiol and myelin basic protein had a synergistic action on inducing mast cell secretion. This effect was more pronounced in Lewis rats, which are susceptible to the development of experimental allergic encephalomyelitis than in Sprague-dawley rats, which are fairly resistant. (Theoharides, T. C., et al., 1993). So, it is likely that MCs can affect BBB permeability thus enhancing the recruitment of inflammatory cells. However a role for MCs in antigen spreading, a key process in autoimmunity (Anne. M., et al., 2006), either early or late during EAE/MS progression, can not be excluded, since MSs proteinases can release immunogenic peptides from myelin and in principle activate T cells thus amplifying the autoimmune reaction (Gregory, N., et al., 1991).

These findings highlight the importance of the role of MC in MS, which can be exerted both early or late during the progression of disease, and suggesting that the modulation of their functions might provide a novel therapeutic tool for the control of this disease. (Dimitriadou, V., et al., 2000).
Figure 6. Activated T cells express α4β1-integrin, which binds to vascular cellular adhesion molecule (VCAM) on the surface of venules in inflamed tissues. This interaction allows the T cells to pass through the endothelial wall and penetrate the extracellular matrix. In multiple sclerosis (upper panel), the T cells re-encounter the cognate CNS antigen presented by MHC class II molecules on either microglial or dendritic cells. This interaction can be inhibited by glatiramer acetate or altered peptide ligands. In addition, statins, angiotensin-converting enzyme (ACE) inhibitors, and PPAR-α agonists can all downregulate the inducible expression of MHC class II molecules. Similarly, cytokines such as interferon-β (IFN-β) downregulate MHC class II molecules and interfere with diapedesis of cells by downregulating metalloproteases. CD28 and CD80/86 interactions can be blocked by the CTLA4-Ig fusion protein. Tolerizing vaccines promote tolerance processes which occur when the T cell/dendritic cell interaction is not optimal. B cells and mast cells are also recruited into the inflammatory infiltrate. Antibody plus complement can produce 'membrane attack' complexes, which can damage the oligodendrocytes and underlying axon. Osteopontin is expressed on the surface of oligodendroglial cells and neurons during active disease, and is pivotal in the disease progression.

THE AIM OF THE THESIS

Recently a role for mast cells in the pathogenesis of multiple sclerosis (MS) has been put forward, based on morphological, biochemical and genetic analysis of human lesions (Brown, M. A., et al., 2007; Toms, R., et al., 1990; Olsson, Y. 1974; Lock, C., et al., 2002; Rozniecki, J.J., et al., 1995; Ibrahim, M. Z., et al., 1996; Steinman, L., et al., 2002) and on animal model of the disease (Bebo, B. F., et al., 1996; Dines, K. C., et al., 1997; Brenner, T., et al., 1994; Secor, V. H., et al., 2000). Mast cells (MC) are present in man and mouse CNS (Maślińska, D., et al., 1999) and their number can increase in diseases (Epari, S., et al. 2006). In mouse most of them are localized into the thalamus and appear to be mainly degranulated in experimental allergic encephalomyelitis (Dines, K. C., et al., 1997). Accordingly to this hypothesis mast cell granule component are found in the liquor of MS patients (Rozniecki, J.J., et al., 1995) and mRNA for mast cell proteins are widely expressed in the MS lesions (Steinman, L., et al., 2002; Steinman, L., 2003). Finally mast cell free mice (W/Wv) developed a very mild experimental autoimmune encephalitis (EAE), while showing the disease as control mice when mast cell population was
Various proposals have been considered for explaining such a role of MC. If activated to degranulate, MC could increase the permeability of the blood brain barrier (BBB) through histamine release, they can also induce the formation of encephalitogenic peptides through proteinase activity (Johnson, D., et al., 1988, Dietsch, G. N., et al., 1991) and even present antigens (Malaviya, R., et al., 1996; Frandji, P., et al., 1996; Villa, I., et al. 2001). About the effect of histamine however conflicting results have been reported. In some experiments histamine was found to ameliorate the clinical scoring in EAE, while in others it can worsen the disease (Pedotti, R., et al., 2003; Steinman, L., 2003; Musio, S., et al., 2006). The finding that also mast cells outside the brain can allow EAE to occur in W/Wv mice (Tanzola, M. B., et al., 2003) suggests that their activation could be achieved directly by injected antigen so, triggering an increment in BBB permeability from outside the brain and enhance T specific lymphocyte entry through it, a process that normally occurs at a low degree (Galea, I., et al., 2007; Krakowski, M. L., et al., 2000). However a recent paper showed that even with a damaged BBB, intracranial antigen Ag presentation occurs in regional lymphonodes and not within the CNS (Walter, L., et al., 2007), supporting the hypothesis that myelin components antigens could directly interact with MC, which can in turn either/both increase BBB permeability or/and activate T lymphocytes in the lymph nodes as antigen presenting cells. These processes should require the activation of the MC functions following the interaction with myelin or myelin components, but nothing is known about this putative event.

In some demyelinating diseases, both in man and animal models, a defect in myelin physiology has been shown to precede the overt disease. In these cases myelin instability was observed which may result in oligodendrocytes (ODC) vesiculation, a process that characterizes remarkably also endothelial cell in MS (Horstman, L. L., et al., 2007), and myelin diffusion into the extracellular environment. A myelin defect is induced for example as a result of the action of peptidyl argininedeiminase (PAD) activity which convert arginyl residues to citrulline. The accompanying loss of positive charge makes myelin susceptible to vesiculation and MBP more susceptible to proteolytic activity (Mastronardi, F. G., et al., 2005). Musse and Harauz (2007) reported in their very recent review that peptidyl-arginine deiminase type 2 (PAD2), which is present in myelinating cells of CNS, can be activated by Ca2+ influx and induce the modification of MBP leading to loss of myelin structure and stability, apoptosis and release of encephalitogenic epitopes. Increased deiminating activity is part of the molecular hallmark of
MS and of an animal model of MS (Mastronardi, F. G., et al., 2006) and deiminated MBP can even induce fragmentation of lipid vesicles (Boggs, J. M., et al., 1999).

Other examples supporting the possibility that myelin instability could precede over demyelinating diseases, are patients with the cerebral form of X-linked adrenoleukodystrophy (Powers, J. M., et al., 1992) and possibly a subset of patients with MS (Lucchinetti, C. F., et al., 1996), the dmy rat (Kuwamura, M., et al., 2004) and the PLP overexpressing Lewis rats which are characterized by a low grade of myelin degeneration and in which EAE induction results in a more severe disease with respect to that induced in wild type animals (Aboul-Enein, F., et al., 2004). Indeed very recently it has been shown that oligodendroglioma cells can shed membrane vesicles in culture (D'Agostino, S., et al., 2006) and possibly induce diffusion of myelin antigens even outside the brain. In Theiler’s virus induced demyelinating disease the immune response is initiated by the presentation of virus antigens by CNS resident APC, to CD4+ T cells. Even in this case a damage to ODC precedes the overt disease (Ercolini, A. M., et al., 2006). A subtle ODC injury could induce myelin vesiculation and diffusion, then the process of inflammation and repair is triggered, which can in turn evoke a cascade of events leading to autoimmunity and both to severe ODC and neuronal lesion. So the possibility that the immune/inflammatory response is itself a reaction to some initiating neurodegenerative process of myelin sheet must also be considered.

The observations reported above suggest that myelin itself may be a factor in the cascade of events which eventually leads to autoimmunity. Accordingly in the mouse EAE model, the clearance of myelin by macrophages could be necessary for the remission phase (Kotter, M. R., et al., 2005; 2006).

We believe that the link connecting MC with the ODC degenerative process and the following inflammatory autoimmune response in the pathogenetic process in MS may be represented by the activation of MC by myelin vesicles. Up to now this possibility has been only occasionally addressed (Theoharides, T. C., et al., 2004; Brenner, T., et al., 1994; Orr, E. L. 1988; Johnson, D., et al., 1988). Some reports support however the hypothesis that MC-myelin interaction can be able to activate MC. Firstly, recently it has been found that anti-myelin antibodies of the IgE class are present in sera of MS patients (Mikol, D. D., et al., 2006), and IgE can be also present in CSF of these patients (Greve, B., et al., 2001). These antibodies, when antigen linked, can directly stimulates MC through FceR-I. Secondly, the heterotypic adhesion of MC with the membrane of different cell types such as T lymphocytes (Mekori, Y. A., et al., 2002), eosinophil (Piliponsky, A, M., et al., 2002) cells of Cajal (Wang, X. Y., et al.,
2007), nerve cells (Furuno, T., et al., 2005), fibroblasts and endothelial cells (Greenberg, G., 1983), induce MC activation and maturation (Blennerhassett M.G., and Bienenstock, J. 1998.). Finally in very early paper, Theoharides et al. (1978) showed that liposomes were able to induce MC activation. So, it should not come to surprise that MC can be activated by membrane derived structures, as myelin is.

On this basis we hypothesized that myelin vesicles, produced by ODC through a degenerative process of an unknown nature, or injected myelin components for triggering EAE, could interact with MC and activate them. The degranulation process which may ensue could trigger a cascade of events capable of starting a positive feedback loop and eventually lead to autoimmunity.

With the aim of evaluating myelin-MC interaction, we plan to investigate about the possible role played by myelin vesicles, prepared from normal or EAE rodents or from human brain autopic biopsies from healthy subjects, in triggering rat peritoneal mast cells (RPMC) activation.

In this paper we show that myelin vesicles interact with a mast cell specific receptor, can activate these cells and can also be ingested by them. These process could be key events in the mast cell function required for inducing EAE in mice and possibly in MS in at least a group of patients where a mild degenerative process may initiate the disease.

MATERIALS AND METHODS

Antibodies and reagents

Phosphate buffered saline (PBS), Tris (Trizma HCl), NaCl, sodium cacodylate, EDTA, SDS and polyacrylamide (SDS gel preparation kit), dextran T500, enzyme substrates (hexosaminidase and chymase), FITC, histamine, poly-D-lysine, poly-ornithine, phorbol-12 myristate-13 acetate (PMA), 48/80 compound, fucoidan, cytochromeC, superoxide dismutase, PERCOLL HEPES, ATP, PMSF, iodoacetate, leupeptin and complete Freund adjuvant were obtained from SIGMA Chemical Co (St. Louis., MO). Glutaraldehyde was obtained from SERVA, Heidelberg, Germany. Blocking peptide, and blocking antibodies anti-Scavenger receptor Class A I/II (SR-AI/II), anti-FASL, anti-TNFα, anti-GFAP as well as Annexin V were purchased from Santa Cruz biotechnology (Heidelberg, Germany). RITC-labelled phalloidin and DAPI were from SIGMA. Secondary antibodies labelled with AlexaFluor 488 (green) or AlexaFluor 594 (red).
were from Invitrogen Molecular Probe (Eugene, Oregon, USA). Anti-\(\gamma\)-tubulin III antibodies was a YOL clone, from Millipore (Billerica, MA, USA). All the other reagents were purchased from Sigma if not indicated differently. All reagents were of the highest quality and purity available. All solutions were made in pyrogen-free distilled water for clinical use (Diaco, Trieste, Italy).

**Animals**

All experimental procedures involving the use of mice or rats were reviewed and approved by Trieste University Animal Care and Use Committee. All animals were housed under specific pathogen-free conditions with light-dark cycles, fed standard animal food, and given free access to autoclaved distilled water from bottles.

**Preparation of rat peritoneal mast cells (RPMC) and rat peritoneal macrophages (RPMAC)**

Wistar male or female rats (200 to 400g) purchased from the animal facility of the University of Trieste, were used throughout. Rats were killed using \(\text{CO}_2\) inhalation. Rat peritoneal mast cells (RPMC) were obtained as follows. The peritoneal cavity was washed with 50 ml PBS containing 1.2 mM \(\text{MgCl}_2\), 5 mM glucose and 0.1% BSA (PBS+). The cell suspension was centrifuged at 200xg for 8 min at 4°C and mast cells were then purified over 0.883g/ml PERCOLL gradient 0.1% BSA 20 min at 1050 rpm. We yield in the pellet fraction a population of pure mast cells. The final cell suspension, in PBS+ containing 0.5 mM \(\text{CaCl}_2\), as judged by differential counts carried out on Diff-Quik System (Medion Diagnostics, Gmbh, Düdingen, CH) stained cytospin specimens (Cytospin 2, Shandon Inc., Pittsburgh, PA), contained >98% mast cells, the remaining cells being macrophages (RPMAC). Peritoneal washings containing even a small number of red blood cells were discharged, since we noted that the presence of these cells did not allow an optimal MC purification. The band at the PBS-PERCOLL interface was enriched in RPMAC. These cells, which accounted for 80%, the remaining cells being neutrophils and lymphocytes were also collected. RPMC and RPMAC were washed and counted electronically (Coulter Counter ZBI, Luton, UK) Disruption of Mast cell suspensions (10^6/ml), for obtaining a sonicate was carried out by Branson sonifier (Danbury, CT; USA). Mast cell incubation medium after degranulation was obtained by stimulating 10^6/ml RPMC
secretion with 48/80 (10 µg/ml final conc) for 30 min, and centrifuging the mixture for 15 min at 2000 xg in an Eppendorf microcentrifuge. The sonicate and the incubation medium obtained from activated RPMC, were stored at -20°C until use.

**Human Neutrophil isolation**

Human neutrophils (HPMN) were isolated from blood of healthy donors collected in citric acid-citrate-dextrose solution (Don Baxter Lab., Trieste, Italy) as previously described (Menegazzi, R., et al.). Briefly, the red cells were removed by dextran sedimentation (1 ml of 4.5% dextran in saline was added to 5 ml of blood). Granulocytes were separated from mononuclear cells by centrifuging the white cell-rich plasma for 20 min at 800 xg on Lymphoprep (RT). A 90sec hypotonic treatment was used to remove residual erythrocytes from the granulocyte rich pellet. The cells were washed once in PBS containing 1.2 mM MgCl$_2$, 5 mM glucose and 0.1% BSA (PBS+), resuspended in the same medium containing 0.5 mM CaCl$_2$, and counted electronically (Coulter Counter ZBI, Luton, UK). The final cell suspension, as judged by differential counts carried out on Diff-Quik System (Medion Diagnostics, GmbH, Düdingen, CH) stained cytospin specimens (Cytospin 2, Shandon Inc., Pittsburgh, PA), contained >95% neutrophils, the remaining cells being eosinophils.

**Neutrophil membrane isolation**

HPMN at a concentration of 2x10$^7$/ml were incubated in PBS$^+$ in the presence of 0.5 µg/ml DFP for 5 min. The cells were then washed twice in PBS$^+$ and then resuspended in 10 mM HEPES, pH 7.4, 100 mM KCl, 3 mM NaCl, 3.5 mM MgCl$_2$, 1 mM ATP, 2 mM PMSF, 2 mM iodoacetate and 10 µg/ml leupeptin (relaxation buffer) at the concentration of 100x10$^6$/ml and disrupted by nitrogen bomb cavitation (Parr Instrument Co, Moline, IL) calibrated at 350 psi for 18 min at 0°C. Nuclei and cellular debris were cleared by centrifuging the homogenate at 850xg 7 min at 0°C. The postnuclear supernatant (PNS) was then layed on sucrose three step discontinuous gradient from the top: 3ml of 1.1200, 3ml of 1.1800 and 2ml of 1.2400 g/ml (d20°C). As previously reported the membrane fraction was obtained at the relaxation buffer-1.1200 interface. The fraction collected from the top of the gradient was characterized by assaying the enzyme
markers of neutrophils subcellular fractions, that is alkaline phosphatise for the plasmamembrane, VitB12BP for specific granules and myeloperoxidase for azurophilic granules. The membrane fraction was specifically enriched with plasma membrane marker while it did not contain significant amount of myeloperoxidase or VB12BP (Vita, F., et al., 1996).

**Oligodendrocyte Primary Culture**

The first step to obtain primary culture of oligodendrocytes is to prepare mixed primary cell culture from neonatal rat brain (Barbarese, E. 1991). After decapitation of neonate rats (d0 or d1) telencephala are dissected and the meninges removed. Pooled tissue was treated with trypsin solution and subsequently cells were separate from debris on 3% BSA (Albumin bovine serum, fraction V, ≥96%, Sigma) cushion. The cells were growth in complete DMEM (Dulbecco’s Modified Eagle’s Medium) containing 10% FCS and 1% penicillin/streptomycin, on poly-D-lysine (0.025%) coated flasks for next two weeks at 37°C, 5% CO2 in a humidified environment, as mixed culture of microglia, oligodendrocytes (ODC), fibroblasts and neurons. After this period we performed the purification of the pure oligodendrocytes. With 5ml of the trypsin-EDTA solution for each flask and with a 5 ml plastic pipet squirt gently over the cells 12 times or more. Take this cell-enriched solution and place in a centrifuge tube that contains an equal volume of 10% FCS medium. Spin 5 min at 100g. The cell pellet is resuspended in 10% FCS medium (7ml) and plate in Petri dish. After 30min the microglia and fibroblasts will have attached but the oligodendrocytes should still be floating. Pellet the cells which should be quite enriched in oligodendrocytes and grow in N2 modified medium (100ml of DMEM, 11mg of piruvate, 5mg transferrin, 1ml BSA 4%, 100μl sodium selenite 0,28mg/ml, 100μl insulin 5mg/ml, 100μl biotin 0,1 mg/ml, 100μl Tri-iodo-L-thyronine sodium salt 10μg/ml, 100μl penicillin/streptomycin and 100μl hydrocortisone 10μM. Cells (5 X 10^4) are seeded into 24-well flat-bottom plates containing coverslips (12 mmm diameter) coated with poly-ornithine (50μg/ml in 0,15M borate buffer pH 8,7). The cells were cultured in N2 modified medium in a 5% CO2 humidified incubator. Differentiated ODC were obtained after 12-14 days. Whole procedure was carried out in sterile environment. These cells were mostly positive for myelin basic protein (MBP) expression and negative for glial fibrillar acidic protein (GFAP); see below.
**Induction of relapsing/remitting EAE**

Experimental autoimmune encephalitis was induced as described by Brown AM et al. (Brown, A. M., and McFarlin, D. E. 1981) with few modifications. At the moment of immunization SJL/JHanHsd female mice were 6 weeks old. Immunization was performed on day 0 and day 7. Day 0: EAE was induced by the injection of an emulsion containing 3.8 ml (76mg) mouse spinal cord homogenate (MSCH), 2ml killed *Mycobacterium bovis-BCG* (50 human adult doses autoclaved in PBS), 7.8ml complete Freund’s adjuvant sonicated at RT at maximum power 6 times for 25 seconds. Mice were injected 0.1 ml on each rear flank subcutaneously. Day 7: same as above without BCG and using incomplete Freund’s adjuvant. The clinical score was evaluated from 25th day to the 56th day. The following scale was used to assign intensity of EAE: 0, normal; 10 slow, sluggish, slightly slower than normal and tail not as responsive as normal; 20, limp tail; 30, limp tail, hind limb unilateral or paw weakness, waddling gait; 40, hind limb or paw paralysis; 50, bilateral hind limb paralysis. Animals that reach this stage were sacrificed immediately to avoid further suffering and their brains were processed for myelin purification.

**Myelin preparation, labelling and modification**

Multilamellar myelin vesicles were isolated on sucrose gradient from adult rat or SJL/JHanHsd female mice brains by following exactly the procedure of Norton and Poduslo (Norton, W. T., Poduslo, S. E. 1973.). The same procedure was employed for isolating myelin vesicles from frozen autoptic human brain samples obtained from normal subjects (kindly provided by Djorje Gveric, University College of London, UK). Typical recovery starting from 2.5g of brain tissue is 10ml myelin suspension at 2 mg/ml of myelin proteins (determine by the Bradford’s method). Myelin membranes were precipitated in TCA( trichloroacetic acid) 10% for 30 min at 0°C. Than they were centrifuged for 20min at 13000 rpm. After this they were washed in ice-cold acetone two times and one time in methanol. The protein fraction was dissolved in sample buffer and analyzed in by SDS-PAGE. Myelin protein samples (determined by the Bradford’s method) were dissolved in reducing and denaturing Leammli sample buffer prior to electrophoresis and boiled two minutes. Samples (30 µg proteins/well) and standards MW (Rainbow RPN 756, GE Bio-Science AB SE-751 84 Uppsala Sweden) were run in Sodium dodecyl-sulphate polyacrylamide gel (SDS-PAGE), 5% stacking and 10% separating, at 120 V
and 35 mA. The gels were stained by Blu Coomassie. The degree of purification was comparable to that reported by Norton and Poduslo, as judged by the presence of three main bands at 24.0 Kd representing proteolipid protein (PLP), at 21.5 KD and 18.5 Kd representing the more abundant isoforms of myelin basic protein (MBP). These two proteins account together for about 80% of the total myelin proteins (Riccio, P., 2000). Preparation of unilamellar myelin vesicles was carried out by the procedure of Lin and Bartlett (Lin, L. F., 1986) by purification on Sepharose 4B column. One milliliter fractions were collected, and absorbance at 280 nm was measured. The yield of unilamellar vesicles from isolated myelin was 25% of the myelin protein loaded. A population of right-side-out vesicles has been obtained by ConA-Agarose affinity column chromatography, from unilamellar vesicles as previously described (Lin, L. F., 1986). Myelin vesicles were delipidated with chloroform/methanol (2:1) solution four times. Then they were centrifuged 3min at 13,000rpm. After this step, water/chloroform solution was added and the mixture was centrifuged again. The procedure was repeated threefold. Delipidated membrane was collected from the water phase. Three phases were made and proteins were collected from the intermediate one. For labelling, myelin vesicles (2mg/ml) where incubated with FITC (1mg/ml) in carbonate/bicarbonate buffer 0.1M pH 9, 1hour at RT protected from the light. Excess of FITC was eliminated by three dialyses against two litres PBS buffer + 0,1NaN₃ each time. Myelin was treated with Proteinase K (0,5 μg/ml), it was incubated 20 min at 37ºC. After incubation, reaction was quenched with 1 mM PMSF to inhibit PK. Myelin was boiled 5-15 min. myelin vesicles were treated with 0,2 M Na₂CO₃ for 30 min at 0ºC to open vesicles and strip them of peripherally associated proteins, leaving behind integral membrane proteins.

### Ultrastructural scanning electron microscope analysis

Samples of ODC cultures on glass coverslip (Sacco Srl Cadorago, Como, Italy), incubated for 30 min at 37°C either alone or in the presence of mast cells, were fixed in 1% glutaraldehyde in 0.1 sodium cacodylate buffer (Electron Microscopy Sciences) (pH 7.4) for 30 min. The coverslips were washed twice in 0.1 sodium cacodylate buffer (pH 7.4), dehydrated in graded 30 to 100% ethanol series, dried in a CO₂ apparatus (Bal-Tec; EM Technology and Application, Furstentum, Liechtenstein), sputter coated with gold in Edwards S150A apparatus (Edwards High Vacuum, Crawley, West Sussex, United Kingdom), and examined with a Leica Stereoscan 430i scanning electron microscope (Leica Cambridge Ltd., Cambridge, United Kingdom).
Ultrastructural analysis on transmission electron microscope

RPSC alone (3 x 10^6 cells), RPMC (3 x 10^6 cells) in the presence of myelin vesicles (50µg/ml) or myelin vesicles alone (50µg/ml) were incubated for 30 min at 37°C, and subsequently fixed for 30 min in a solution of 1,5% glutaraldehyde (Serva, Heidelberg, Germany) in 0.1 sodium cacodylate buffer (pH 7.4) containing 0.03 M CaCl₂. The samples were washed twice with sodium cacodylate buffer (pH 7.4) and then postfixed with 1% osmium tetroxide for 1h at 4°C. Postfixed cells were dehydrated with an ascending ethanol series ending with 100% ethanol and then embedded in Dow epoxy resin (DER332; Unione Chimica Europea, Milan, Italy) and DER732 (Serva, Heidelberg, Germany), as previously described (Zabucchi, G., et al., 1986, Lungarella, G., et al., 1992) Ultrathin sections were prepared with an Ultrathome III (LKB, Pharmacia) and then were double stained with lead citrate and uranyl acetate and observed with a transmission electron microscope (EM208; Philips, Eindhoven, The Netherlands). Micrographs were taken with a Morada Camera (Olympus Soft Imaging Solutions (OSIS), Munster, Germany)

Adhesion and Secretion assay

ELISA plates (96-well Maxisorp, Falcon, Becton-Dickinson, Labware Europe, Le Ponte De Claix, France) were coated with myelin vesicle suspension (50µg/ml protein concentration) in 0.1M bicarbonate buffer ( 0.1mM Na2CO3/NaHCO3) pH 9 for about 4 hours at 4 ºC. Control wells were coated with neutrophil membrane, BSA 3%, skim milk 5% or left uncoated. Well binding capacity was blocked by BSA 3% in overnight incubation at 0°C. Preliminary experiments showed that by adding 2.0 micrograms of myelin proteins to one well, 0.84 were found linked to the plastic well on the average, accounting for about 41.8% of the proteins.
added. Excess of myelin was removed by washing extensively with PBS Tween 0.1%; control wells were washed as well with the same buffer. Subsequently RPMC (10^4 cells in a final volume of 250 µl) or 2x10^4 human PMN were added to and incubated in the coated wells for 30min at 37°C. When indicated RPMC were preincubated 10 min at 37 C with either antibody anti SR-A 10 µg/ml or its competitor fucoidan 100 µg/ml. After the incubation time non adherent cells were withdrawn and pelleted. The supernatant was carefully collected and the cell pellet resuspended in the same volume of the assay. Then the wells were washed twice more with PBS+. β-hexosaminidase activity was measured in the supernatants, while chymase activity was measured in adherent and non-adherent cells. The assay solution contain PBS (phosphate buffer 10mM, Ph 7.4, NaCl 154mM, KCl 27mM), 0,1% BSA, 0,1% CTAB (cetyltrimethylammonium-bromide, Sigma) and enzyme substrates, N-Succinyl-Ala-Ala-Pro-Phe-p-nitroanilide (0.2mM finale) for chymase and 4-Nitrophenyl N-acetyl-β-D glucosaminide (0.5mM finale) for β-hexosaminidase. The enzymatic activity was read in a plate ELISA reader (Bio-tek instruments INC) at 450 nm. The chymase activity (due to the cell binding property of the secreted enzyme) was prefered as an adhesion marker. The extent of adhesion, that is the percentage of RPMC remaining well-associated after washing, taking the number of added cells as 100%, was calculated from a calibration curve made up for the enzyme in each experiment. The extent of secretion, that is the percentage of β-hexosaminidase activity free in the supernatant taking the total enzyme activity of added cells as 100%, was calculated from a calibration curve made up for the enzyme in each experiment. Since the amount of adherent MC was calculated from the content of chymase remaining into the well, we always checked if the amount of enzyme released could allow an underestimation of the adhesion process. In any condition tested we can exclude that a significant underestimation occurred, while when it could be possible, that is in the presence of fucoidan, we could confirmed the competitive effect by FACS analysis and morphological analysis. The same assay based on chymase enzymatic activity was employed for evaluating RPMC adhesion on ODC monolayer. Chymase activity ion ODC was undetectable. Myeloperoxidase assay (Vita, F., et al., 1996) was employed as an enzyme marker for evaluating human PMN adhesion on coated wells by referring to a calibration curve.

**SDS-PAGE and Western Blotting**
Mast cell and macrophage were sonicated 3 times 20 seconds. An enriched membrane fraction was obtained at the interface of a one-step sucrose gradient (PBS-1.1200 g/ml d20ºC). Protein samples (determined by the Bradford’s method) were dissolved in reducing and denaturing Laemmli sample buffer prior to electrophoresis and boiled two minutes. Samples (50 µg proteins) and standards MW (MagicMark, Invitrogen Carlsbad, CA, USA) were run in Sodium dodecyl-sulphate polyacrylamide gel (SDS-PAGE), 5% stacking and 10% separating, at 120 V and 35 mA. Running buffer contain Tris-HCl 50 mM, pH 8.3, glycine (minimum 99% TCR, Sigma) 384mM. Proteins were blotted onto PVDF membrane (Amersham Biosciences, UK) at 25V and 90 mA for 90 min. Blots were blocked in solution contained TBS (Tris-HCl 50 mM pH 7.6, NaCl 154 mM) Tween 0.1%, BSA 3%, milk 5% over night. The day after they were incubated with goat IgG anti Scavenger receptor A I/II (10µg/ml) for 1 hour at RT. After washing blots were incubated with rabbit anti-goat IgG conjugated with peroxidase (1:40,000) for 1 hour. The bands of interest were detected using enhanced chemiluminescence (Amersham, Buckinghamshire, UK) and band images were captured using Kodak autoradiographic films (Sigma).

**Immunofluorescence of oligodendrocytes**

For immunofluorescence analysis of ODC, the cells were fixed with 4% PAF in PBS for 20 min at room temperature and permeabilized in blocking buffer (5% BSA plus 0.1% Triton-X100 and 2% foetal calf serum (FCS)) for 30 min at RT. Then blocked in FCS 5% for 30 min and incubated with antibodies. To distinguish mature oligodendrocytes from other cells, we performed immunocytochemistry for myelin basic protein (MBP) with an rabbit polyclonal antibody against mouse and rat MBP (200µg/ml; Sigma) diluted 1:500 and a FITC-conjugated goat anti rabbit polyclonal antibody (1:100). Most of the cells in the monolayer were MBP positive. We have used also Lectin-FITC (100µg/ml) type II for 40 minutes at 37°C, from Bandeiraea simplicifolia (Sigma) which recognize galactocerebrosides (galactose) on oligodendrocytes. For monitoring the presence of astrocytes in oligodendrocyte cultures we employed mouse monoclonal anti-GFAP diluted 1:600 and FITC-conjugated goat anti mouse polyclonal antibody (1:100); occasionally few cells were shown to be GFAP positive.

For analyzing the assembly of tubulin, the specimens, including ODC alone or ODC treated with RPMC, were fixed in paraformaldehyde (4% diluted in PBS) for 20min at room temperature (RT), blocked and permeabilized for 20min at RT in a solution containing 5% BSA, 0.1 Triton-
X100 (Merck, Darmstad, Germany) and 2% fetal calf serum (Gibco, Invitrogen). Then the cells were incubated with a monoclonal rat antibody against α-tubulin III (YOL clone, Seromed) for 1 h at RT. Immunofluorescence reaction was visualized using secondary antibodies AlexaFluor 488 (green), (dilution 1:500 in blocking buffer, for 45 min at RT, Invitrogen Molecular Probes). To visualize actin, cells were incubated with phalloidin RITC-conjugated (1:200 in blocking buffer, 45 min at RT, Sigma). Nuclear DNA was counterstained with DAPI (4,6-Diamidino-2-phenylindole dihydrochloride; 1:1000 diluted in PBS; 10 min at RT). Samples were analysed with a standard fluorescence microscope (Zeiss) equipped with a CCD camera and MetaVue software. Cells stained with the secondary antibody only showed no immunostaining. Fluorescence was detected using appropriate filter sets: green fluorescence = ex488nm; red fluorescence = ex594nm and blu fluorescence (DAPI) = ex395nm.

**Immunofluorescence of mast cells and macrophages**

The cells were blocked in PBS+( phosphate buffer 10mM, Ph 7.4, NaCl 154mM, KCl 27mM, BSA 0.1%), goat serum 5% for 1 hour and incubated with antibodies. After that cells were washed and fixed in 4% PAF and cytospin 4min 400 rpm. For detection of Scavenger Receptor Class A (SR-A) we employed goat polyclonal antibody against a peptide mapping near N-terminus of SR-A of mouse origin (10µg/ml), 1 hour incubation and, anti-goat FITC conjugate antibody developed in rabbit (4.5mg/ml) diluted 1:100.

**FACS analysis**

Two assays were carried out by cytofluorimetric analysis. The first was performed for showing the presence of SR-AI/II on RPMC surface. Briefly, 10⁶ cells were resuspended in 1ml PBS+( phosphate buffer 10mM, Ph 7.4, NaCl 154mM, KCl 27mM, BSA 0.1%) containing NaN₃ 0.1% (Merck, Darmstadt, Germany). Antibody anti SR-A in concentration of 15µg/ml was added and incubated with RPMC at 4°C for one hour; thereafter the cells were washed three times. and anti-goat FITC conjugate antibody developed in rabbit (4.5mg/ml) diluted 1:600 was added and the cells were washed two more times before FACS analysis. The second analysis was performed for studying the interaction between RPMC and FITC-labelled myelin in suspension. For this purpose, 10⁶ RPMC were challenged with FITC-myelin 25 µg/ml and incubated for 30 min at 37°C, either in the absence or in the presence of fucoidan 100µg/ml,
blocking peptide 15µg/ml (N-terminal segment of SR-A receptor used for immunization and production of Ab anti SR-A) and 15µg/ml SR-A as competitors. Myelin ingestion process was monitored by adding Trypan Blue 0.5 mg/ml final concentration as FITC-quenching agent. The cells were analyzed using Cytomics FC500 instrument (Beckman-Coulter, Inc., Fullerton, CA) equipped with an argon laser (488 nm, 5 mW) and using a photomultiplier tube fluorescence detector for green (525 nm) or orange (610 nm) filtered light. The flow rate was kept at a data rate below 100 events/second to avoid cell coincidence. For each sample, at least 10,000 events were acquired. Data analysis was performed with the WinMDI software (J. Trotter, Scripps Research Institute, La Jolla, CA).

**Assay of Superoxide Anion (O2-) Production**

10^5 RPMC or RPMAC in 50 µl PBS+, were added to 96-wells plastic plates (tissue culture treated polystyrene 96 wells, Corning, NY) and subsequently superoxide (O2-) production was measured by the superoxide dismutase-inhibitable cytocrome C reduction assay as previously described (54). Briefly, aliquots (50µl) of 0.36 mM cytocrome C (final concentration 0.12 mM) and 50 µl of PBS+ alone or containing either 25 µg/ml myelin (protein content) or phorbol 12-myristate 13-acetate (PMA) as positive control (50 ng/ml final concentration) were added to the cell-containing wells. The mixtures were then incubated at 37°C in a humidified incubator, and the plate was read at 550 nm and 540 nm in a microplate reader (Multiskan MCC/340; Gruppo Flow SPA, Milan, Italy) every 10 min for 90 min. The amount of reduced cytochrome C was calculated from the absorbance difference between 550 nm and 540 nm, using 0.037 optical density (OD) units for 1 nmol reduced cytocrome C as a standard.

**Apoptosis of oligodendrocytes**

For apoptosis assay cells were incubated with anti-cleaved Caspase 3 Asp175 (dilution 1:100, Cell Signaling Technology, Beverly, MA, USA) for 1 hour at RT. Samples were analysed with a standard fluorescence microscope (Zeiss) equipped with a CCD camera and MetaVue software. Cells stained with the secondary antibody only showed no immunostaining. Fluorescence was detected using appropriate filter sets: green fluorescence = ex488nm; red fluorescence = ex594nm and blu fluorescence (DAPI) = ex395nm.
Videoimaging

Videoimaging, experiments were carried out on cells plated on glass coverslips coated with poly-ornithine. Fura-2 pentacetoxymethylester (fura-2 AM) was used as Ca\(^{2+}\) indicator. The cells were loaded by incubating (30min, 37ºC) in a physiological external saline solution (20mM Hapes, 150mM NaCl, 2mM CaCl, 5mM KCl, 0,9 mM MgCl and 0,1% BSA) and 1µM fura-2 AM. The digital fluorescence-imaging microscopy system was built around an inverted microscope (Zeiss Axiovert 135, Oberkochen, Germany) equipped with an intensified CCD camera (Hamamatsu Photonics, Hamamatsu, Japan). Loaded cells were alternatively excited at 340 and 380nm using a modified dual wavelength microfuotometer (Jasco CAM-230, Tokyo, Japan). Fluorescence images were collected with the CCD camera and the analog output was digitized and integrated in real time using an image processor. Image acquisition was performed at 4 frames/s. Calculations of ratio and the temporal plots of the fluorescence signal were calculated off line. The temporal plots, i.e. the variations in the mean value of fluorescence intensity, were calculated from ratio images in areas of interest. A temperature-controlled microincubator chamber (Medical System Corporation, Greenvale, NY, USA) maintained the temperature at 37ºC during the videoimaging experiments. Resting mast cells or other stimuli like histamine, mast cell release and sonicate, macrophage sonicate or 48/80 were gently applied to the bathing solution by loading appropriate volumes of concentrated solution into a 2ml syringe connected to the microincubator chamber via a small tube.

RESULTS

Myelin characterization
Compared to other membranes, myelin is unusual in that it has a low protein to lipid ratio and relatively simple protein composition. Proteolipod protein (PLP) and myelin basic protein together comprise 80-90% of total myelin proteins. Fig. 1 shows typical electrophoretic pattern of myelin proteins. Electroforesis was carried out on 10% polyacrilamid gel according to Leamli. The gel was stained with Coomassie Brilliant Blue. Lane 1, molecular weights.; lane 2, mice myelin vesicles (30µg of protein); lane 3, rat myelin vesicles (30µg of protein).

**Figura 1. Electrophoretic pattern of myelin proteins**

**Multilamellar and unilamellar myelin vesicles**
We have prepared unilamellar and multilamellar myelin vesicles using the protocol reported by Lin et al. (1986). Later we have performed different experiments of adhesion using both of this vesicles. As we have not noted any difference between these two types of vesicles we decided to use multilamellar vesicles, which are easier to prepare, for all the other experiments.

Figura 2. Electron microscopy analysis of multilamellar (left) and unilamellar (right) myelin vesicles.

Rat peritoneal mast cells

Mast cells were purified from rat peritoneal wash (see materials and methods) and characterized by electron microscopy and light microscopy (Diff Quik System).

Figura 3. Electron microscopy (left) and light microscopy (right) of mast cells

Mast Cell interaction with immobilized myelin
Fig 4a shows that rat peritoneal mast cells (RPMC) adhere to myelin coated wells (right panel), while the adhesion was weaker when the wells were coated with BSA (left panel). We quantized the extent of adhesion by biochemical means using chymase as mast cell marker and by referring to a calibration curve. Fig. 4b shows that mast cells adhered strongly on either rat or mouse myelin coated vesicles, but only weakly on uncoated wells or on wells coated with BSA, skim milk or neutrophil-membrane fraction. In the range of the amounts of myelin tested for coating the plate wells (10-50 μg/ml/well) the extent of cell adhesion was independent on the myelin amount used in the coating procedure and reached already the maximum value after 30 min of incubation. Of note, the myelin vesicles prepared from EAE mice induced an extent of adhesion which was not significantly different from that showed by the myelin isolated from normal mouse brains. RPMC showed a comparable extent (25 ± 5.0%) of adhesion on well coated with myelin vesicles prepared from human brain autoptic samples (Fig. 4c).

Rat myelin de-lipidation strongly reduced the adhesion of Mast Cells to the wells coated with modified myelin, suggesting that myelin allows mast cells adhesion through a component which can be washed out by organic solvents. Carbonate buffer treatment reduced adhesion. On the other hand adhesion remains on control level with boiled myelin and myelin treated with proteinase K (Fig.4d). During myelin purification oligodendrocytes are disrupted and the myelin derived vesicles could reseal both in inside out (ISO) or right-side out (RSO) conformation, and appear as multilamellar or unilamellar structures (see the “myelin characterization” paragraph). Multilamellar and unilamellar vesicles were separated and shown to exert un-distinguishable pro-adhesive properties for rat mast cells (not shown). Conversely RSO vesicles were pro-adhesive (40.2 ± 6.0%), while ISO had a very low pro-adhesive property (22.7± 3.0%) which was comparable to that of BSA-coated wells. Since RSO myelin-coated wells stimulate a RPMC adhesion which were not significantly different from that found for multilamellar myelin (shown in Fig.1b), we used the latter preparation, from rat brain, for all the other experiments if not differently indicated.

The interaction with myelin vesicles strongly stimulates mast cell secretory process evaluated by assaying free β-hexosaminidase activity in the myelin coated wells (Fig.4e) contemporaneously to the adhesion assay. Human PMN also adhere to rat and mouse myelin as well (Fig.4f), but their adhesion did not trigger the secretory process of the azurophilic marker myeloperoxidase (not shown).
Figura 4a

![Bar chart showing percentage of adhesion for different plate coatings: uncoated, BSA, milk, mouse myelin, and EAE myelin.](image)

Figura 4b

MC adhesion on myelin coated wells

![Bar chart showing percentage of adhesion for uncoated well, BSA, mPMN, mouse normal myelin, mouse EAE myelin, human normal myelin, and human MS myelin.](image)

Figura 4c
Fig.4. Morphological and biochemical evaluation of mast cells (MC) adhesion on myelin-coated wells. a. Light microscope appearance of BSA-coated (left) and myelin-coated (right) wells after RPMC addition. Diff-Quik staining system; original magnification 40x. b,c,d. Extent of RPMC adhesion on coated wells. Values are the percentage ± SD of at least ten experiments and were calculated from the RPMC number remaining adherent after washing the wells and were calculated from a calibration curve of chymase content; mPMN = human PMN membrane; milk = skim milk; PK = proteinase K e. Extent of RPMC secretion when in contact with coated wells. Values are the percentage ± SD of at least ten experiments and were calculated from the percentage of free β-hexosaminidase activity in the supernatant of the RPMC incubated within the coated wells, by referring to a calibration curve of this enzyme. f. Extent of human PMN adhesion on coated wells. Values are the percentage ± SD of at least three experiments and were calculated from the PMN number remaining adherent after washing the wells and were calculated from a calibration curve of myeloperoxidase content.

Mast cell interaction with myelin in suspension

With the aim of further confirming the mast cell myelin interaction with a more precise assay, the myelin vesicles were labelled with FITC and employed for cytofluorimetric (FACS) analysis after incubation with RPMC. Fig.5b shows that FITC-labelled myelin clearly sticks to RPMC in suspension after 30 min of incubation, thus confirming the data obtained by the amount of chymase remaining bound to the myelin coated wells after washing out non adherent cells. Having showed the strong myelin-mast cell interaction, we asked which receptor could be involved.

For this purpose we evaluated the role of β2 and β1 integrins which are known to be expressed by MC by using 60.3 antibody (α-β2) and peptide (RGD), a ligand for β1 integrins. We also tried to inhibit MC adhesion on myelin by the combining action of antibodies α-ICAM and 60.3. ICAM is ligand of β2 integrins and is expressed by astrocytes. It is still unknown if it is expressed by oligodendrocytes. The results show that integrins are not involved significantly in mast cell adhesion on myelin. Fig 5a

Since it is known that the scavenger receptors type A (SR/A type I/II) mediate the myelin macrophage interaction (Da Costa, C. C., et al., 1997) and “in vivo” data suggest its presence on
mast cells (Brown, J. M., et al., 2007), we analyzed if this receptor could also being involved in myelin-mast cell interaction.

Firstly we showed that the receptor is present on mast cell surface using immunofluorescence (Fig. 5c). We confirmed the presence of SR-AI/II on mast cells with FACS (Fig.5d), and by western immunoblotting analysis a strong signal was also found in RPMC membrane enriched fraction (lane 1), comparable to that found in rat peritoneal macrophages (RPMAC) lysate (lane 2, insert in fig 5d). For testing if this receptor could be responsible of myelin recognition, we challenged RPMC with FITC-labelled myelin vesicles and evaluated their interaction by FACS analysis after including in the assay medium blocking antibodies against SR-AI/II. As shown in Fig.5e the association of mast cells with myelin was strongly reduced under these experimental conditions. A stronger reduction of myelin binding to mast cells was found when fucoidan, a compound which is known to bind SR-A (Santiago-Garsia, J., et al., 2003) or the N-term peptide used to produce anti-SR-A antibodies, were included in the medium (Fig. 5f). Interestingly, while the increased secretion induced by myelin from RPMC, evaluated in the myelin-coated well model, was also inhibited by the blocking antibodies, fucoidan strongly stimulated the process (Fig.5g) suggesting that the two competitors act in a different way. The inhibition of RPMC-myelin interaction in the presence of fucoidan was confirmed by light microscope analysis (Fig. 5h).
**Figura 5b**

MC + Ab-SR  
MC + Ab-non specific

**Figura 5c**

Contol  
Secondary Ab  
Ab α-SR-A
Figura 5d

Ab α-SR-A

30 min of incubation

Figura 5e

binding of myelin-FITC (FACS)

Figura 5f

Figura 5g
Cytofluorimetric, biochemical and morphological evaluation of the interaction between rat myelin vesicles and MC free in suspension.

a. Attempt to inhibit mast cell adhesion on myelin coated wells with antibodies anti integrins and anti ICAM.

b. Cytofluorimetric quantitative analysis of RPMC association with FITC-labelled rat myelin vesicles. From left to right: thin continuous line delimiting gray peak = RPMC alone; thick line = 10 min incubation of RPMC with FITC-labelled myelin vesicles; thin line = 30 min incubation of RPMC with FITC-labelled myelin vesicles; at least 10,000 events were scored in each assay.

c. Presence of SR-A I/II on MC surface shown by immunofluorescence (left). On the right panel aspecific binding of the secondary Ab is shown. For more details see Matherials and methods.
d. Cytofluorimetric analysis of SR-AI/II presence on RPMC surface; From left to right: thin continuous line delimiting gray peak = RPMC alone; thin line = RPMC in the presence of FITC conjugated secondary antibody; thick line= RPMC with both anti-SR-A and FITC conjugated secondary antibody; at least 10,000 events were scored in each assay; Inset: western blotting analysis of SR-AI/II in RPMC (lane 1) and RPMAC (lane 2). 50 µg proteins were loaded.

e. Cytofluorimetric quantitative analysis of RPMC association with FITC-labelled rat myelin vesicles. From left to right: thin continuous line delimiting gray peak = RPMC alone; thick line =30 min incubation of RPMC with anti-SR-A antibody and FITC-labelled myelin vesicles; thin line =30 min incubation of RPMC with FITC-labelled myelin vesicles; at least 10,000 events were scored in each assay

f. Cytofluorimetric quantitative analysis of RPMC association with FITC-labelled vesicles; at least 10,000 events were scored in each assay. Values are the percentage of RPMC associated with FITC-labelled myelin in the presence of SR-A competitors, taken the total number of RPMC scored as 100%. *= p<0.05 (Student’s t analysis for paired data)

g. Evaluation of the extent of secretion from RPMC in contact with coated wells in the presence of SR-A competitors. Values are the percentage ± SD of at least three experiments and were calculated from the percentage of free β-hexosaminidase activity in the supernatant of the RPMC incubated within the coated wells, by referring to a calibration curve of this enzyme.

h. Left panel: morphological appearance of rat myelin vesicles-RPMC association in suspension. A cell population partial enriched in RPMC was used. Right panel: morphological appearance of rat myelin vesicles-RPMC association in suspension in the presence of 50 µg/ml fucoidan as SR-A competitor. Diff Quik staining original magnification x100.

i. Cytofluorimetric quantitative analysis of RPMC association with FITC-labelled rat myelin vesicles. From left to right: thin continuous line delimiting gray peak = RPMC alone; thick line =30 min incubation of RPMC with FITC-labelled myelin vesicles after the addition of Trypan blue 0.5 mg/ml (final concentration) as quenching agent; thin line =30 min incubation of RPMC with FITC-labelled myelin vesicles; at least 10,000 events were scored in each assay.
Myelin is ingested by mast cells and triggers superoxide production

In the cytofluorimetric analysis the trypan blue quenching showed that a significant part of mast cell interacting myelin vesicles were ingested in MC during the 30 min incubation (Fig.5i). This latter aspect of mast cell-myelin interaction was also documented at the transmission electron microscope (Fig.6a-f). Fig6a shows the appearance of the isolated myelin vesicles. Following myelin contact some mast cells emitted long and thin pseudopodia (Fig.6b) which could embrace and subsequently ingest the membrane vesicles (Fig.6c and d) and eventually imprisoned them in a tight phagosome (Fig. 6e). Granule secretion inside the myelin-containing phagosome and outside was also frequently observed (Fig.6d and f). Human neutrophils, on the contrary, while adhering on myelin coated wells did not ingest the myelin vesicle in suspension (not shown). We also found that RPMC exposed to myelin vesicles produced a lot of superoxide anion which was produced and released outside the cell. The amount of superoxide production was lower but comparable with that produced by PMA stimulated neutrophils, while higher than that produced by myelin stimulated macrophages. As expected, human neutrophils challenged by myelin did not produced superoxide (Fig.7).
Figura 6
Fig. 6. Transmission electron microscopy. Ultrastructural appearance of the interaction between rat myelin vesicles and MC free in suspension; bar = 1 μm.

a. Myelin vesicles alone; b-d early stages of myelin-RPMC association. Arrow in b shows embraced myelin vesicles; arrow in d shows a granule discharging its content in the phagosome forming around myelin vesicles; bar = 1 μm.

e. RPMC showing some engulfed myelin vesicles packed in a tight phagosome (arrow).

f. RPMCs undergoing compound exocytosis after being challenged with myelin vesicles.

Fig. 7 Superoxide production by RPMC, peritoneal macrophages and human PMN following rat myelin interaction, evaluated by superoxide dismutase inhibitable cytochrome C reduction. Values are expressed as nmoles ± SD (5 experiments) of superoxide produced and released from 10^5 MC/well in 10 min of incubation. White columns = resting cells; gray columns = myelin activated cells; black column = HPMN activated by PMA. RPMC = rat peritoneal mast cells; RPMAC= peritoneal macrophages; HPMN= human PMN. Rest = resting conditions; MyR= rat myelin;
Mast Cell adhesion on the oligodendrocyte surface

Having shown that RPMC can strongly interact with myelin we evaluated by light and ultrastructural analysis if they can also interact with whole oligodendrocytes (ODC). We prepared oligodendrocytes primary culture, whose characterization was performed by using lectin type II from Bandeiraea simplicifolia (Fig.8a) and antibody anti myelin basic (Fig.8c) protein which recognise mature oligodendrocytes. Lectin type II specifically recognise galactocerebrosides on oligodendrocyte membrane. A significant positivity for GFAP was not found in our primary culture (Fig. 8d). For more details see Materials and Methods. Figure 9 shows Light and Electron microscope appearance of oligodendrocytes primary culture. Fig.10a and 10b shows that resting mast cells bind the whole oligodendrocyte surface. Noteworthy, mast cells did not adhere on the ODC-free substrate of the culture slide and adhere only sometimes on the cell body, but very frequently on the long processes of ODC (Fig.10a,b). Fig.10c and 10d shows that also RPMAC were shown to be able to adhere on ODC monolayer, however the extent of their adhesion was lower than that of MC. Fig. 10e shows that the extent of adhesion of mast cells was not significantly increased by activating them with 48/80 compound but many secreted granules were seen free and stuck on the ODC surface. The ultrastructural analysis of RPMC-ODC interaction revealed that adherent resting mast cells can undergo single granule secretion (Fig.11), however only a small extent of secretion was measured by assaying free β-hexosaminidase release with respect to the myelin-coated wells model (not shown).

As for myelin vesicle-RPMC interaction, anti-scavenger receptor antibodies and the N-term peptide of SR-A, significantly inhibited the adhesive interaction evaluated either by morphological or biochemical means (Fig.12a,b,c).
Figura 8 . Characterization of oligodendrocytes primary culture. Oligodendrocytes were positive for FITC-lectin (upper left). Lectin specifically recognise galactocerebrosides on oligodendrocyte membrane. Mature oligodendrocytes are positive for myelin basic protein (MBP) but negative for aspecific astrocytes protein.
Figura 9. Oligodendrocytes primary culture from rat brain. Light microscope appearance, original magnification X400, Diff Quik staining (a, b). Scanning electron microscopy of oligodendrocytes; bar=2 µm, (c, d).
Figura 10. Electron and Light microscope appearance of oligodendrocytes (ODC) in culture exposed to either MC or peritoneal macrophages. a. and c. bar = 10 μm; b, d and e (original magnification x400, Diff Quik staining). a. and b. ODC monolayer exposed for 30 min to RPMC. Note that RPMC adhere mainly on the ODC processes. c. and d. ODC monolayer exposed for 30 min to RPMAC. Note that RPMAC adhere mainly on the ODC processes. e. ODC monolayer exposed for 30 min to RPMC in the presence of 10 μg/ml 48/80 compound. Note many RPMC granules adhering mainly on ODC processes.
Figura 11. Scanning electron microscopy. Higher magnification of ODC-adhering RPMC for showing the secretory process going on by single granule exocytosis; bar=2 μm;
Figura 12. Involvement of the scavenger receptor-A I/II in the interaction between MC and oligodendrocytes. a. many RPMC adhere on ODC monolayer; Diff Quik staining; original magnification x40; b. in the presence of anti-SR-AI/II a strong inhibition of RPMC adhesion on ODC monolayer is evident; Diff Quik staining; original magnification x40; c. quantisation of RPMC adhesion on ODC monolayer in the presence of SR-A competitors by assaying chymase content remaining cell associated after washing. The extent of adhesion was calculated by referring to a calibration curve of the enzyme content in RPMC. No significant chymase activity was detected in ODC. Values are the mean percentage adhesion ± SD of three experiments.
Cytoskeletal modification

Fig. 13b,d shows that ODC monolayer underwent a significant morphological alteration starting from 90 min after the addition of 30,000 RPMC with respect to untreated monolayer (Fig.13a,c). The effect was already detectable when 10,000 RPMC were added to the culture well (not shown). This alteration, observed in all the six experiments carried out, was characterized by a cell-body contraction leaving long and thin cell to cell connecting processes. The cell bodies and the nuclei appeared smaller with respect to untreated ODC. Noteworthy, the modification involved all the cells of the monolayer and not only those bearing adherent MC (Fig.13b,d) suggesting an effect of soluble factors released from RPMC. The same contraction of the ODC monolayer was obtained by adding to the ODC culture a mast cell sonicate, or the incubation medium of 48/80 stimulated RPMC (Fig.14b and c). Of note the relative amount of 48/80 alone was without effect on ODC (not shown). Fig.14d shows that pure histamine 0.1 mM induced the same effect. The morphological alteration of ODC could ascribed to cytoskeletal modification.

Fig. 15b,c,d show that ODC underwent a strong modification of both actin microfilament network and of interacting microtubules following RPMC adhesion (10, 20, 40000 RPMC added/well) with respect to untreated cells (Fig.15a). The actin network appeared packed around the nucleus, while the tubulin assembly appeared more diffused and abundant than in untreated cells where it was found arranged in well defined clusters along the structure of the microtubule network. Of note the extent of this effect on actin microfilaments was proportional to the number of MC added to the ODC culture starting from 10,000 MC/well and reached its maximum level at 30,000-40,000 RPMC/well.
Figura 13. Morphological alteration of the oligodendrocytes culture following exposure to $3 \times 10^4$ RPMC for 90 min at 37°C.  
a,c. Untreated ODC monolayer; Diff Quik staining; original magnification x250 (a) and x100 (c).  
b,d. ODC monolayer appearance after RPMC challenge; original magnification x 250 (b) and x100 (d). Note that an evident contracture causes the formation of thinner processes and of a smaller cell body as compared to untreated cells.
Figura 14. Morphological alteration of the oligodendrocytes culture following exposure to RPMC granule components. Diff Quik staining; a. Untreated ODC monolayer; original magnification x 100. b. ODC monolayer appearance after the addition of incubation medium derived from 48/80 activated $3 \times 10^4$ RPMC; original magnification x 100. c. ODC monolayer appearance after the addition of RPMC sonicate derived from $3 \times 10^4$ RPMC; original magnification x 100. d. ODC monolayer appearance after the addition of 0.1 mM pure histamine; original magnification x 100.
Fig. 15: Trichromic immunofluorescence analysis of oligodendrocyte monolayer exposed to MC. Red fluorescence: phalloidin-labelled filamentous actin; green fluorescence: α-tubulin; blue fluorescence: DAPI labelled nucleus. See Material and Methods section for more details. a. untreated ODC monolayer; original magnification x 250; b,c,d. ODC monolayer after 60 min incubation with 10,000 (b), 20,000 (c) and 40,000 (d) RPMC; original magnification x 250;
Mast cells can induce apoptosis in oligodendrocytes

Having shown that MC can induce a modification of microtubule assembly, which can trigger apoptosis in ODC (Mastronardi, F. G and Moscarello, M. A., 2005), we tested if after MC challenge ODC undergo programmed cell death. After 2 hours of incubation in the presence of MC, many ODC nuclei are caspase positive (Fig. 16b) and appeared smaller and more brilliant in DAPI staining (Fig. 16d) with respect to controls (Fig. 16 a,c).

![Figura 16. Bichromic immunofluorescence analysis of oligodendrocyte monolayer exposed to MC. Red fluorescence: phalloidin-labelled filamentous actin; green fluorescence: anti-cleaved Caspase 3 Asp175; labelled nuclei: blu fluorescence (DAPI). See Material and Methods section for more details. a,c. untreated ODC monolayer; original magnification x 250; b,d. ODC monolayer after 2 hours incubation with 20,000 RPMC; original magnification x 250;](image-url)
Mast cells are able to increase oligodendrocyte intracellular level of Ca$^{2+}$

In video imaging assay we observed that after only a few minutes of incubation with oligodendrocytes, resting mast cells are able to increase intracellular level of Ca$^{2+}$ (Fig. 17). For this “action” of mast cells it is not necessary that mast cells adhere on oligodendrocyte monolayer. Fig. 18 shows that also mast cell sonicate and mast cell incubation medium (48/80) is able to induce ODC response. Histamine as a one of components of mast cell granules induced the same phenomenon. Macrophage sonicate had no effect on oligodendrocytes (Fig. 18).

Figure 17. Videoimaging of oligodendrocytes
Figura 18. Percent of oligodendrocytes able to respond in the presents of different stimuli.

Figura 19. C-kit inhibitor Gleevec® (Novartis) is able to reduce symptoms in relapsing–remitting form of Experimental Autoimmune Encephalomyelitis
DISCUSSION AND CONCLUSIONS

The aim of this investigation was that of evaluating the effect of myelin vesicle and oligodendrocytes interaction with mast cells. We showed that myelin vesicles can interact with rat peritoneal mast cells (RPMC) and stimulate their functions. Firstly we found that RPMC adhere to myelin immobilized on plastic surface and, when in contact with it, secreted the content of their granules. Under these experimental setting we showed that the same number of RPMC adhered on multilamellar or unilamellar myelin coated wells, however when the pro-adhesive activity of ISO and RSO vesicles were tested we found that only the latter induced RPMC adhesion. This finding suggests that RPMC adhesion is stimulated through myelin components which are exposed on its surface and hence that RPMC adhesion is a specific process. The specificity of the reaction is also supported by the finding that RPMC did not adhere on wells coated with neutrophil membrane vesicles.

The cytofluorimetric analysis confirmed the interaction of mast cells with myelin vesicles free in suspension, thus making any methodological artefact very unlikely. Furthermore we showed that when mast cells come in contact with free myelin vesicles they produce a significant amount of superoxide, even higher than that produced by macrophages. We also show by ultrastructural analysis that RPMC can ingest myelin vesicles, even in the absence of opsonines, secrete into the phagosome single granule content and the content of many granules into the extracellular environment by compound exocytosis. Eventually the ingested myelin became imprisoned in a very tight phagosome. These findings suggest that myelin vesicles could be a strong stimulus for triggering mast cell activation consisting in oxygen derived free radicals production and granule enzymes release. Considering the already reported capacity of mast cells to present antigens to lymphocytes (Frandji, P., et al., 1993; Malaviya, R., et al., 1996) the myelin engulfing properties of mast cells can even enhance/trigger the autoimmune reaction in EAE. Human neutrophils (HPMN) cannot ingest free myelin vesicles in the absence of opsonine nor they release the content of their granules. The myelin-coated wells adhesion model confirmed that HPMN did not initiate a secretion process even if they adhere to myelin to an extent comparable to that of RPMC. The different outcome of HPMN-myelin interaction, besides to show once more the specificity of the myelin-RPMC association, clearly indicate that challenging the cell with free or immobilized myelin represent two different experimental models which can engage different cell receptors.

We actually proved that mast cells can interact also with oligodendrocytes. Resting RPMC can in fact adhere to oligodendrocytes monolayer and, after activation with 48/80, many
granules were found ODC associated. The finding that the heterotypic adhesion of whole mast cells took place mainly on ODC long processes and only sometimes on the cell body suggest that myelin, which is enriched in the cell processes (Bruce, D., et al., 2004), is mainly involved into this interaction and so, that the interaction is mostly specific. Scanning electron microscope observation revealed that adhering mast cells underwent single granule secretion, however in this experimental setting we failed to measure a strong release reaction as found with myelin vesicles free in suspension, suggesting that ODC can in some way limit the secretory process of mast cells as suggested to happen in mast cells surrounding some brain tumours (Theoharides, T. C., and Conti, P. 2004.).

At 60-120 min from RPMC addition to ODC culture, an evident contraction of the monolayer was found associated with a strong alteration of the cytoskeletal actin and tubulin assembly, which increased in extent by increasing the number of RPMC added (from 10,000 to 40,000). This unexpected morphological alteration was well evident in all the experiments carried out It could very likely depend on the release of granule content from RPMC since the incubation medium obtained after challenging these cells with a strong secretory stimulus had the same effect. Since also histamine induced this effect, it may be one and perhaps the most active compound in this sense. A cytoskeletal network modification is associated with the ODC contraction induced by RPMC. The actin network appears increased and embracing the nucleus, while the tubulin assembly appears less organized even if the well defined clusters which characterize untreated ODC analyzed by anti-α-tubulin, were still evident. At present we don’t know the underlying molecular mechanism of this cytoskeletal modification. We believe that it could be dependent on a transmembrane signal induced by a factor released from adhering RPMC. The finding that MC 48/80 released is able to increase intracellular level of Ca\(^{2+}\) in oligodendrocytes (videoimaging assay) supports this hypothesis. It has been shown that the cytoskeleton is important for myelin formation and maintenance and that targeting microtubule network in ODC initiate the apoptotic program in these cells (cited in Mastronardi, F. G., and Moscarello, M. A. 2005.), hence the strong alteration induced on the cytoskeletal structure of ODC by RPMC, suggests that these cells could be cytotoxic against ODC. Accordingly our findings showed that oligodendrocytes undergo to programmed cell death when incubated with RPMC. Even a vesiculation process is likely to be triggered or amplified following the cell contraction, which in turn could induce myelin vesicles diffusion in the surrounding environment. If this will be shown to be the case, than mast cell could not only represent a starting element for triggering EAE, but also an element of a positive feed-back mechanism.
which can amplify the ODC degeneration and vesiculation, giving raise to mtelin vesicleles capable of further MC activation.

Having shown that MC interacts with myelin we asked which receptor could be the candidate capable of mediating this interaction. A good candidate was the scavenger receptor type A, the receptor that in macrophages mediate the ingestion of myelin. After showing by western blotting and by cytofluorometric technique that SR-AI/II is expressed by MC, we showed that blocking this receptor, by specific antibodies or by N-term peptide of SR-A, resulted in a significant inhibition of RPMC-myelin interaction both in suspension and in coated wells. Furthermore also the MC degranulation was inhibited under these experimental conditions. As expected, the SRAI/II-competitor fucoidan was shown to inhibit the adhesion reaction, but at the same time strongly stimulated the secretory process in RPMC. Since we measured the adhesion reaction by the amount of chymase remaining well-associated after washing away non-adherent cells, so the strong secretory reaction, besides to confirm that MC bear on their membrane the SRAI/II, was unsuitable to show the competitive action of these compounds in myelin-coated wells model. The competitive action of fucoidan was however confirmed by both cytofluorimetric and morphological analysis. We also show by cytofluorimetric analysis that mast cells can ingest myelin vesicles in the absence of opsonines, thus confirming the ultrastructural findings. By exploiting the quenching activity of Trypan blue for FITC we found that most of the RPMC-associated myelin is ingested and that the engulfment process is inhibited by anti-SR-AI/II blocking activity. This finding, which cannot exclude that other receptors may be involved, indicate that SR-AI/II is required also for myelin ingestion. Scavenger receptor-A family is involved in cell adhesion, phagocytosis and very likely in triggering adaptive immunity (Santiago-García, J., et al., 2003; Greaves, D. R., and Gordon, S. 2005), all of these functions could be active in RPMC challenged with myelin suggesting that SR-A may be a good candidate for being considered a therapeutic target for hindering, in the early phase, the progression of autoimmune demyelinating diseases. However the subject deserves further investigation, since macrophages through SR-A can clear myelin debris and promote re-myelination. May be that SR-A can have two opposite effects: early it may trigger or amplify demyelination, while, lately it may favour the remission phase through improvement of macrophage-mediated myelin clearance.

The RPMC interaction with myelin appears to be mediated by a lipid dependent component since delipidation inhibits it. Accordingly to other papers on myelin structure analysis, changing its lipid structure can also affect the protein-lipid interaction (Lin, L. F., et
al., 1986) and possibly the availability of a putative myelin ligand for SR-AI/II of RPMC surface. So the myelin component responsible of the interaction with SR-AI/II remains to be identified.

In conclusion we put forward the hypothesis that in the pathogenesis of EAE and perhaps MS in man, a key role may be played by the interaction of mast cells with myelin which in various ways can trigger and enhance the immune reaction to myelin components and stimulate disease progression.

One possible scenario could be that myelin vesicles produced by olygodendrocytes via a vesiculation process determined by myelin instability can precede and strongly influence the progression of EAE/MS by stimulating mast cells functions. Mast cells can in turn increase the BBB permeability and allowing more (myelin) specific lymphocytes to reach the brain interior (Engelhardt, B., 2006; Archambault, A. S., et al., 2005) furthermore they can present myelin antigen to T lymphocytes and further enhance, through cytoskeletal modification and apoptosis, the process of ODC vesiculation.

Myelin could be a key factor in mast cell activation within CNS which could explain why W/Wv mice are protected from EAE, being mast cell deficient. However it was shown that reconstituting MC population in W/Wv mice cannot allow the recruitment of MC in the brain but allow anyway to re-establish the susceptibility to develop EAE (Tanzola, M. B., et al., 2003). Assuming that BBB cannot hinder the exit of myelin vesicles from the brain, after a degeneration process leading to ODC vesiculation, these myelin vesicles may be able to interact with mast cells even outside the SNC. Even in this condition mast cells can activate T lymphocytes in the lymph nodes and enhance the entry of these cells into the brain interior and hence EAE triggering.
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