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THESIS

THERAPEUTIC EFFICACY OF ADIPOSE-DERIVED MSC IN CHRONIC EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

S.S.D. MED/26

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ABSTRACT

Mesenchymal stem cells (MSC) represent a promising therapeutic approach for neurological autoimmune diseases; previous studies have shown that treatment with bone marrow-derived MSC induces immune modulation and reduces disease severity in experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis.

Here we show that intravenous administration of adipose-derived MSC (ASC) before disease onset significantly reduces the severity of EAE and decreases spinal cord inflammation and demyelination by immune modulation. ASC preferentially home into lymphoid organs, but migrates also inside the central nervous system (CNS). Most importantly, administration of ASC in chronic established EAE significantly ameliorates the disease course and reduces both demyelination and axonal loss, and induces a Th2-type cytokine shift in T cells. Interestingly, a relevant subset of ASC expresses activated α4 integrins and adheres to inflamed brain venules in intravital microscopy experiments. Bioluminescence imaging confirms that α4 integrins control ASC accumulation in inflamed CNS. After penetration within EAE lesions, ASC induce a significant increase of the number of endogenous oligodendrocyte progenitors. As for the mechanisms responsible for such effect, we found that ASC cultures produce vascular endothelial growth factor, insulin growth factor-I, basic fibroblast growth factor, brain-derived growth factor and platelet-derived growth factor-AB both in basal condition and after inflammatory stimulus. Interestingly, these molecules are all involved in the proliferation of both oligodendrocyte precursors and ASC themselves. In conclusion, we show that ASC display clear therapeutic effect by a bimodal mechanism, by suppressing the autoimmune response in early phases of disease as well

as by inducing local neuro-regeneration by activating endogenous progenitors in animals with established disease.

Overall our data suggest that ASC represent a valuable tool for stem cell-based therapy in chronic inflammatory diseases of the CNS.

INTRODUCTION

MULTIPLE SCLEROSIS

Etiology and pathogenesis of MS

Multiple sclerosis (MS) is a chronic inflammatory demyelinating autoimmune disease of the central nervous system (CNS) of unknown aetiology and heterogeneous clinical symptoms and course.

The most frequent symptoms are weakness and/or sensory disturbances in one or more limbs, optic neuritis, ataxia, bladder dysfunction, fatigue and cognitive deficits (O'Connor,'02). The most common clinical pattern, affecting about 80% of patients, encompasses relapsing and remitting clinical signs and symptoms at the beginning of disease (RRMS), which are characterized by acute attacks followed by complete or partial recovery and a lack of disease progression in between two relapses. After 10–20 years, the majority of these patients develop a progressive disease course (secondary progressive MS, SPMS) with creeping deterioration of mainly motor function of the lower limbs. Up to 15% of MS patients show disease progression from the beginning, which is defined as primary progressive MS (PPMS) and which most likely represents a slightly different underlying pathology with more diffuse demyelination and axonal damage and less inflammatory contribution (Weiner '04; Hafler et al.,'07; Debouverie et al., 2008) (Figure 1).

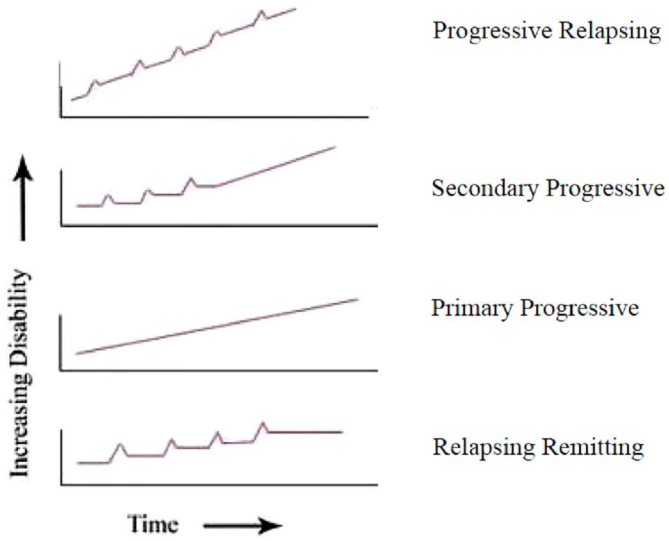

Figure 1: Clinical subtypes of MS

Much has been done to understand the aetiology of MS, with a major focus on the role of the immune system. The autoimmune reaction has been shown to involve the activation of both T and B lymphocytes infiltrating the brain and the spinal cord. In general, T cell responses are beneficial and protect us against pathogens; however, their unrestrained activation can be deleterious and cause autoimmune diseases.

Naive T cells differentiate into separate functional effector subsets specializing in producing cytokines in different combinations. Initially, two polarized subsets were described: T helper 1 (Th1) cells produce interferon-γ (IFN-γ), tumor necrosis factor-α (TNFα), and/or interleukin-2 (IL-2), and play a critical role in protection against intracellular pathogens. T helper 2 (Th2) cells, which instead produce IL-4, IL-5, IL-9, and IL-13, but not IFN-γ, are involved in protection against helminthes, as well as in allergic responses and modulating Th1-mediated inflammatory responses (Holmoy,'07; McFarland et al,'07).

A more recently described subset of $CD4^+$ effector Th cells has been named Th17, because of its ability to produce IL-17 in addition to other cytokines. Th17 cells

apparently protect against extracellular infections by gram-negative bacteria and fungi, partly by recruiting neutrophils. Differentiation of naive $CD4^+$ T cells into Th1 cells requires IL-12, IFNγ, or both, whereas Th2 cells are polarized by IL-4 in the absence of IL-12 and IFN-γ, and Th17 cells require IL-23 for their terminal differentiation. Recently, it has been identified a minor subpopulation of $CD4^+$ cells that selectively express forkhead box P3 (FoxP3) capable of preventing the development of autoimmunity, thus exerting a regulatory effect (Treg). Tregs seems to modulate both Th1 and Th2 effector responses during infection, as well as in models of autoimmunity and allergy; the impact of Tregs on Th17 responses in autoimmunity and infection requires more detailed study (O'Connor et al.,'09) (Figure 2).

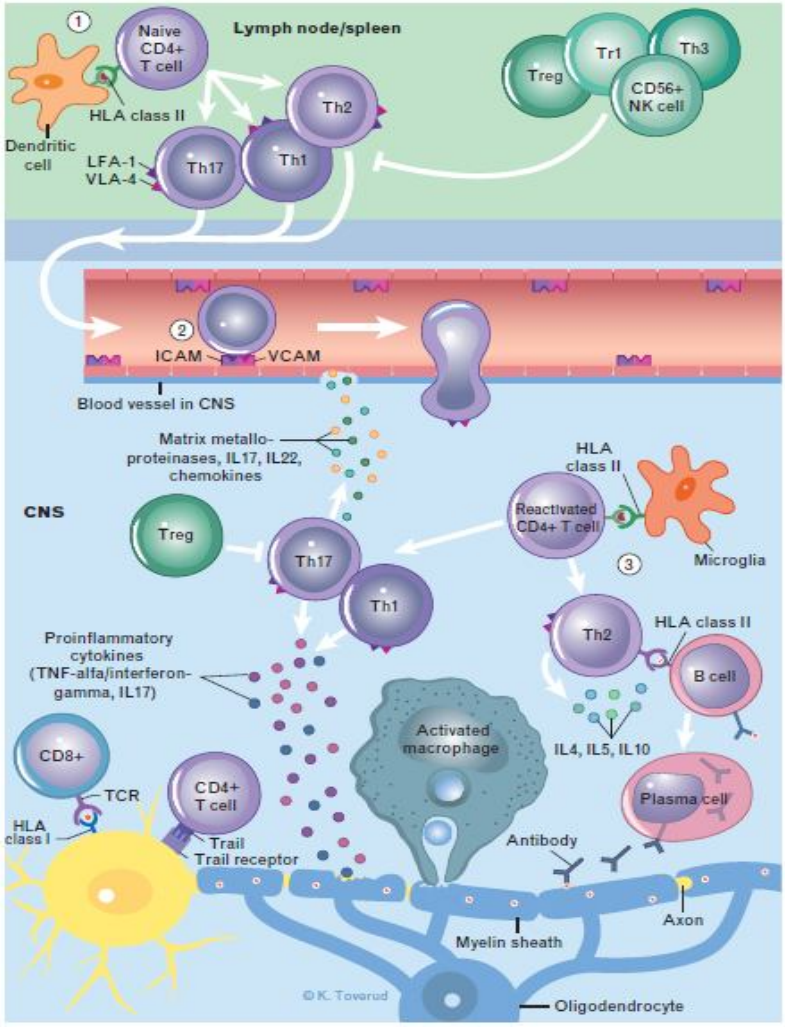

Figure 2: Immunopathogenesis of MS (Holmoy et al.,'08).

It is important to understand the individual contributions of the various T cell subsets to disease progression to define suitable targets for future therapies. The activation of $CD4^+$ autoreactive T cells are crucial events in the initial steps of the pathology: it was thought that acute lesions were initiated only by $CD4^+$ Th1, but mounting evidence now indicates that also Th17 cells play a significant role in MS pathogenesis; some studies have shown that Th1 and Th17 cells have been implicated in the animal model of MS, experimental autoimmune encephalomyelitis (EAE), and have also been detected in brains of MS patients, especially in acute lesions (Korn et al., 2007; Bettelli et al., 2008; Tzartos et al., 2008).

It has been suggested that myelin-specific auto-reactive IFN-γ secreting Th1 cells and/or IL-17 producing Th17 cells are primed in periphery by unknown factors (autoantigens or foreing, eg. viral), after which they migrate to CNS through ICAM-1, VCAM-1 and E-selectin mechanisms. Once antigen-specific T cells arrive in the CNS, they interact with local antigen presenting cells (APC) capable of presenting a MHC-peptide complex. In MS, there is reason to believe that the peptide presented by the local APC is a breakdown product of myelin and this molecular mimicry may trigger either an enhanced immune response against the bound antigen or down-regulation of immune response (anergy), depending on the type of signalling that results from the interactions between the surface co-stimulatory molecules and their ligands. In case of positive interaction, the activation leads to the production of pro-inflammatory cytokines such as IL-12, IFN-γ, TNF-α and IL-17, that results in the proliferation of the Th1 and Th17 cells and ultimately in immune-mediated injury to myelin and oligodendrocytes.

In case of anergy, T cells release a different subset of cytokines such as IL-1, IL-4 and IL-10, which counteract pro-inflammatory cytokines leading to the proliferation of anti-inflammatory $CD4^+$ Th2 cells. Th2 cells may send anti-inflammatory signals to local APCs and stimulate pathologic or repair-enhancing antibody-producing cells. The balance

between pro-inflammatory and immunomodulating cytokines is important in regulating disease activity and an imbalance favouring pro-inflammatory cytokines may result in final demyelination, axonal loss and subsequent neurological disability (Sospedra and Martin, 2005).

Damage of the target tissue, the CNS in our case, is, however, most likely mediated by other components of the immune system, such as antibodies, complement and factors produced by innate immune cells. The observation that IgG are elevated in the cerebrospinal fluid (CSF) of MS patients has been the most important and earliest evidence suggesting an involvement of B cells and antibodies in the pathogenesis of MS. The role of the humoral immune response in MS pathogenesis is supported by several observations: EAE studies have shown that B cells are involved in disease initiation (Raine et al., '99) and can also modify disease phenotype and severity (Svennson et al.,'02); moreover, high numbers of chronically activated B cells, plasma cells and increased IgM and IgG are present in the CSF and meninges of MS patients. Furthermore, B-cell clonal expansion and oligoclonal IgG production can be detected in brain plaques and CSF from patients with MS (Colombo et al.,'00).

Recent studies have suggested that the innate immune system also plays an important role both in the initiation and progression of MS by influencing the effector function of T and B cells (Weiner,'08). The effector cells, in turn, express cytokines and activation markers that further activate innate immune cells (Monney et al.,'02).

Dendritic cells (DCs) are professional antigen presenting cells that play an important role in promoting activation and differentiation of naïve T cells. The interaction of DCs with T cells is crucial in determining T cell differentiation into either effector T cells (Th1, Th2 and Th17 cells) or regulatory T cells (natural and induced Tregs cells). DCs can also affect NK cells, promoting pro-inflammatory T cell responses in MS and are also involved in determination of the RR and SP disease phases (Gandhi et al.,'09).

Microglial cells comprise 10–20% of glial cells and are the most common immune cells in the CNS. Microglial cells are considered resident macrophages of the nervous system, being involved in phagocytosis, antigen presentation and production of cytokines (Benveniste,'97). Microglial cells are rapidly activated in response to injury, neuro-degeneration, infection, tumors and inflammation. Microglial/macrophage cell activation contributes to MS and EAE pathology through antigen presentation with MHC-II and costimulatory molecules (CD83 and CD40) and secretion of pro-inflammatory cytokines. Persistent activation of microglial cells has also been observed in the chronic phase of relapsing-remitting EAE and a correlation has been observed between activated microglial cells and loss of neuronal synapses. In addition, macrophages and microglial cells are involved in demyelination and phagocytosis of the degraded myelin (Rasmussen et al.,'07).

Natural killer (NK) cells contribute to both effector and regulatory functions of the innate immune system via their cytotoxic activity mainly against viral infected cells or tumor cells and through their ability to secrete different cytokines (Moretta et al.,'08). Several reports have highlighted the importance of NK–DC interactions during the early stages of the immune responses (Ferlazzo et al.,'02). NK cells can stimulate DC maturation and increase the amount of DC-produced cytokines (Gerosa et al.,'02; Piccioli et al.,'02). However, the actual role of NK cells in CNS autoimmunity is still not clear.

Besides the critical role of immune system in MS pathogenesis, it remains to be elucidated how the immune attack leads to neuronal damage. Probably, once in the perivascular space, autoreactive T cells get reactivated by local APCs, and thereby gain access to CNS parenchyma through glia limitans. Pro-inflammatory cytokines released by activated T cells and activated microglia lead to further inflammatory cell

recruitment, finally resulting in an aggressive immune attack against the myelin sheath. Following myelin disruption, unprotected axons were affected by metabolic dysfunction and showed higher vulnerability to neurotoxic mediator, resulting in progressive and massive axonal degeneration (Herz et a.,'09).

Remyelination processes are in most cases insufficient, leading to irreversible disability. Different factors account for this repair deficit: local inhibitors of the differentiation of oligodendrocyte progenitor cells (OPCs) or a defect in the recruitment of OPCs toward the demyelinated area may be involved in inefficient remyelination (Piaton et al.,'09).

Although the disease course is highly variable, 50% of patients will not be able to walk independently within 15 years of onset. Current treatments for MS include immunomodulatory and immunosuppressive drugs. Interferon beta and glatiramer acetate reduce the number of relapses, but if these therapies are not successful or the disease develops into a progressive phase there are no effective treatments for modification of the course of the disease. Mitoxantrone showed the clinical progression of secondary progressive MS in a randomised clinical trial, although its long-term clinical effect is unknown. The limited effectiveness of these treatments justifies the assessment of alternative therapeutic strategies in patients with MS with aggressive clinical course (Blanco et al.,'05).

EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS (EAE)

Due to the limited availability of human brain tissue during active disease, especially in early disease stages, a broad variety of animal models have been developed to get a deeper understanding of the molecular mechanisms and kinetics of neuronal cell death and axonal degeneration during inflammatory autoimmune CNS disease.

EAE is possibly the best animal model to study autoimmune diseases and in particular demyelinating diseases of the CNS such as MS (Furlan et al.,'09).

Today the most common forms of EAE are:

1. active EAE induced by subcutaneous injection of an encephalitogenic peptide, mostly myelin oligodendrocyte glycoprotein (MOG)35-55 or proteolipid protein (PLP)139-151, which is emulsified in complete Freund's adjuvant (CFA) containing mineral oil and Mycobacterium tuberculosis, followed by intraperitoneal injection of pertussis toxin;

2. adoptive transfer EAE (AT-EAE) induced by intravenous injection of myelin-reactive CD4+ Th1 lymphocytes into naïve animals (Gold et al.,'06; Mix et al.,'08).

Depending upon the species, the antigen and the mode of sensitization, EAE may have a chronic relapsing (Figure 3 A) and a severe attack followed by incomplete recovery and a secondary progressive stage (Figure 3 B) course that mimics human MS.

The classical picture of acute EAE is characterized by perivascular inflammation mainly represented by $CD4^+$ and $CD8^+$ T lymphocytes, macrophages within the spinal cord and less consistently in the brain and extensive demyelination, axonal and neuronal damage.

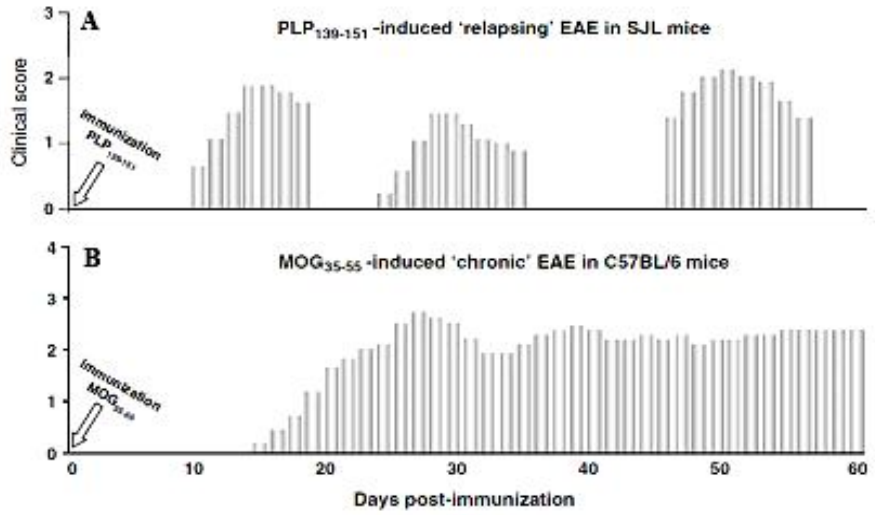

Figure 3: EAE clinical course (Furlan et al.'09)

The environment exerts a major effect on EAE, leading to the activation of autoaggressive T cells which consequently home to the brain and induce disease. Activation state is the necessary prerequisite for T cells to migrate through the blood-brain barrier (BBB) irrespective of their antigen specificity and the migration through the BBB requires the involvement of adhesion molecules on both T cells (LFA-1 and VLA-4) and endothelial cells (ICAM-1 and VCAM-1). Following migration, neuroantigen-specific $CD4^+$ T cells are reactivated in situ by fragments of myelin antigens presented in the framework of MHC class II molecules on the surface of macrophages and microglia.

These events are associated with the release of a vast amount of cytokines that could have a pro-inflammatory effect (TNF-α, TNF-β, IFN-γ and IL-12) and consequently a disease-promoting role, or anti-inflammatory cytokines (TGF-β, IL-10 and possibly IL-4), which may protect from disease (Furlan et al '09; Herz et al., '09).

This immunopathological scenario make chronic EAE a suitable model to test therapeutic approaches which target simultaneously both inflammation and neuro-degeneration.

The complexity of MS pathogenesis and the poor efficacy of approved therapies, challenged researchers to keep open minds and to develop future plans of attack from multiple fronts, as stem cells-based therapies.

STEM CELLS

Origin and characteristics

Stem cells have captured the popular imagination with the promise of enhanced tissue repair, the treatment of degenerative diseases and even the amelioration of dysfunction associated with normal ageing. Stem cells are defined as cells that have the ability to renew themselves continuously and possess the ability to differentiate into many cell types. It is important to do some distinction in the wide and heterogeneous world of stem cells: stem cells are not homogeneous but exist instead as part of a developmental continuum.

The most primitive of the cells, the embryonic stem cell, is totipotent. This cell has the potential to develop into a complete embryo, to form any type of cell, but this unique property is evanescent. It appears with fertilization of the egg and disappears by the time the embryo reaches the 4- to 8-cell stage. With subsequent divisions, embryonic stem cells lose the ability to generate an entire organism, however, they are capable of differentiating into cells present in all 3 embryonic germ layers, ectoderm, mesoderm, and endoderm, and on this basis are called pluripotent.

With subsequent divisions, cells become more and more restricted in their ability to differentiate into multiple lineages. They are then called multipotent and they are capable of forming a limited number of cell types. This is the property of adult stem cells, also referred to as somatic stem cells, which are able to self-renew during the

lifetime of the organism and to generate differentiated daughter cells. In the adulthood, tissues are in a perpetual state of flux under homeostatic conditions. Even in the absence of injury, they are continuously producing new cells to replace those that have worn out. For this reason, adult stem cells can be found in a metabolically quiescent state in most specialized tissues of the body, including the brain, bone marrow, liver, skin, and fat (Figure 4).

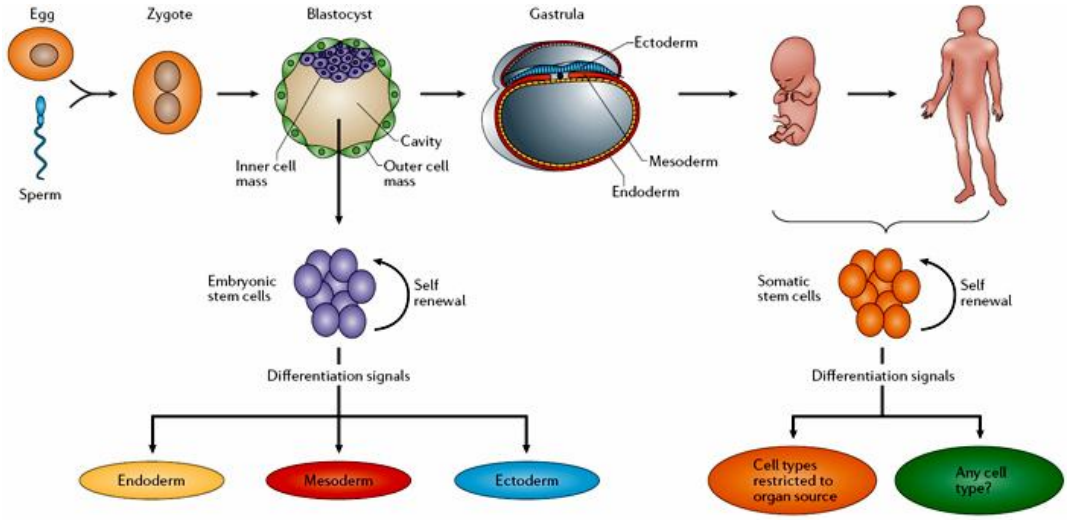

Figure 4: Different stem cells

Embrionic Stem Cells

In 1981, pluripotent cells were found in the inner cell mass of the mouse embryo, and the term "embryonic stem cell" was coined (Martin et al.1981; Evans and Kaufman, 1981). Embryonic stem cells (ESC) are derived from the blastocyst, the pre-implantation embryo that develops 5 days after the fertilization of an egg by a sperm. It contains all the material necessary for the development of a complete human being. The inner mass of the blastocyst is composed of 30-34 cells that are referred as pluripotent

because they have the potential to produce all of the body's cell types. ESC can be expanded in vitro for prolonged periods of time without loss of pluripotency.

The possible use of ESC in regenerative medicine is strongly limited, in addition to ethical considerations, by the observation that transplanted ESC lead to the induction of teratomas, formed by the uncontrolled growth of cells. Moreover, their clinical application is limited because ESC represent an allogenic resource and thus have the potential to evoke an immune response (Hipp et al.'08).

Foetal Stem Cells

An alternative source for ESC-based treatments may be represented by cells established from the foetus proper or from the supportive extra-embryonic structures (amniotic fluid, umbilical cord, Wharton's jelly, amnion and placenta) (foetal stem cells; FSC). Several studies indicate that stem cells derived from these foetal sources exhibit many features of ESC regarding the expression of stem-cell markers and their self-renewal capacity, while their spectrum of differentiation potential, either in vivo and in vitro, recapitulates features of plasticity residing between the pluripotent ESC and the multipotent adult stem cells. Moreover, FSC do not seem to form teratomas (Pappa et al.,'09). Among foetal tissues, umbilical cord blood has served as a source of hematopoietic stem and progenitor cells, including mesenchymal stem cells, for successful repopulation of the blood cell system in patients with malignant and nonmalignant diseases or for further expansion. Umbilical cord blood stem cells (UCB-SC) exhibit replating efficiency indicative of self-renewal potential, as well as multi-differentiation potential (Broxmeyer HE, et al.,'02).

Induced Pluripotent Stem Cells

Ethical difficulties regarding the use of human embryos, as well as the problem of tissue rejection following transplantation have induced researchers to look for a way to circumvent these issues: in 2006 Takahashi and Yamanaka demonstrated the possibility to generate pluripotent cells directly from the patients' own cells.

Induced pluripotent stem cells (iPS) were first produced from somatic mouse cells and in 2007 from somatic human cells, after retroviral transduction with the four transcription factors Oct3/4, Sox2, c-Myc and Klf4. The generated iPS were remarkably similar to naturally-isolated pluripotent stem cells: morphology, cell surface antigenic markers, genes expression and capability of differentiation into fully differentiated tissues were similar to ESC.

This has been cited as an important advancement in stem cell research, as it may allow researchers to obtain pluripotent stem cells, without the controversial use of embryos (Takahashi and Yamanaka,'06).

In addition to ESC, FSC and iPS, tissue specific stem cells could be isolated from various tissues of more advanced developmental stage:

Adult Stem Cells

More restricted stem cells can also be obtained from a variety of adult tissues and could be indicated as adult or somatic stem cells (ASC). ASC tend to be a tissue specific, self-renewing populations of cells which can differentiate into cell types associated with the organ system in which they reside; they are quite rare, on the order of 1 in 10,000 cells within the tissue of interest. Currently, it is known that niches of stem cells exist in many tissues, such as bone marrow, brain, liver, skin, skeletal muscle, the gastrointestinal tract, the pancreas, the eye, blood, fat and dental pulp (Hipp et al.,'08).

For many years, the general belief was that adult tissue-derived multipotent stem cells were developmentally restricted to the tissue where they reside. However, in the last years, several studies showed that some populations of adult stem cells endowed with the capacity to trans-differentiate into cells belonging to different germ layers, including neural cells (Bjornson et al.,'99).

Neural Stem Cells

Among ASC, multipotent neural stem cells (NSC) could represent an interesting tool in cell transplantation, especially in neurological disorders.

NSC are an heterogeneous population of immature, self-renewing, multipotent, progenitor cells of adult mammalian CNS capable of driving neurogenesis and gliogenesis throughout adulthood within certain specialized regions of the brain (i.e. the sub-ventricular zone (SVZ), the olfactory bulb and the dentate gyrus of the hippocampus), which therefore behave as highly specialized tissue niches (Figure 5) (Gage,'00).

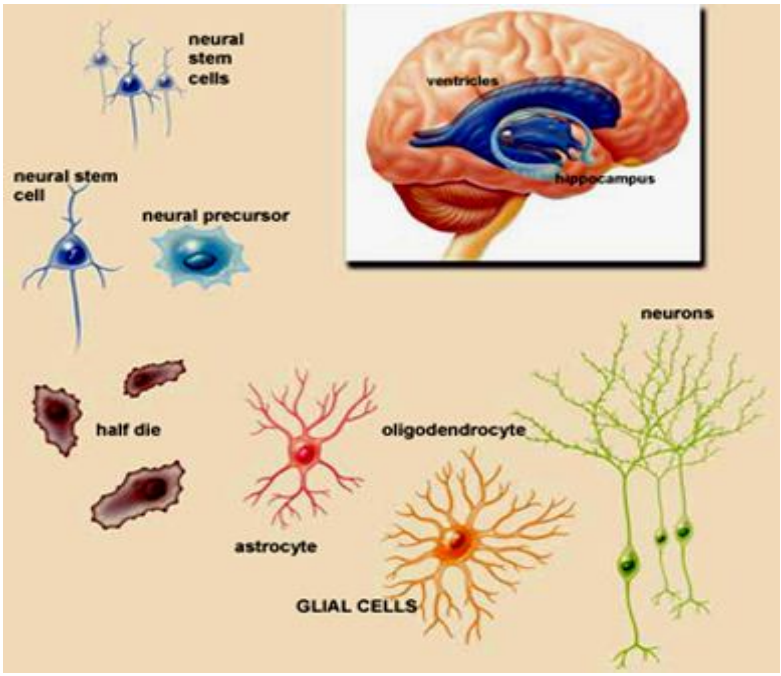

Figure 5: Neural stem cells; origin and differentiation.

Adult NSC can undergo extensive in vitro expansion upon epigenetic stimulation and possess the capacity to generate a progeny of differentiated functional daughter neural cells like neurons, oligodendrocytes and astrocytes (Kim et al.,'04).

It has been recently shown that endogenous NSC may sustain neurogenesis and gliogenesis in response to several different injuries such as those occurring during inflammatory, ischemic, or traumatic events (Picard-Riera et al.,'02). These pathogenic events might trigger a cascade of cellular and molecular signals – possibly via the release of soluble mediators (e.g. cytokines, chemokines, metalloproteases, adhesion molecules, etc.) – that are capable of supporting neurogenesis and gliogenesis that, in turn, drive brain repair. In vivo experiments aiming at repairing injured CNS by transplanting multipotent adult NSC, have shown that these cells might survive to transplantation procedures within the host CNS, can display notable migratory properties from the site of grafting due to the expression of VLA-4, the counter-ligand of VCAM-I, and maintain their multipotency. Notably, NSC transplants did not induce tumor formation in immunodeficient mice, thus strongly suggesting that the tumorigenic potential in vivo of such a potent cell source is minimal. However, transplanted NSC might exert their therapeutic effect not only by differentiating into lineage-restricted daughter cells but continuing to release neurotrophic growth factors. This latter evidence suggests that NSC might repair brain damage also remaining in their undifferentiated state and acting as bystander regulators of neuron and/or oligodendrocyte rescuing via the constitutive or induced release of neurotrophic molecules (Pluchino et al.,'03-'05). Thus, in vitro cultivated NSC represent a reliable cell source for transplantation experiments.

However, the invasiveness associated with harvesting of NSC and the small amount of cells that can be obtained may limit their clinical application. As the efforts to bring

experimental advances to the clinic are mounting, the source of these cells is becoming a crucial issue.

A potential alternative to NSC is represented by mesenchymal stem cells (MSC).

Mesenchymal Stem Cells

Historically, bone marrow (BM) has been considered an elective source of adult stem cells. The adult bone marrow contains a heterogeneous population of cells including hematopoietic stem cells, macrophages, erythrocytes, fibroblasts, adipocytes and endothelial cells. In addition to these cell types, the bone marrow also contains a subset of non-hematopoietic stem cells which posses a multilineage potential, representing 0,01-0,001% of total BM cells (Figure 6).

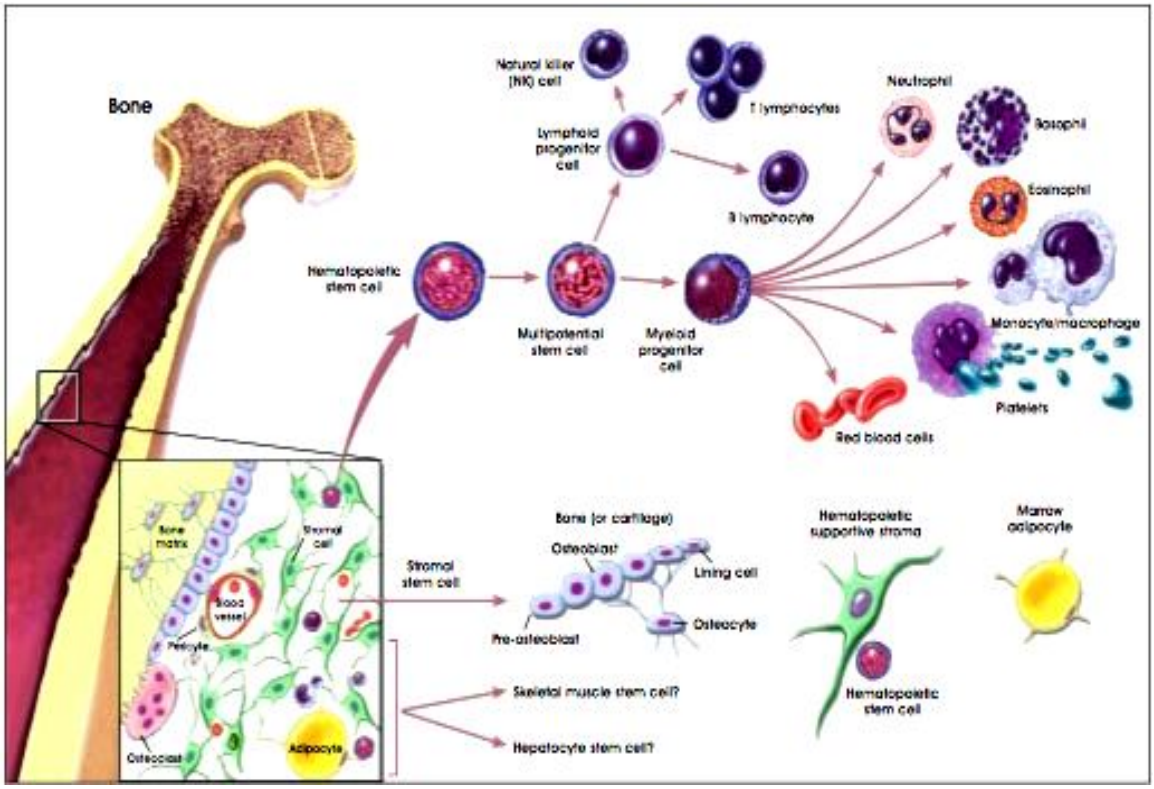

Figure 6: Bone marrow cells

The presence of this non-haematopoietic stem cells was first suggested by the observations of the German pathologist Clonheim 130 years ago. His work raised the possibility that BM may be the source of fibroblasts that deposit collagen fibres as part

of the normal process of wound repair. Evidence that BM contains cells that can differentiate into other mesenchymal cells, as well as fibroblasts is now available, starting with the work of Friedenstein and colleagues (Friedenstein et al.,'74). He described the isolation of large, polygonal, spindle-shaped, clonogenic cells in monolayer cultures, which he defined as colony-forming unit fibroblasts (CFU-Fs). CFU-F-derived stromal cells can serve as feeder layers for the culture of HSC and they can differentiate into adipocytes, chondrocytes and osteocytes both in vitro and after transfer in vivo (Prockop et al.'97).

MSC have now been isolated from various sites other than BM, almost from every type of connective tissue, and also in other tissues including, spleen, thymus, adipose tissue, amniotic fluid and so on.

Since a specific and unique marker has not yet been identified, MSC could be defined according to 4 criteria:

- MSC show he ability to proliferate in vitro as plastic-adherent cells;
- MSC express CD29, CD44, CD71, CD73, CD90, CD105, CD271 and Stro-1;

The variable expression level of these markers that has been observed probably arises from species differences, tissue source and culture conditions.

- MSC do not express the haematopoietic markers CD45, CD34 and CD14 and co-stimulatory molecules CD80, CD86 and CD40; in addition, MSC do not express HLA class II antigens and therefore transplantation into an allogenic host may not require immunosuppression.

In addition, another criteria to identify supposed MSC population, is their capacity to be induced to differentiate in vitro into chondrocyte, osteoblast and adipocyte (Dominici et al., '06).

The rationale for the use of MSC in cell transplantation therapy is the consideration that these cells couple an immunomodulatory activity with the ability to penetrate into damaged tissue, where they could release trophic factor or undergoing trans-differentiation resulting in directly regenerative effect. Probably the different relevance of these effects depends on the pathological conditions.

The immunomodulatory effect of MSC has only been recently described, following the observation that BM-MSC, the largely studied population of MSC, strongly inhibited T-cell responses to mitogens and alloantigens.

In vitro and in vivo studies indicate that BM-MSC exert their effect on T-cells activation, effector function and proliferation. Several studies have demonstrated BM-MSC exert their effect preferentially on T-cell proliferation: in the presence of MSC, virtually no cell division could be detected within 72 hours (Glennie et al., '05). BM-MSC induced the arrest of T cells in the G0/G1 phase of the cell cycle, but do not promote T-cell apoptosis. Inhibition of T-cell proliferation by MSC has been reported to lead to decreased IFNγ production both in vitro and in vivo and to increased IL-4 production by Th2 cells, which indicates a shift in T cells from a pro-inflammatory (IFNγ-producing) state to an anti-inflammatory (IL-4-producing) state.

It has also been shown that MSC have immunomodulatory properties impairing CD8+ T-cell cytotoxicity and directly inducing the proliferation of regulatory T cells, a specialized subpopulation of T cells that suppress activation of the immune system and thereby help to maintain homeostasis and tolerance to self antigens. (Uccelli et al.'06-'08; Tyndall et al.,'09).

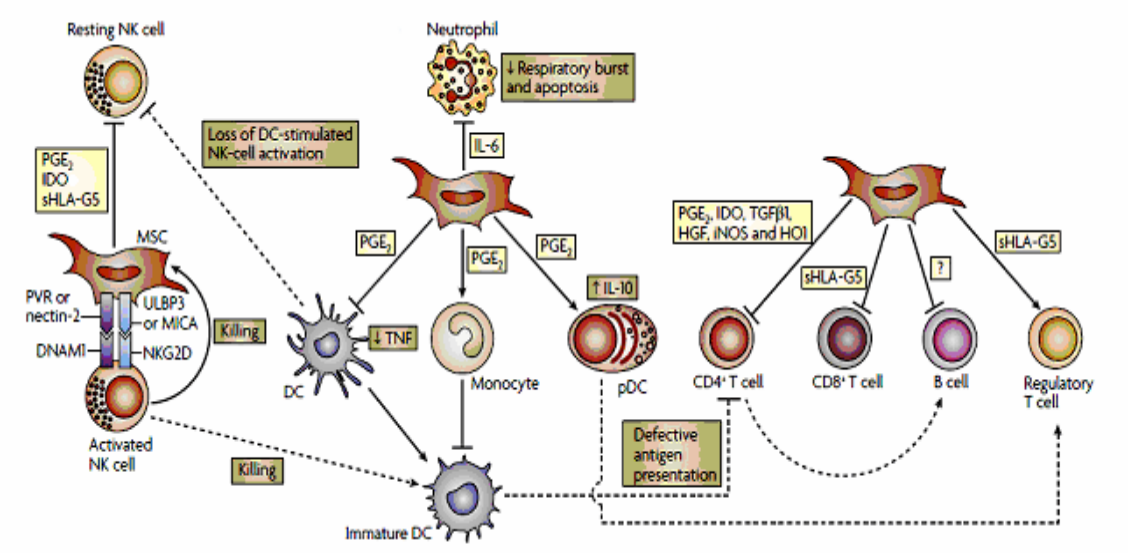

Figure 7: Immunomodulatory effect of BM-MSC (Uccelli et al.,'08)

As regards the interaction between BM-MSC and B cells, several groups have demonstrated that MSC inhibit in vitro human B cells proliferation, differentiation to antibody-secreting cells and chemotaxis (Corcione et al.,'06). However, other in vitro studies have shown that MSC could support the survival, proliferation and differentiation to antibody-secreting cells of B cells (Traggiai et al., '08). Probably, these different results may, at least in part, be accounted by different experimental conditions.

In addition to adaptive immune system, in the last years has been demonstrated that MSC exert immunomodulatory effect also on innate immune system.

Dendritic cells are the most potent APC and play a critical role in the initiation of primary immune responses. BM-MSC have been shown to inhibit the initial differentiation of monocytes into $CD1a^+$ DC and in case of mature DC, MSC were able to down-regulate the expression of co-stimulatory molecules CD80 and CD86 and decrease IL-12 secretion, thus impairing the antigen-presenting function of the DC. These data suggest that MSC might modulate the immune system, not only acting

directly on T cells, but also at the very first step of the immune response through the inhibition of DC (Jang et al.,'05).

The effect of MSC on NK cell-mediated cytotoxicity is controversial and related to different experimental approach. Few studies have demonstrated that the proliferation of IL-2 or IL-5 driven NK cells was inhibited by BM-MSC (Spaggiari et al., '06). But, consistent with the low level of surface expression of MHC-I, MSC were highly susceptible to NK-cell-mediated cytotoxicity and the incubation of MSC with IFNγ, which up-regulate the expression of MHC-I, partially protected them from NK-cell-mediated cytotoxicity. Finally, the interplay between MSC and NK cells in strictly related to microenvironment.

As regards the interactions between MSC and neutrophils, another important cell type of innate immunity, BM-MSC have been shown to dampen the respiratory burst and to delay the spontaneous apoptosis of resting and activated neutrophils through an Il-6-dependent mechanism (Figure 7) (Raffaghello et al., '08).

As regards the possible mechanisms of MSC-mediated immune modulation, several aspects have been evaluated. Soluble factors secreted by MSC, or by immune cells in response to MSC, and contact-dependent mechanisms has been proposed to collaborate for the MSC-mediated immune regulation. The interaction between MSC and their target cells involves cell-cell contact mediated by adhesion molecules as observed in several in-vitro studies. Moreover, soluble factors such as hepatocyte growth factor, prostaglandin E2, TGF-β1, indoleamine 2,3 dioxygenase (IDO), nitric oxide and IL-10 have been involved, while other factors remain unknown (Rasmusson et al.,'04; Aggarwal et al.,'05; Krampera et al.,'06).

The use of stem cells for tissue repair requires that cells can easily access to target organ, in this case the CNS, to exert their therapeutic effect. Several works have

demonstrated that MSC have the ability to home into damage brain migrating from the blood toward inflamed tissues where they had a protective effect (Vellieres ’03; Chen ’03; Phinney ’05; Mahmood ’06; Ruster ’06). Transplantation experiments have shown that MSC distribute to a wide variety of tissues following systemic administration and may be capable of participating in ongoing cellular turnover and replacement within an engrafted organ (Devine et al.,’03). Systemically administered MSC seem to preferentially home to the site of injury, where they support functional recovery.

There is much evidence to support the theory that MSC can home to injured or inflamed tissue involving migration across endothelial cell layers. The mechanism by which MSC can migrate and extravasate from the blood vessels is probably due to the expression by injured tissue of specific receptors or ligands to facilitate trafficking, adhesion and infiltration of MSC, as in the case of recruitment of leukocytes. Chemokine receptors and their chemokine ligands are essential components involved in the migration of immune cells into sites of inflammation, and it has been recently demonstrated that MSC also express some of these molecules. MSC show coordinated rolling and adhesion behaviour on endothelial cells through a p-selectin and VCAM1-dependent mechanism (Ruster et al.,’06; Chamberlain et al.,’07). In addition, MSC formed close contacts to the endothelial cells and were also able to integrate into the endothelial layer. Finally, they were able to leave the intact endothelium and finish their transmigration (Schmidt et al.,’06), thus showing that the systemic delivery of MSC could be a useful means to administer these cells in the clinic.

After the engraftment into damaged tissue, it has been proposed that MSC could support tissue repair through multiple mechanisms, as inhibition of scar formation, decrease apoptosis, promotion of angiogenesis, and stimulation of intrinsic cells to support the re-establishment of the complex neurological pathways (Chen et al.,’03; Caplan et al.,’06).

MSC may have anti-inflammatory and anti-proliferative effects on microglial cells and astrocytes, resulting in the induction of a neuroprotective microenvironment (Li et al.,'05; Lanza et al.,'09). In addition, the implantation of MSC could stimulate proliferation, migration, and differentiation of the endogenous precursors that survived as more differentiated cells. This effect is mediated by the release of trophic molecules like nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), vascular endothelial growth factor (VEGF), fibroblast growth factor 2 (FGF-2) and the polycomb family transcriptional repressor (BMI-1) (Munoz et al., '05; Zhang et al.'05; Rivera et al.,'06).

It has also been proposed that MSC could support tissue regeneration by direct trans-differentiation into cells of mesenchymal and non-mesenchymal lineages: several studies have demonstrated that MSC can also differentiate, under appropriate conditions, to form cells of various tissue lineages, including neurons (Brazelton et al.,'00; Mezey et al.,'00; Woodbury et al.,'00). In this regard, a large body of evidence has established the neural differentiation potential of MSC derived from BM. The treatment of BM-MSC with different molecules and growth factors induced very rapid morphological changes that are typical of neural cells together with the expression of neural markers, such as nestin, neurofilaments, MAP-2, or Neu-N (Winslet-Gendebien et al.,'05; Krampera et al.,'07). In addition, the data about the neuronal differentiation of A-MSC in terms of electrophysiological properties have provided not convincing results (Hung et al.'02): the use of chemical differentiation protocols, which promoted neural changes, could not support the acquisition of neuron-like excitability, which has been observed after co-culture with mature neural cells or transplantation into the brain (Wenisch et al.,'06).

All these features make MSC a suitable candidate for cellular transplantation in the regenerative treatment of a variety of diseases. Much attention has been paid to MSC-

based therapies for the treatment of autoimmune disease characterized by widespread degeneration and inflammation, like multiple sclerosis.

Previous studies have shown that BM-MSC were effective in ameliorating both chronic and relapsing-remitting EAE. In these studies, BM-MSC were injected before the onset of disease or on the day of clinical symptom onset and the main mechanism was related to the immune-suppression on autoreactive B and T cells, and large amounts of MSC have been detected in lymphoid organs where T-cell priming occurs. As regards their effects within the inflamed CNS, this is still a matter of debate and the mechanisms of BM-MSC contribution to neuro-regeneration in chronic EAE are not clearly understood. In fact, murine BM-MSC do not express α4 integrins, and the study of BM-MSC homing in EAE lesions has provided conflicting results regarding the capacity of BM-MSC to migrate into the inflamed CNS (Zhang et al.,'05; Zappia et al.,'05; Gerdoni et al.,'07; Kassis et al.,'08).

More recently, human BM-MSC have been shown to be effective on chronic EAE, by inducing Th2-polarized immune response, reducing IFN-γ and Th17 producing cells and promoting oligodendrogenesis and inhibiting astrogliosis in EAE (Bai et al.,'09). Interestingly, recent data show that BM-MSC inhibit key mechanisms responsible for EAE induction by a paracrine conversion of CCL2 chemokine from agonist to antagonist of the Th17 cell function (Rafaei et al.,'09). Although BM has been the main source for the isolation of multipotent MSC, the harvest of BM is a highly invasive procedure and the number, differentiation potential, and the maximal life span of MSC from BM decline with increasing age. Therefore alternative sources to isolate MSC are subject to intensive investigations.

Adipose Stem Cells

In the last years, much attention has been paid to adipose-derived mesenchymal stem cells (ASC) because adipose tissue is an abundant and appealing source of donor tissue. The adipose tissue is a highly complex tissue and consists of mature adipocytes, preadipocytes, fibroblasts, vascular smooth muscle cells, endothelial cells, resident monocytes/macrophages, and lymphocytes. The stromal-vascular cell fraction (SVF) of the adipose tissue has come more and more into the focus of stem cell research, since this tissue compartment provides a rich source of pluripotent adipose tissue-derived stem cells.

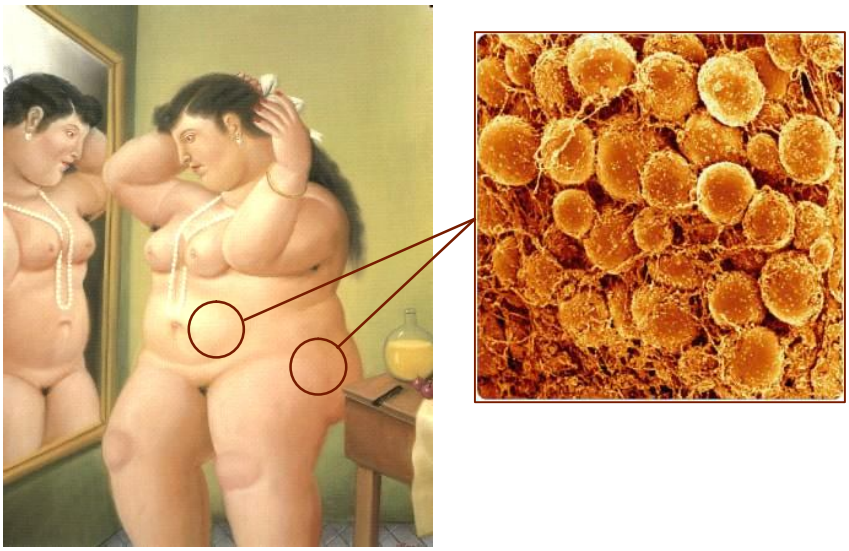

Figure 8: Adipose tissue

ASC were firstly identified by Zuk and co-workers in 2001, who isolated a population of stem cells, termed processed lipoaspirate cells (PLA), from human lipoaspirates. They characterized phenotype, heterogenicity and growth kinetics of PLA and found that PLA were capable of multiple mesodermal lineage differentiation, as shown by the expression of several lineage-specific genes and proteins, and in addition could also be induced to express markers consistent with a neurogenic phenotype, suggesting an ectodermal potential (Zuk et al.,'01-'02).

The simple surgical procedure, donor tissue is isolated under local anesthesia with a minimum of patient discomfort, the easy and repeatable access to the subcutaneous adipose tissue and the uncomplicated enzyme-based isolation procedures make this tissue source for MSC most attractive. Furthermore, cultured ASC seem to display an increased in vitro proliferative potential compared with BM-MSC, and could generate a clinically effective cell dose more rapidly than the same number of marrow cells: 1g of adipose tisse yields approximately $5x10^3$ stem cells, which is 500-fold grater than the number of stem cells isolated in 1 g of bone marrow (De Ugarte et al, 2003; Fraser et al, 2004; Mitchell et al.,'06). In addition, ASC can be obtained in large quantities due to the quick proliferation kinetics and slow senescence ratio in vitro (Lin et al.,'08). Therefore, ASC do represent an alternative source of autologous adult stem cells that can be obtained repeatedly in large quantities.

Most importantly, a comparative analysis of MSC obtained from bone marrow and adipose tissue clearly showed that ASC were not different regarding morphology, immune phenotype, success rate of isolating MSC, colony frequency, and differentiation capacity (Kern et al.,'06; Schaffler et al.,'07).

As regards the cell surface marker expression, a detailed analysis showed that ASC, like BM-MSC, expressed CD13, CD29, CD44, CD58,CD90, CD105, CD73 and Stro-1 and were negative for endothelial and hematopoietic markers. However, certain key differences in surface marker expression between marrow and adipose tissue-derived cells were also detected. Interestingly, each of these differences was in the expression of adhesion molecules with known function in homing and mobilization of hematopoietic stem cells. Thus, ASC expressed CD49d (α4 integrin) which forms an heterodimer with CD29 to create very late activation antigen-4 (VLA-4) while cells derived from bone marrow did not. Unlike BM-MSC, very low expression of CD106 (VCAM-I) was observed in ASC samples. Expression of CD106 in BM-MSC is

functionally associated with hematopoiesis and the low level of CD106 on ASC is consistent with the localization of these cells to a non-hematopoietic tissue (Zuk et al.,'02; DeUgarte et al.,'03).

In addition to phenotype, also immunomodulatory properties of ASC are similar to BM-MSC. In this respect, several studied have demonstrated that ASC did not generate in vitro proliferation of allogeneic T cells and suppressed the lymphocyte proliferative response to mitogens and alloantigens in a dose-dependent manner. This inhibition persisted when ASC were separated from lymphocytes by a permeable membrane thus suggesting the involvement of soluble factors in addition to cell-cell contact mechanisms. Moreover, has been observed an inhibition of the production of inflammatory cytokines (TNFα, IFNγ, and IL-12) of T cells stimulated by nonspecific mitogenic and by allogeneic stimuli (Puissant et al.,'04; Yanez et al.,'06; McIntosh et al.,'06).

Another feature required for the use of ASC in cellular therapy is the ability to migrate and access to damaged tissue. It has been demonstrated that ASC, after intravenous, intra-peritoneal or sub-cutaneous infusion, were able to migrate to a wide range of tissues, including lungs, lymphoid organs, CNS and liver, and persist up to 75 days. No evidence of clonal expansion has been identified within any organ examined (Meyerrose et al.,'07).

Similar to other stem cells population, ASC have shown to differentiate not only into osteoblasts, adipocytes and chondrocytes, but also, ASC can be induced to differentiate into endodermic and neuroectodermic lineages.

Type of differentiation	Differentiation factors
Adipogenic	Insulin, IBMX, dexamethasone, rosiglitazone, indomethacin
Chondrogenic	BMP-6, BMP-7, FGF-2, TGF-β_1, TGF-β_2, TGF-β_3, dexamethasone, IGF-1
Osteogenic	$1,25(OH)_2D_3$, β-glycerophosphate, ascorbic acid, BMP-2, dexamethasone, valproic acid
Myogenic differentiation	Specific microenvironment?
Cardiomyogenic differentiation	IL-3, IL-6, SCF
Vascular/endothelial	Specific microenvironment?
Neurogenic	Valproic acid, insulin, hydroxyanisole, hydrocortisone, EGF, FGF
Pancreatic/endocrine	Activin-A, exendin-4, pentagastrin, HGF, nicotinamide, high glucose concentration
Hepatic	HGF, OSM, DMSO
Hematopoietic	Specific microenvironment?

Abbreviations: $1,25(OH)_2D_3$, 1,25-dihydroxy-cholecalciferol; BMP, bone morphogenetic protein; DMSO, dimethyl sulfoxide; EGF, epidermal growth factor; FGF, fibroblast growth factor; HGF, hepatocyte growth factor; IBMX, 3-isobutyl-1-methylxanthine; IGF, insulin-like growth factor; IL, interleukin; OSM, oncostatin M; SCF, stem cell factor; TGF, transforming growth factor.

Table 1: ASC transdifferentiation media (Schaffler et al.,'07)

In this regard, several groups have demonstrated that under exposure to particular chemical medium, ASC could be induced to differentiate into specific cell lineages, such as neurons, endocrine pancreatic cells, hepatocytes, endothelial cells, and cardiomyocytes, thus showing a trans-differentiation potential (Woodbury '02; Safford et al.,'04; Schaffler '07).

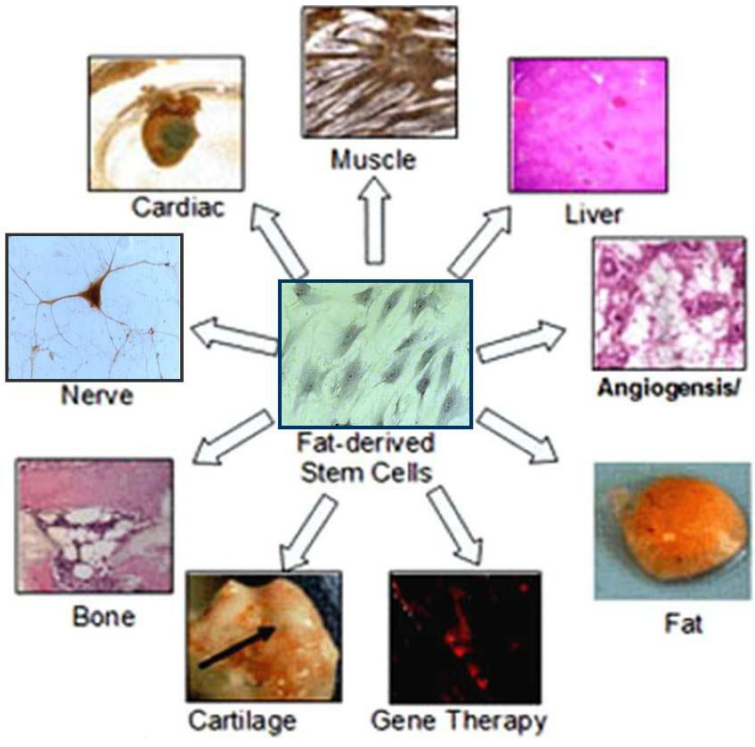

Figure 9: Trans-differentiation potential of ASC

As far as neural trans-differentiation is concerned, several studies have shown that using a complex neurogenic differentiation protocol with retinoic acid and BDNF, both murine and human ASC develop a neuronal morphology and immunocytochemical profile of neuronal cells, including the expression of TH and GABA-A. Electrophysiological properties are also suggestive of initial neuronal differentiation: differentiated ASC displayed resting membrane potential close to –60 mV, delayed-rectifier type K^+ currents, as well as voltage-dependent Na^+ currents absent in basal conditions; however, these electrophysiological properties were not enough to fire an action potential (Krampera et al.,'07; Anghileri et al.,'08). However, this trans-differentiation potential may be relevant for neuroregeneration.

Based on these features, the abundant and practical donor tissue, immunophenotype, immunomodulatory properties and trans-differentiation potential, ASC represent an highly promising tool for cell therapy and tissue engineering.

AIM OF THE STUDY

Multiple sclerosis and its animal model, EAE, are autoimmune disorders targeting the CNS, where the inflammatory response leads to demyelination and axonal degeneration. Chronic EAE is a suitable model to test therapeutic approaches which target simultaneously both inflammation and neuro-degeneration. MSC represent a promising therapeutic approach for neurological autoimmune diseases because they can differentiate in vitro into multiple mesenchymal and non-mesenchymal lineages and can efficiently induce the proliferation, migration and differentiation of neural endogenous progenitors through the secretion of neural growth factors, thus representing an attractive therapy for regenerative medicine (Munoz et al.,'05). In addition, MSC have relevant immune modulatory effects both in vivo and in vitro, as they can suppress many functions of immune cells (Uccelli et al.,'08).

Among MSC, ASC represent a very attractive tool due to the abundant and easily accessible donor tissue. Moreover, ASC display multi-lineage plasticity in vivo an in vitro, have a much higher frequency in the adipose tissue than in the bone marrow and exerted immunomodulatory activity comparable to BM-MSC (Puissant et al.,05). In addition, a subpopulation of ASC expressed VLA-4 that plays a key role in the migration of cells into the inflamed CNS in EAE/MS (Pluchino et al.,'03). Finally, several groups including ours have found evidence for neural trans-differentiation of ASC in vitro (Anghileri et al.,'08), a property which may be relevant for neuroregeneration.

On the basis of these considerations, we decided to investigate the efficacy of ASC in chronic EAE with a preventive and a more interesting therapeutic treatment protocol, focusing the attention on clinical and histological effects, homing and persistence of ACS into CNS as well as the molecular mechanisms mediated by injected cells.

MATERIALS AND METHODS

EAE induction and treatment protocols

Chronic EAE was induced in 6-8 weeks old C57Bl/6 female mice purchased from Harlan Italy (S. Pietro di Natisone, Italy), by subcutaneous immunization with 200 µg of MOG_{35-55} peptide in incomplete Freud's adjuvant containing 0.8 mg/ml Mycobacterium Tuberculosis, as previously described (Constantin et al., 1999). Pertussis Toxin (50ng; Sigma Aldrich, Milan, Italy) was injected at the day of immunization and after 48 hours. Body weight and clinical scores were daily registered according to a standard 0-5 scale: 0 = healthy, 1 = limp tail, 2 = ataxia and/or paresis of hindlimbs, 3 = paraplegia, 4 = paraplegia with forelimb weakness or paralysis, 5 = moribund or death animal (Figure 10). All animals were housed in pathogen-free conditions. The experiments received authorization from the Italian Ministry of Health, and were conducted following the principles of the NIH Guide for the Use and Care of Laboratory Animals, and the European Community Council (86/609/EEC) directive.

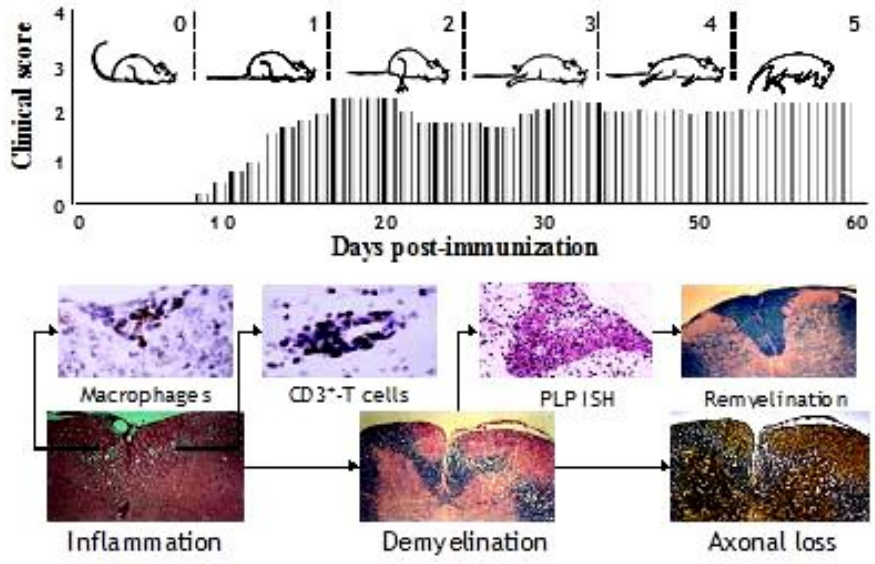

Figure 10: EAE clinical and histological evaluation

Mice developed the first clinical signs of EAE at 12.7 ± 1.3 days post immunization (dpi), reached a peak at 14.0 ± 1.2 dpi and then presented a stable disease course, typical of this chronic model. EAE is characterized by extensive perivascular inflammation mainly represented by $CD4^+$ and $CD8^+$ T lymphocytes and macrophages within the spinal cord and less consistently in the brain. Inflammatory events are associated with widespread demyelination and progressive axonal loss (Figure 8) .

To evaluate the clinical and pathological efficacy of ASC in chronic EAE, cells were administered with two distinct protocols: preventive and therapeutic. In the preventive set of experiments, ASC (either from wild type mice or from animals transgenic for the GFP fluorescent protein) were injected intravenously (iv) at 3 and 8 days post-immunization (dpi), i. e. before the disease onset. In the therapeutic protocol, ASC were administered iv after the peak of disease severity, when the clinical score was stable (23 and 28 dpi). For each injection, $1x10^6$ MSC were resuspended in 1 ml of PBS without Ca^{2+} and Mg^{2+} and injected through the tail vein; each iv administration was divided in two injections of $0.5X10^6$ ASC in 0.5ml vehicle. Control mice received only vehicle.

Mesenchymal stem cell cultures

Murine ASC were obtained from 6-8 weeks old C57Bl/6J mice, as well as from C57Bl/6-Tg(UBC-GFP)30Scha/J mice expressing the GFP fluorescent protein. The isolation of stromal-vascular fraction from adipose tissue was carried out on 10 ml of lipoaspirates, as previously described (Anghileri et al., 2008). Briefly, after washing in HBSS, extracellular matrix was digested at 37°C with collagenase A (Sigma), centrifuged at 1200g and the pellet was resuspended in NH_4Cl; the stromal fraction was then collected by centrifugation and filtration. Murine BM-MSC were collected by flushing femurs and tibias with medium and filtered as previously described (Krampera

et al., 2003; Krampera et al., 2007). Cells were then cultured in DMEM, glucose, GLUTAMAX I^{TM}, 15% heat-inactivated adult bovine serum (Celbio), penicillin and streptomycin (Invitrogen Corp. Carlsbad, CA). After 72 hrs, non-adherent cells were removed. When 70-80% adherent cells were confluent, they were trypsinized, harvested and expanded by using culture medium with 50ng/ml HB-EGF (R&D System, Minneapolis, MN). All the experiments were performed using MSC at 17-23 passages.

MSC immunophenotyping and adhesion molecules expression

Murine MSC were recognized by immunophenotype using mAbs specific for CD106 (VCAM-1), CD9, CD44, CD80, CD138 and Sca1. In addition, the absence of haematopoietic markers (CD45, CD11c and CD34) and endothelial markers (CD31) was assessed as previously described (Krampera et al., 2003). All mAbs were purchased from Pharmigen/Becton Dickinson (Palo Alto, CA, USA). For the study of adhesion molecule expression, ASC were labelled with fluorescent antibodies for α4 integrins (PS/2 clone, kindly provided by Dr. Eugene Butcher, Stanford University), LFA-1 (anti-aL-chain; clone TIB213 from American Type Culture Collection/ATCC, VA, USA), PSGL-1 (clone 4RA10, kindly provided by Dr. Dietmar Vestweber, Max Plank Institute, Germany), L-selectin (Mel-14 clone, ATCC) and CD44 (IM/7 clone, ATCC). Isotype-matched antibodies were used as controls. For immunophenotypic analysis and adhesion molecule expression, MSC were detached using trypsin/EDTA (Sigma), washed and resuspended at 10^6 cells/ml. Cell suspension was incubated with 15% adult bovine serum, followed by incubation with the specific mAbs at 4°C for 30 minutes. At least 10,000 events were analyzed by flow cytometry (FACScalibur, Becton Dickinson) using the Cell Quest software.

ImageStream data acquisition and analysis

ASC were prepared for immunostaining as described above and then incubated with 10 μg/ml of anti-α4 integrins mAb for 30 min on ice. After washing, cells were stained with goat anti-rat IgG-PE conjugated (Caltag Laboratories). Stained cells were re-suspended in PBS and images were acquired on the ImageStream® imaging cytometer System 100 (Amnis Corporation, Seattle, WA, USA). Images of fixed cells were collected and analyzed using ImageSteam Data Exploration and Analysis Software (IDEAS) (George et al., 2006). α4 integrin clustering was evaluated analyzing the distribution on the cells surface of the fluorescence pattern. Uniform (uniform distribution of fluorescence), Clustered (small spots of fluorescence) and Caps (big clusters of fluorescence) cells were gated using the Area feature Vs the Delta Centroid XY feature (Bolomini-Vittori et al., 2009). The area feature was calculated for channel 4 (PE-specific emission; area of fluorescence), applying to the images a Threshold mask, previously created; this feature allowed us to discriminate between cells with larger fluorescence area (high area values) and smaller fluorescence area. The Delta Centroid (DC) XY feature calculates the distance between the center of the PE fluorescence image and the center of the brightfield image for each image pair. This feature distinguished images with globally distributed staining (lower DC values) from those with capped staining (higher DC values). When plotted versus the Area feature, DC XY permits to distinguish between punctate and uniform staining at the same time (Bolomini-Vittori et al., 2009). In our analysis, cells with Area values higher than 600 and Radial Delta Centroid values lower than 16 were considered "Uniform" cells for their fluorescence distribution. Cells with Area values lower than 600 and Radial Delta Centroid values lower than 16 were considered "Clustered" cells (small spots of fluorescence). Cells with Radial Delta Centroid values higher than 16 were considered "Caps" cells (highly polarized fluorescence).

Proliferation assays

MOG_{35-55} activated T cells were obtained from draining lymph nodes of mice immunized with antigen as previously described (Constantin et al., 1999). $CD4^+$ T cells ($2x10^5$/well) were co-cultured with 5,000, 2,000 and 1,000 irradiated ASC in 96-well microtiter plates in the presence of 30 mg/ml antigen peptide and $8x10^5$ APC (irradiated splenocytes) for 3 days. [^{3}H] thymidine (1mCi) was added in each well 18h before the end of cultures. [^{3}H] thymidine uptake was determined in a Microplate Scintillation Counter and expressed as counts per minute (CPM). In separate experiments we studied the ex vivo proliferation of T cells from peripheral lymph node cells isolated from mice immunized with MOG_{35-55} peptide and treated or not with ASC. Total draining lymph node cells were isolated 7 days after disease onset from mice treated with ASC in the pre-clinical phase of disease (3 and 8 dpi). Total draining lymph node cells were isolated at the end of the experiment (72 dpi) in mice receiving ASC after disease stabilization (at 23 and 28 dpi). For all ex-vivo proliferation experiments, $1x10^6$ cells/well were cultured in 96-well microtiter plates in the presence of 10-30 μg/ml MOG peptide or 1 μg/ml anti-CD3 plus 2 μg/well anti-CD28mAb. After 72 hrs of incubation, cultures were pulsed for 18 hrs with 1 μCi per well of [H^3]-thymidine, and proliferation was measured from triplicate cultures.

ELISA and Bio-Plex assays

Supernatants from lymph node cells isolated from mice treated with ASC in the pre-clinical phase of disease or after disease stabilization, or derived from in vitro co-cultures between MOG_{35-55} activated T cells and ASC were used for Bioplex cytokine assays (BioRad), following manufacturer's instructions. Briefly, anti-cytokine conjugated beads were plated in 96-well microtiter plates and then removed by vacuum filtration. Samples were then added in each well, and the plate was incubated for 30

min by mixing at 300 rpm. Bio-Plex cytokine assays were then washed and the detection antibody was added to each well for 30 min. After washing, streptavidin-PE was added and samples were then analyzed immediately by a Bioplex array-system. Unknown cytokine concentrations were automatically calculated by Bio-Plex software using a standard curve derived from a recombinant cytokine standard. To determine the production by ASC of bFGF, BDNF, CNTF, PDGF-AB, VEGF and IGF-I, supernatants were obtained from ASC ($1x10^5$) in basal condition and/or after incubation with TNFα (50U/ml) for 24 hrs and analyzed by Quantikine® ELISA Immunoassay (R&D), following the manufacturer's instructions. Briefly, cells were grown in 24-well plates and the supernatants were harvested and centrifuged for 10 min to remove cell debris. Samples were added in 96-well pre-coated plates and incubated for 2 hrs at RT. After washing, a specific polyclonal antibody followed by substrate solution were added and the colour development was measured at 450nm (BioRad Microplate Reader). The concentration of growth factors was calculated using the standard curve.

Cell transfection and biolumiscence in vivo imaging

Murine ASC were transiently transfected with a plasmid encoding for the firefly luciferase (*Photinus pyralis*), under the control of the SV40 early enhancer/promoter region (pGL4.13 internal control vector; Promega Corporation, USA). Transfection was performed by lipofection with the Lipofectamine^{TM} 2000 reagent system (Invitrogen), according with the manufacture's instructions; cells were transfected in a 12 well plate (600,000 cells/well) and the Lipofectamine (μl)/DNA (μg) ratio used was 2.5/1. After lipofection, cells were washed and kept for 18 h at 37°C in complete medium. The transfection efficiency was evaluated transfecting with the same protocol ASC with a plasmid encoding for eGFP, under the control of a CMV promoter (pEGFP-N1 vector; Clontech). Cells were analyzed by flow cytometry for the eGFP fluorescence:

efficiency was always higher than 55% in all the experiments. The day after transfection, 1.2 x10^6 ASC were transplanted iv into healthy or EAE mice (7 to 9 days after disease onset). For anti-α4 integrin treatment, mice were injected iv with 500 μg of anti-α4-integrin mAb every other day, whereas cells were incubated before transplantation with 100 μg anti-α4 mAb in 200 μl PBS, for 30 min on ice. Bioluminescent signal generated by luciferase activity was measured using the In Vivo Imaging System IVIS® 200 (Xenogen Corporation, USA). An aqueous solution of the luciferase substrate D-luciferin (150 mg/kg; Xenogen) was injected iv 12 min before imaging. Animals were under general anesthesia (2.5% isoflurane in oxygen). Images were acquired using a CCD camera and analyzed with the Living Image 2.6 software and the Living Image 3D (Xenogen). Acquisition time was of 6 min (binning factor: 8; field of view: 12,8; f/stop 1; open filter). A pseudo-color image representing light intensity (blue, least intense; red, most intense) was created for each mouse.

Intravital microscopy

ASC were labelled with green 5-chloromethylfluorescein diacetate (Molecular Probes, Eugene, OR). To mimic brain inflammation in early phase of EAE, C57Bl/6 female mice were injected i.p. with 12 μg of LPS (*Escherichia coli* 026:B6; Sigma-Aldrich) 5–6 hrs before the intravital experiment (Piccio et al., 2002). Briefly, animals were anesthetized and the preparation was placed on an Olympus BX50WI microscope and a water immersion objective with long focal distance (Olympus Achroplan) was used. A total of 1x10^6 fluorescent labelled cells/condition was slowly injected into the carotid artery by a digital pump. The images were visualized by using a silicon-intensified target video camera (VE-1000 SIT; Dage MTI) and a Sony SSM-125CE monitor and recorded using a digital VCR (Panasonic NV-DV10000). ASC that remained stationary on venular wall for ≥30 s were considered adherent.

Histology and immunohistochemistry

At sacrifice, samples of brain, lumbar, dorsal and cervical spinal cord were snap-frozen in isopentan immersed in liquid nitrogen and stored at -80°C until use. Fifteen μm-thick cryostat sections were serially obtained from spinal cord and brain samples and processed for H&E, Spielmeyer stainings and immunohistochemistry to evaluate the presence of inflammatory cells, demyelination, axonal loss and oligodendrocyte progenitors, according to standard protocols and as previously described (Lovato et al., 2008; Lolli et al., 2005). Histological assessment of spinal cord demyelination (Spielmeyer) and inflammatory infiltrates (H&E) in lumbo-sacral segments was performed calculating affected areas in at least three sets (100 μm apart) of six sections for each animal. For immunohistochemistry, primary antibodies for macrophages/monocytes, CD3, CD4, CD8 T cells (Serotec, Oxford, UK), neurofilaments (MAB 5448; 1:1000; Chemicon, Temecula, CA) or PDGFαR (Sigma) were incubated overnight. After washing, anti-rat biotinylated secondary antibody was added; the reaction was visualized with ABC kit (Vector Laboratories, Burlingame, CA) and diaminobenzidine (Sigma). Images of immune-peroxidase were obtained with Zeiss Axiophot microscope and Axiocam camera with Axiovision software. Lesions were identified on digital images of Spielmeyer-stained or H&E sections; the lesion area was determined following a manual outline of the lesion border and expressed as percentage of the total spinal cord section area. For the determination of axonal loss, the number of neurofilament-reactive profiles was manually counted within the lesions (on sections adjacent to Spielmeyer-stained section) and their density was calculated by dividing the manual counts by the lesion area. The assessment of neural precursors was performed by counting the number of PDGFαR$^+$ cells on eight random 40X fields in the entire spinal cord section. In order to evaluate the tissue distribution, the relationship with inflammation and the differentiation by GFP$^+$ ASC in lymphoid organs and in

spinal cord, sections were incubated with DAPI and then with anti-CD3, GFAP (Dako), O4 (MAB345; 1:100; Chemicon) or PDGFαR mAbs overnight (Bonetti et al., 1997); the signal was then detected by appropriate secondary biotinylated antibodies and Streptavidin Texas Red (Vector). Slides were viewed under a Zeiss MC80 microscope or Leica TCS SP5 tandem confocal scanner with acquisition of images at different wave-length (DAPI 455nm, GFP 509nm, Texas Red 615nm). The proportion of GFP^+ MSC undergoing neural differentiation was calculated by dividing the number of GFP^+ MSC expressing neural phenotypic markers by the total number of GFP^+ MSC in lesion areas. All the above counts were performed in three sets (four sections 100 μm apart) for each animal. All data are expressed as mean percentage ± SD.

Statistical analysis

Statistical analysis using a two-tailed Student's *t*-test was performed to evaluate differences between A-MSC-treated and control conditions for several parameters: T cell proliferation, cytokine production, pathological alterations and number of oligodendrocyte progenitors in EAE lesions.

RESULTS

Preventive administration of A-MSC reduces the severity of chronic EAE

Clinical and neuropathological effects of A-MSC-based therapy

The preventive administration of ASC significantly ameliorated the subsequent clinical course of EAE. The mean clinical score at disease peak was significantly lower in ASC injected mice compared to controls (mean ± SD: 1.1 ± 0.7; vs 2.5 ± 0.7 in control mice; $p<0.002$) and also daily clinical score were significantly lower in treated mice compared to controls starting from 13 dpi until sacrifice (Figure 11 and Table 2).

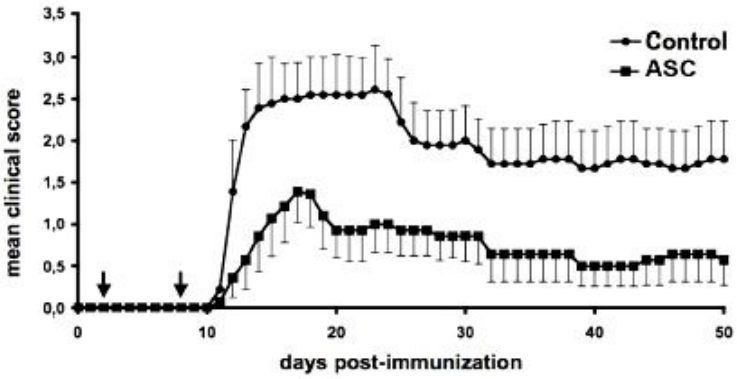

Figure 11: Preventive protocol EAE - clinical course

Table 2: Clinical and pathological features of EAE – preventive protocol

Treatment (n. animals)	Disease Onset (dpi)	Mean Maximum Score	Mean Cumulative Score	Inflammatory Area (%)	Demyelinated Area (%)	Axonal Loss (%)
Controls (10)	12.7±1.3	2.5±0.7	77.9±36.2	18.9±9.5	17.7±8.5	21.8±4.0
ASC (10)	13.7±2.1	1.1±0.7*	32.8±21.7*	8.1±7.7**	9.1±7.6**	9.2±5.1**

*$p<0.002$; **$p<0.0005$;

The pathological analysis at 50 dpi of spinal cord sections in all control mice showed the presence of demyelinated areas and inflammatory infiltrates, composed of T lymphocytes and monocyte/macrophages (Figure 12), while, mice injected with ASC showed a significant reduction of the areas of inflammation, demyelination and axonal loss (Table 2).

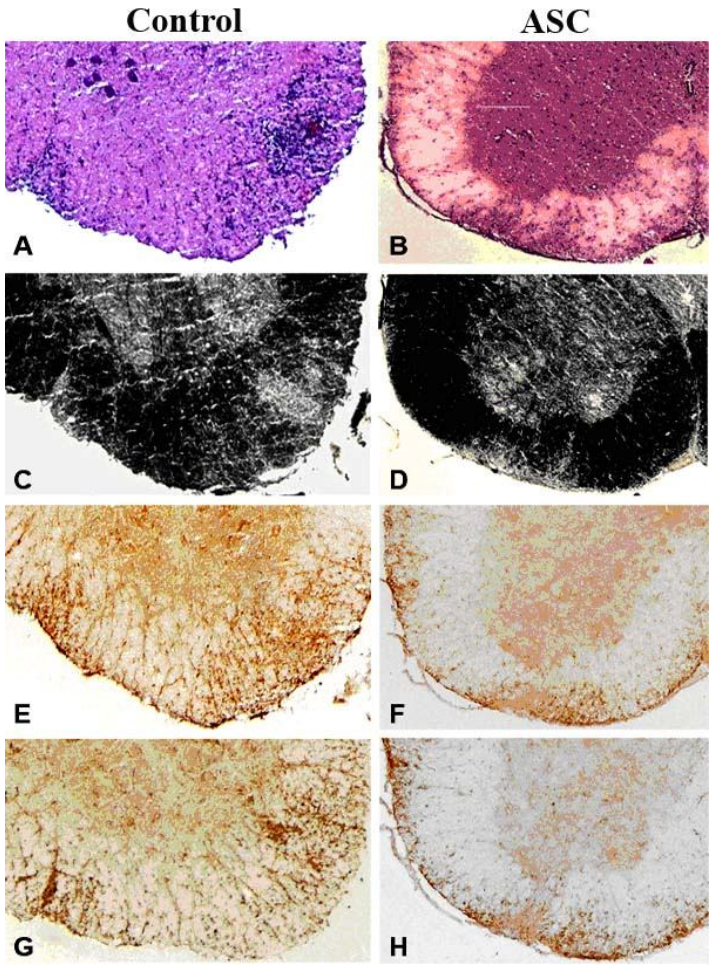

Figure 12: Histological analysis of spinal cord – preventive protocol

Hematoxylin and eosin (Figure 12 A, B) and Spielmeyer (Figure 12 C, D) stainings of lumbar spinal cords showed reduction of inflammation and demyelination in ASC-treated mice, as compared to control mice. Immunohistochemistry for CD3 T

lymphocytes (Figure 12 E, F) and macrophages (Figure 12 G, H) on serial sections showed decreased number of inflammatory cells in EAE lesions from ASC-treated mice in comparison to controls.

The composition of inflammatory infiltrates, as well as the apoptotic rate of perivascular cells, were similar in both groups. GFP^+ ASC had similar clinical and pathological effects on EAE as compared to wild type ASC, suggesting that fluorescent ASC are suitable for mechanistic studies in vivo. No anti-GFP autoreactivity was detected in the serum from ASC treated EAE animals by performing immunocytochemistry on GFP^+ ASC cultures. In addition, no evidence of GFP reactivity was seen in $CD11b^+$ macrophages in all EAE lesions examined with both treatment protocols.

Mechanisms of the beneficial effects of the preventive treatment with A-MSC in EAE: Immune regulation

Previous reports have shown a significant inhibition of proliferation of autoreactive immune cells by BM-MSC (Zappia et al, 2005). In agreement with these results, we found that ASC inhibited MOG-specific T lymphocyte proliferation both in vitro and in vivo. In fact, ASC co-cultured in vitro with $CD4^+$ T cells in the presence of MOG_{35-55} peptide exerted a dose-dependent inhibition of T cell proliferation (Figure 13) and induced a significant decrease of IFNγ (Figure 14 A), GM-CSF, IL-17, IL-4 and IL-5 production (data not shown). No effect was observed on the production of TNF-α and IL-1β, which were produced in low amounts after stimulation in vitro with MOG (data not shown).

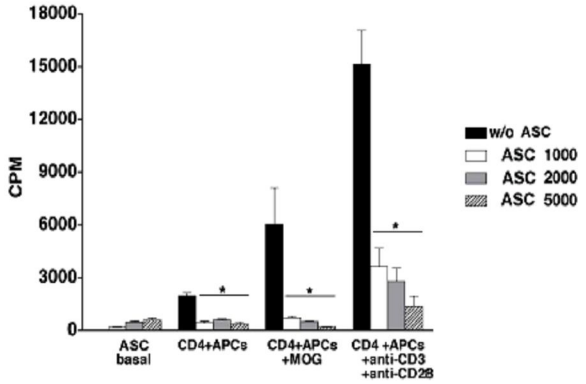

Figure 13: Effect of ASC on the proliferation of autoreactive T cells in vitro.

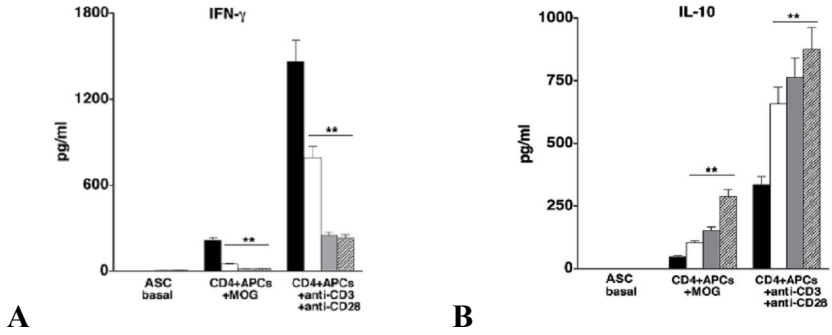

Figure 14: Effect of ASC on cytokine production in vitro.

Interestingly, we observed a selective and significant increase of IL-10 production as compared to control condition ($p<0.02$), suggesting that ASC promote the generation of T lymphocytes with regulatory activity in vitro (Figure 14 B).

We next asked whether the effects observed in vitro were also responsible for the in vivo activity of ASC. Ex vivo analysis of peripheral lymph node cells isolated from mice treated with ASC showed a significant reduction of proliferation in the presence of MOG_{35-55}, when compared to control mice (Figure 15 A). In addition, the production of both pro-inflammatory and anti-inflammatory cytokines by peripheral lymph node cells isolated from mice treated with ASC and stimulated with MOG_{35-55} was generally suppressed in comparison with control EAE animals (Figure 15 B, C).

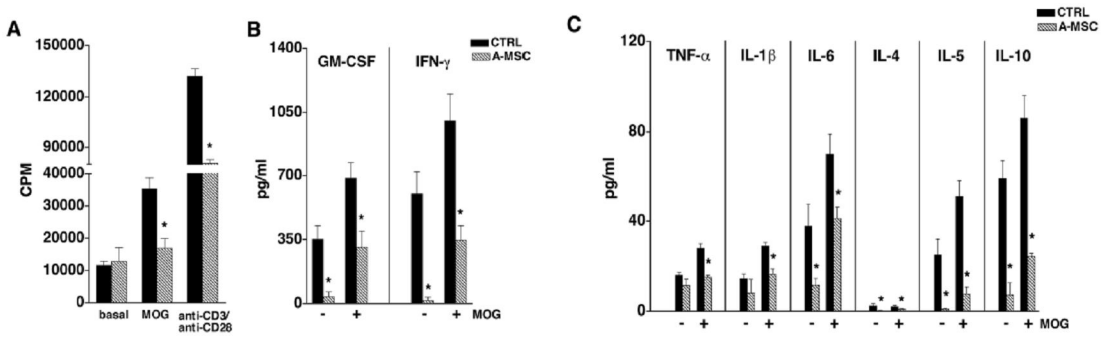

Figure 15: Proliferation and cytokine production – preventive protocol

ASC homing and local neurogenesis in EAE lesions

GFP$^+$ ASC distributed in normal mice mainly in the spleen and lymph nodes; by contrast, in EAE mice ASC displayed an increased migration to lymphoid organs but also penetrated into the spinal cord (Figure 16). The number of ASC gradually decreased in time in spleen and lymph nodes, while it persisted almost unchanged within the spinal cord up to 93 dpi. We next investigated the localization of ASC within the normal and inflamed spinal cord. In basal conditions, a very limited number of ASC was found in the sub-meningeal spaces of both brain and spinal cord (0,7 ± 0,3 cells/mm^2).

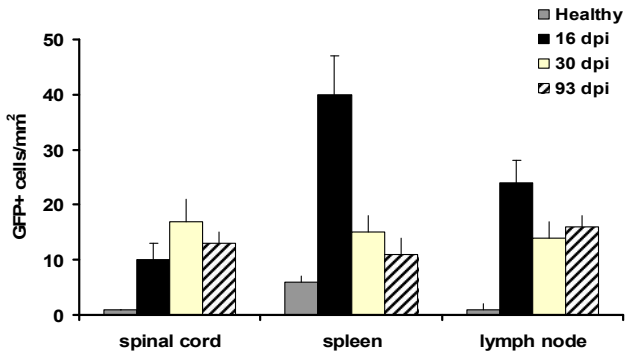

Figure 16: Distribution of ASC – preventive protocol

In spinal cord from mice with EAE the vast majority of ASC was detected mainly within the lesions, both associated with inflammatory cuffs and sparse in the white matter parenchyma. Confocal microscope image with DAPI (blue), CD3 (red) and GFP (green) showed the presence of GFP+ ASC in EAE spinal cord at 16 (Figure 17 A) and 30 dpi (Figure 17 B); only few GFP^+ ASC were observed either in regions of normal appearing white matter or in the gray matter. GFP+ ASC are detected in white matter spinal cord up to 93 dpi (Figure 17 C, D).

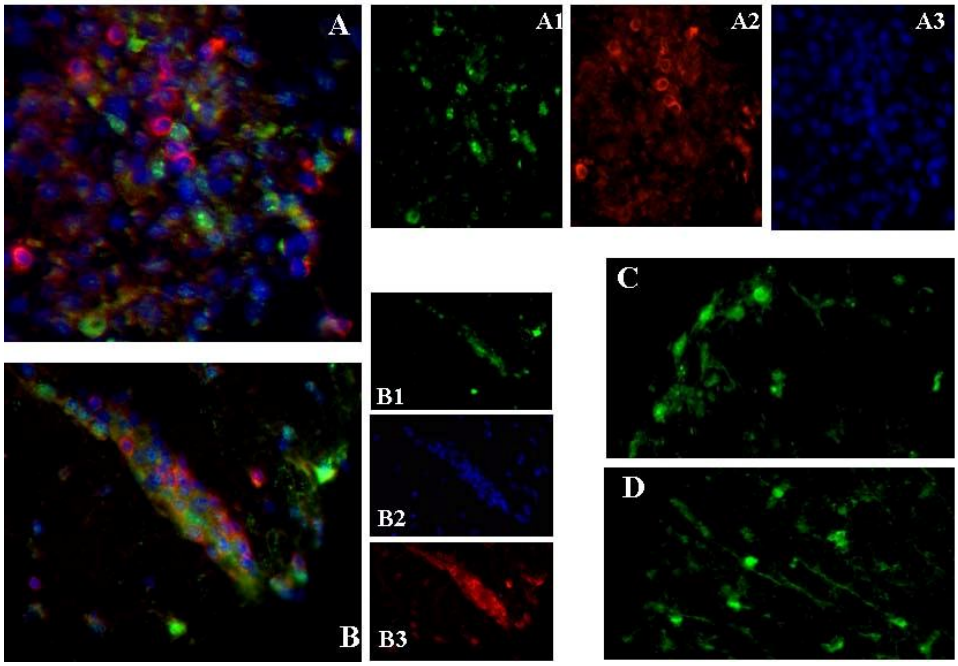

Figure 17: Distribution of ASC during the pre-clinical phase of EAE

To explore the mechanisms sustaining the homing of ASC to inflamed CNS we evaluated their adhesion molecule profile. Flow cytometry studies were performed in order to characterize on ASC and BM-MSC the expression of adhesion molecule repertoire, namely anti-leukocyte function-associated antigen (LFA-1), α4 integrins, L-selectin, P-selectin glycoprotein ligand (PSGL)-1, CD44. The results show up to 25% of ASC, but not BM-MSC, express α4 integrin, suggesting that a sub-population of

ASC might gain access to the inflamed brain (Gronthos et al.,'01; Rafei et al.,'09) (Figure 18).

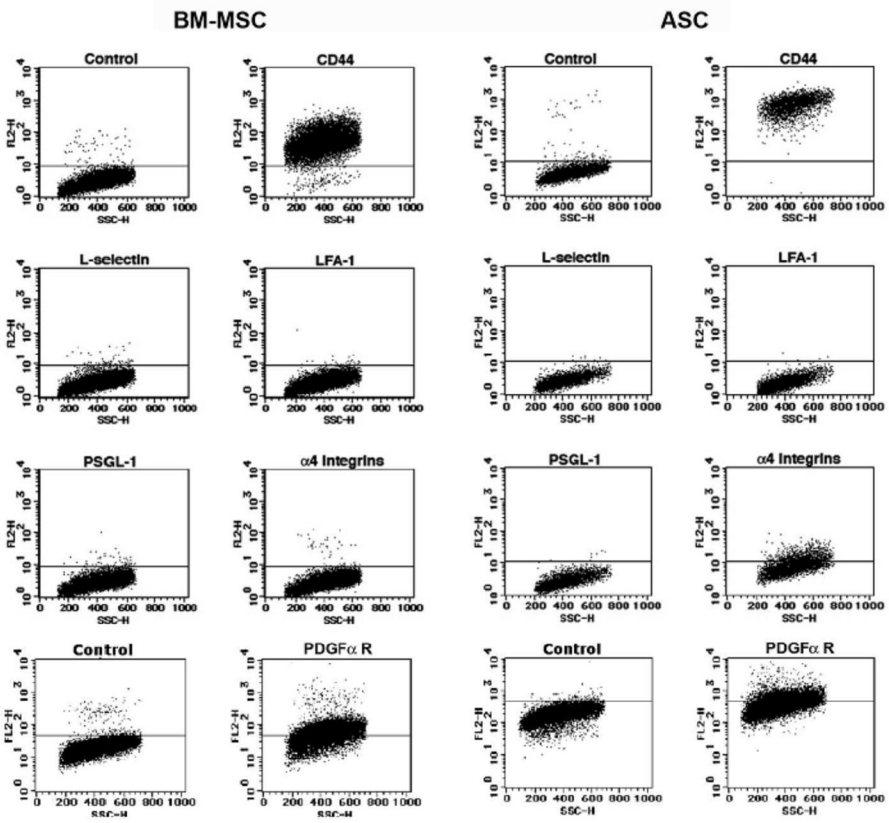

Figure 18: Adhesion molecule profile of BM-MSC and ASC.

These results, together with previous data showing that MSC express β1 integrins, suggest that ASC express VLA-4 (α4β1) integrin. Moreover, ImageStream analysis revealed that the vast majority of VLA-4+ cells express activated VLA-4. ASC were stained with anti-α4 integrin antibody followed by anti-rat IgG-PE. Analysis of α4 integrin clustering was evaluated by analyzing the distribution on the cells surface of the fluorescence: Uniform (uniform distribution of fluorescence), Clustered (small spots of fluorescence) and Caps (big clusters of fluorescence) (Figure 19 A).

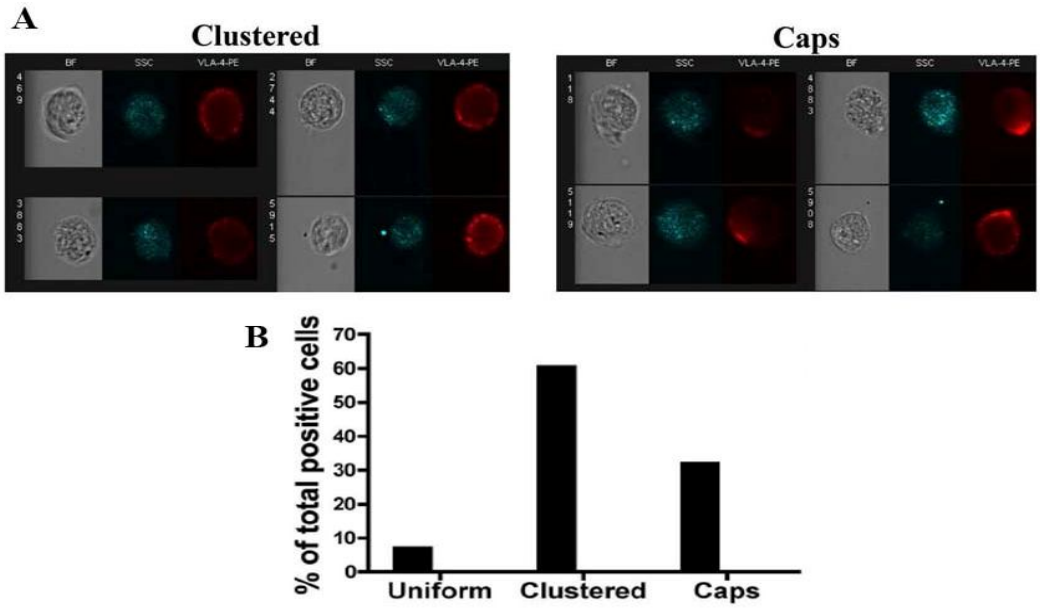

Figure 19: VLA-4 expression

The quantification of the three different populations of ASC, according with α4 integrin clustering on their surface shows that 92% of ASC displayed functionally active α4 integrin (Figure 19 B).

To assess their ability to interact with inflamed brain endothelium, we performed intravital microscopy experiments in an experimental model in which brain endothelium expresses high levels of VCAM-1, the endothelial ligand for VLA-4 integrin (Constantin et al.,'00; Piccio et al.,'02). We found that fluorescently labelled ASC arrested in inflamed brain venules in intravital microscopy experiments. Cells are the bright intravascular dots (arrows) inside blood vessels labelled with fluorescent dextrans (Figure 20). The results obtained confirmed that ASC were able to efficiently adhere to inflamed brain vessels in vivo.

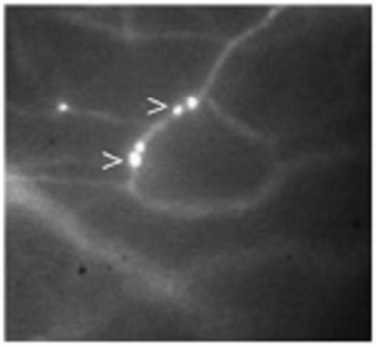

Figure 20: Intravital microscopy.

To definitively demonstrate that ASC accumulate in the inflamed brain through a VLA-4-dependent mechanism, we performed bioluminescence *in vivo* assay. ASC transfected with the luciferase reporter gene were transplanted iv in healthy or EAE mice (7 to 9 days after disease onset).

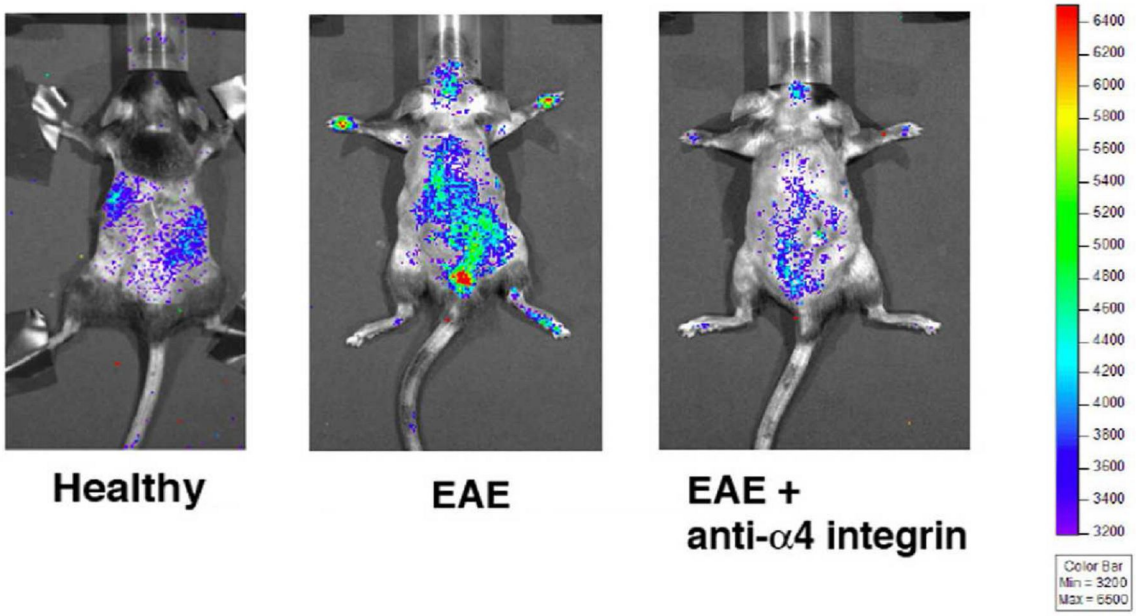

Figure 21: ASC migration into CNS of EAE mice.

Bioluminescent signal by luciferase activity generated by transfected ASC in transplanted mice was visualized by the IVIS® 200 imaging system, 7 days after ASC

injection. Images show the bioluminescent signal detected in one representative animal per group (Figure 21: left: healthy mouse; middle: EAE; right: EAE + anti-α4 integrin antibody). The colour scale next to the images indicates the signal intensity, with red and blue representing high and low signal intensity, respectively. The results showed that ASC accumulate preferentially in spleen and liver in healthy mice, whereas in EAE mice the bioluminescent signal is prevalent in areas corresponding to dorsal-lumbar spinal cord. Interestingly, the pretreatment with anti-α4 integrin antibody dramatically inhibited ASC accumulation in inflamed CNS (Figure 21).

The fate of ASC after iv injected was assessed by immunofluorescence in the spinal cord of healthy and EAE mice, by evaluating the number of GFP+ cells expressing the glial phenotypic markers GFAP for astrocytes, O4 for mature oligodendrocytes and PDGFα R for oligodendroglial precursors (Figure 22 A, B).

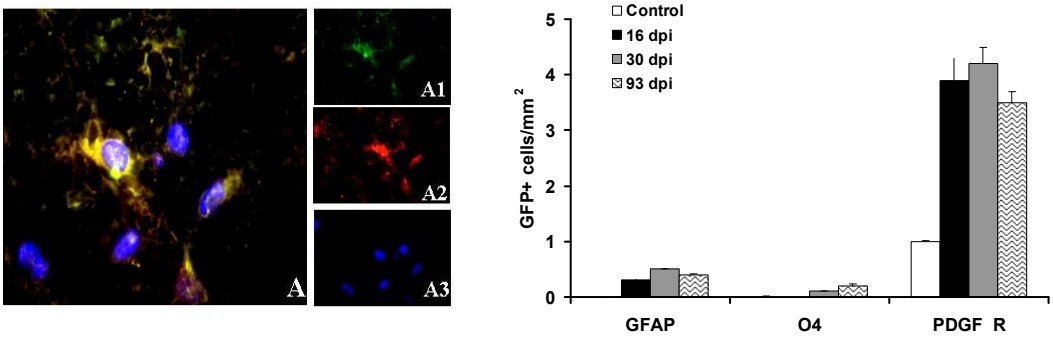

Figure 22: A: Confocal microscope image with DAPI (blue), PDGFαR (red) and GFP (green); B: quantification of glial markers expression upon ASC into the spinal cord.

We then assessed whether ASC could favour remyelination and regeneration when administered in the pre-clinical phase of disease. Given the relevance of the PDGF pathway in both oligodendrogenesis and MSC self-renewal (Lachapelle et al.,'02; Tokunaga et al.,'08), we investigated adhesion molecule profile of ASC and we first assessed by flow cytometry the expression of PDGFα R on ASC cultures: 28% of them displayed PDGFα R (Figure 18). We found that ASC treatment promoted local neurogenesis by inducing a three-fold increase of PDGFα R^+ oligodendrocyte progenitors (15.0 ± 2.1), when compared to untreated mice (5.4 ± 1.1) (Figure 23 A, B).

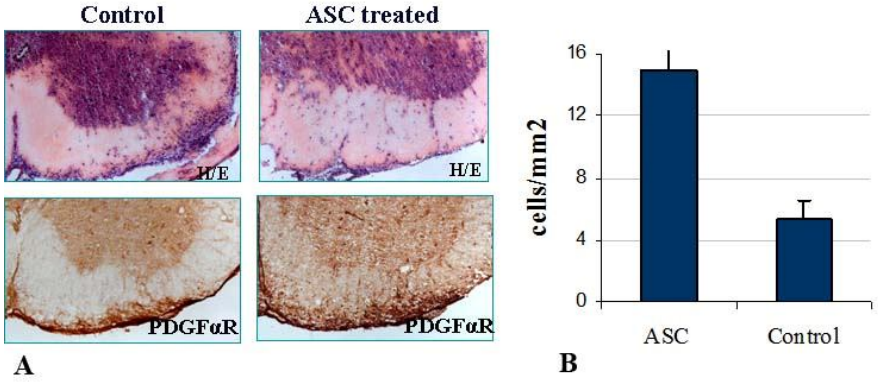

Figure 23: Increase of oligodendrocyte precursors, identified with anti PDGFαR, in ASC treated mice compared to controls.

We then asked whether the increase in oligodendrocyte progenitors derived from local precursors or from PDGFα R^+ ASC penetrated in the spinal cord. In this regard, the comparative analysis between the total number of PDGFα R^+ cells and the number of GFP+/PDGFα R^+ ASC showed that about 40% of oligodendrocyte precursors derived from ASC (Figures 16 and 22 B), a proportion similar to that found in culture.

We next investigated whether GFP^+ ASC migrated in the parenchyma underwent mature glial differentiation. As summarized in Figure 22 B, the double staining with glial phenotypic markers showed that a very limited subset of GFP^+ ASC displayed mature glial differentiation markers (i. e. GFAP or O4) in EAE lesions. No evidence of

binucleated GFP^+ cells expressing such markers was observed in all samples examined. Thus, these results suggest that ASC penetrated and persisted into inflamed spinal cord, where they contributed to activate oligodendroglial progenitors. To better support this point, we then assessed by ELISA assay the secretion by ASC of growth factors, which may influence their self-renewal as well as the process of oligodendrogenesis.

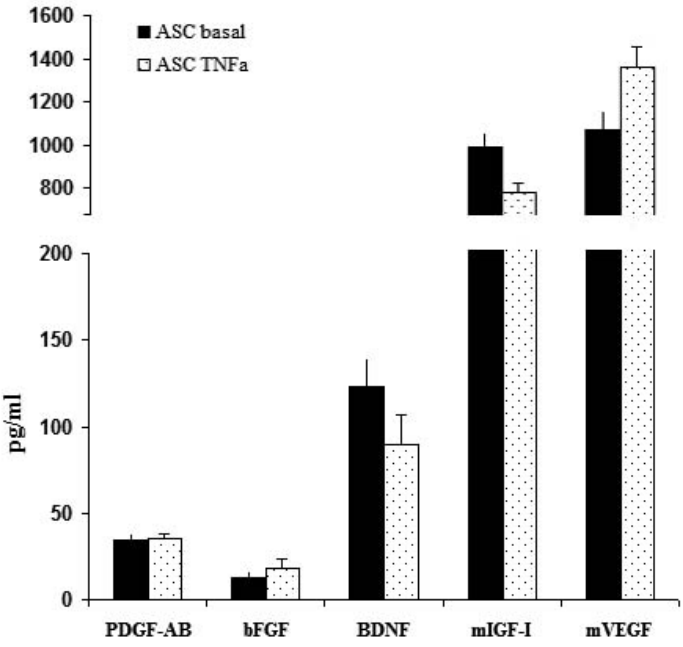

Figure 24: Secretion in the supernatant of growth factors by ASC

As summarized in Figure 24, we found that ASC both in basal conditions and after TNF-α stimulation produced detectable amounts of bFGF, PDGF-AB and BDNF and considerable amounts of IGF-I and VEGF which have been shown to activate circulating and resident stem cells tissue and promote angiogenesis (Crisostomo et al.,'08); no production of CNTF was observed in any condition (data not shown).

Therapeutic administration of ASC ameliorates the severity of chronic EAE

Clinical and neuropathological effects

Since ASC are able to exert a beneficial effect in the preventive protocol, more interestingly and in contrast with the results obtained with BM-MSC (Zappia et al.,'05), we found that therapeutic administration of ASC at 23 and 28 dpi, when disease entered an established clinical course, significantly ameliorated the disease, thus displaying a true therapeutic effect. Daily mean of the clinical scores shows that after about two weeks ASC-treated animals displayed a significant amelioration of the clinical course in comparison to control EAE mice (Figure 25 and Table 3). The effect started to be clinically significant at 41 dpi and increased progressively until sacrifice at 72 dpi. The pathological analysis at sacrifice confirmed the beneficial effect of ASC, in terms of reduction of either demyelination, inflammation and axonal loss (Table 3).

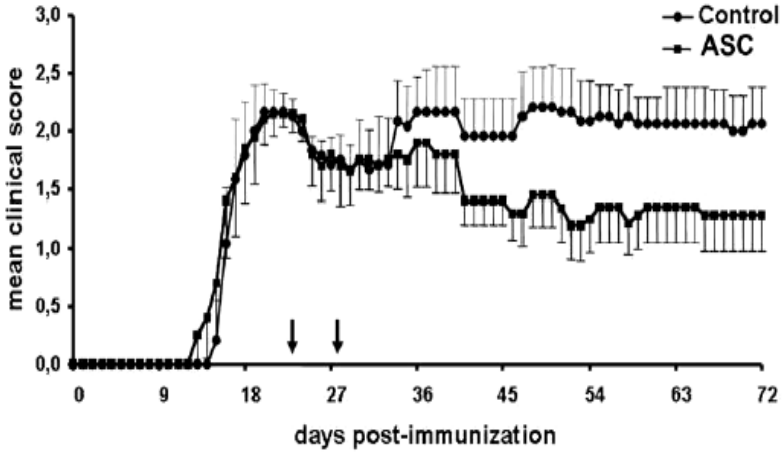

Figure 25: Therapeutic protocol EAE- clinical course

Table 3: clinical and pathological features of EAE – therapeutic protocol

Treatment (n. animals)	Mean Maximum Score	Mean Maximum Score (0-28 dpi)	Mean Cumulative Score (29-72 dpi)	Inflammatory Area (%)	Demyelinated Area (%)	Axonal Loss (%)
Controls (10)	2.2±0.4	25.2±5.3	92.2±25.1	14.2±6.9	14.6±6.6	22.6±6.0
ASC (10)	2.3±0.4	24.8±6.5	63.0±29.6*	7.7±2.3*	7.1±3.1*	3.9±1.1**

*p<0.002; **p<0.0005.

Hematoxylin and eosin (Figure 26 A, B) and Spielmeyer (Figure 26 C, D) stainings of lumbar spinal cords confirmed that clinical amelioration was accompanied by significant reduction of both inflammation and demyelination in ASC-treated animals, in comparison to control mice. Strikingly, axonal density in spinal cord of ASC-treated animals was almost comparable to normal healthy mice (Table 3).

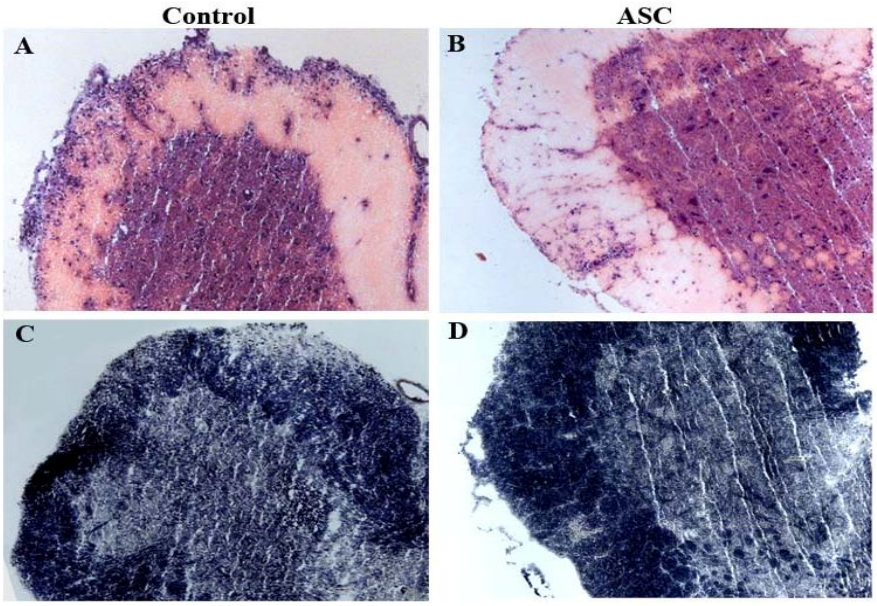

Figure 26: Histological analysis of spinal cord – therapeutic protocol

Mechanisms of the beneficial effects of the therapeutic treatment of EAE with ASC: Immune regulation

The therapeutic effect of ASC on established EAE induced us to seek for their underlying mechanisms of action. Interestingly, homing of ASC into lymph nodes was lower in mice receiving cells after the disease onset, when compared to animals treated in the pre-clinical phase of disease. In fact, the number of ASC injected with the therapeutic protocol detected in lymph nodes was only two times higher than that observed in healthy mice and about five times lower than mice receiving ASC in the pre-clinical phase of disease (Figure 27).

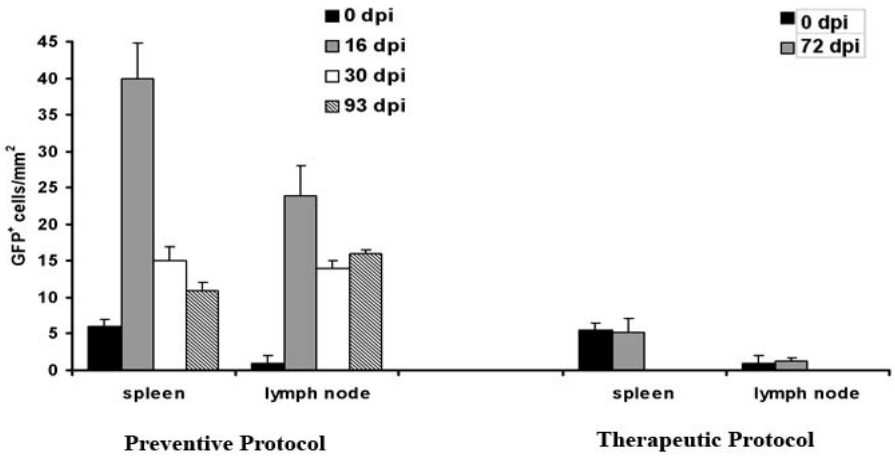

Figure 27: Quantification of GFP$^+$ ASC in lymphoid organs.

The proliferation of MOG-specific T cells isolated from mice injected in a chronic phase of EAE and the production of pro-inflammatory cytokines was not different from control mice at 72 dpi (Figure 28 A).

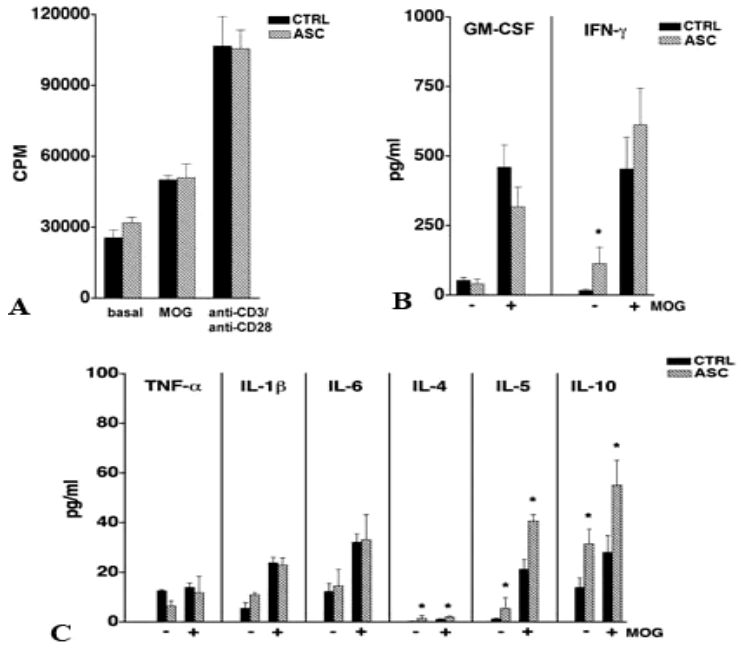

Figure 28: Cytokine production – therapeutic protocol

However, cytokine contents in supernatants of lymph node cells showed that basal production of IFNγ was increased in cells obtained from mice treated with ASC (Figure 28 B). Interestingly, we observed an enhanced production (p<0.02) of IL-4, IL-5 and IL-10 by T cells both in the absence and in the presence of antigen (Figure 28 C), suggesting that ASC injected in mice with established disease induced a shift towards a Th2 phenotype, contributing to disease amelioration.

ASC homing to CNS and induction of local neurogenesis in EAE lesions

In addition to lymphoid organs, the analysis of the distribution of GFP+ ASC in spinal cord from EAE mice sacrificed at 72 dpi confirmed the ability of these cells to home into the inflamed CNS when injected in mice with established disease. In this regard, their distribution pattern and quantity (10.1 ± 2.2 GFP^+ cells/mm^2 in EAE lesions) were comparable to those observed with the preventive protocol (Figure 29).

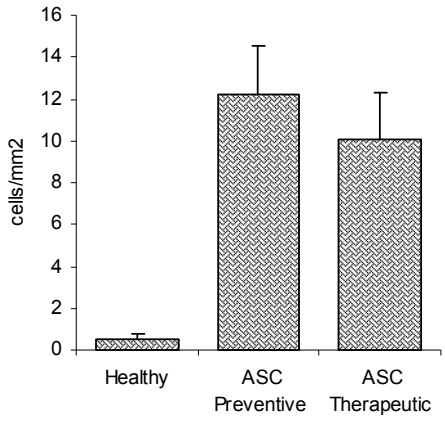

Figure 29: Homing of ASC into spinal cord during EAE.

We next sought for the mechanisms responsible for the therapeutic effect of ASC. We first evaluated the number of oligodendrocyte precursors, which may contribute to neuroregeneration. As observed in mice receiving ASC during the pre-clinical phase of EAE, ASC penetration in EAE lesions induced a three-fold increase of the total number of PDGFα R^+ cells, when compared to EAE lesions from control mice (Figure 30 A).

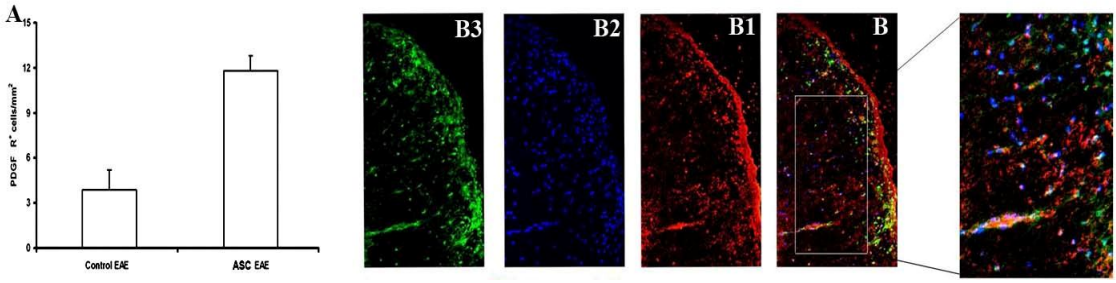

Figure 30: A: Increase of PDGFαR$^+$ cells in ASC-treated mice; B: subset of GFP$^+$ cells (green, B3) express PDGFαR (red B1), as evident in merge image (B and inset); nuclei are stained with DAPI (blue B2).

The comparative analysis of the total number of PDGFα R^+ cells and of GFP^+/PDGFα R^+ ASC showed that about 20% of oligodendrocyte precursors derived from ASC (a proportion similar to that expressing PDGFαR on ASC before injection, Figure 18), whereas the majority originated from local precursors (Figure 30 B).

As shown also for mice treated in the pre-clinical phase of disease, we observed that a very limited number of GFP^+ cells expressed the markers of mature glial cells (i. e. GFAP or O4) (Figure 31 A); in particular, mature oligodendrocyte derived from ASC were less than 2%, suggesting that ASC do not significantly contribute directly to the process of remyelination. As in preventive protocol, no evidence of binucleated GFP^+ cells expressing neural markers was observed in all samples examined.

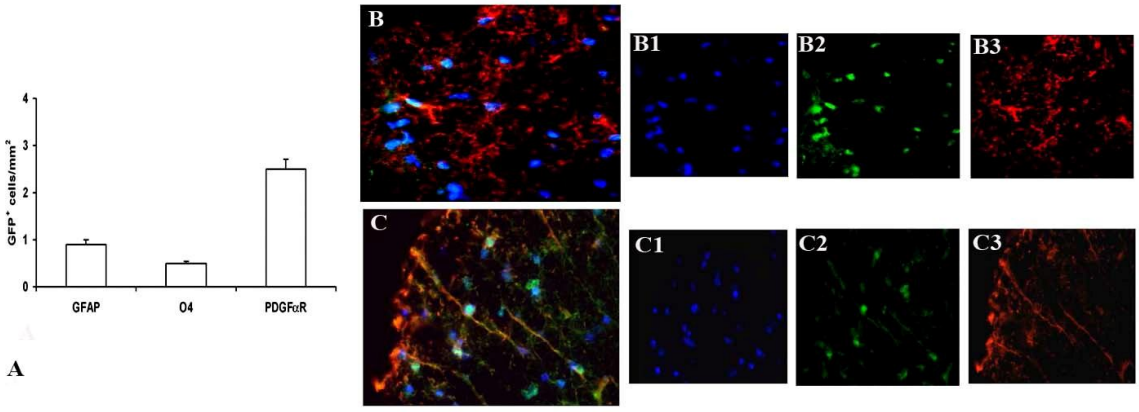

Figure 31: A: Differentiation of ASC injected during established EAE; B: examples of GFP^+ cells (B2, C2) expressing the markers of $O4^+$ mature oligodendrocytes (B3) or $GFAP^+$ astrocytes (C3), as visible in merge images (B,C); DAPI staining in B1 and C1.

DISCUSSION

The rapid scientific progress in the last years in stem cell biology has generated growing enthusiasm for the development of therapeutic strategies in the field of regenerative medicine and tissue regeneration will be a major challenge for degenerative diseases and even for the amelioration of normal ageing. As outlined in the Introduction, stem cells can be obtained from various sources, including embryos, foetal tissues, umbilical cord blood and adult organs. Once isolated, these cells may be easily induced to expand and differentiate into functional progenies suitable for cell replacement. Stem cells-based therapies could be used to cure degenerative disorders such as hematologic, cardiovascular, muscular, gastrointestinal pathologies, chronic hepatopathies, and neurological diseases (Boucherie et al.,'09).

Stem cells represent a promising approach in the treatment of neurological disorders and, in particular, for autoimmune diseases of the CNS. Multiple sclerosis is the most frequent inflammatory CNS pathology, which is sustained by anti-myelin autoreactive cells; in addition to inflammatory-mediated demyelination, a distinctive feature of this disease is the concomitant presence of axonal/neuronal damage, which strictly correlates with irreversible neurological deficits that eventually leads to important limitation to MS patients. These features explain the challenge that MS represents for many scientists working with stem cells; for these reasons, MS is the prototypic disease where to test therapeutic tools that couple neuro-regeneration and immune modulation. Among adult stem cells, NSC probably represent the gold standard in neurological disorders, because of their capacity to generate a progeny of differentiated functional daughter neural cells and their ability to penetrate into the CNS, where they display also potent anti-inflammatory effects. These features made NSC an attractive cell source for the treatment of autoimmune neurological diseases characterized by widespread inflammation,

demyelination and axonal loss like MS and its animal model (Pluchino et al.,'03, '05). However, the source and the availability of stem cells is becoming a crucial issue for their clinical application and the invasiveness associated with isolation of NSC may limit their use in human applications. In this regard, BM-MSC have been considered an elective source of adult stem cells. In addition to self renewal potential, BM-MSC couple an immunomodulatory activity with the ability to penetrate into damaged tissue, where they could release trophic factors or undergo trans-differentiation resulting in directly regenerative effect (Chamberlain et al.,'07).

In consideration of these features, several groups have tested the efficacy of BM-MSC in the treatment of autoimmune neurodegenerative disorders; in particular, BM-MSC have been shown to ameliorate both chronic and relapsing-remitting EAE, reducing inflammatory infiltrates, demyelinated areas and axonal loss (Zappia et al.,'05; Zhang et al.,'05; Gerdoni et al.,'07). However, the beneficial effect in chronic EAE was evident only when murine BM-MSC were injected before the disease onset (Zappia et al.,'05). In both chronic and relapsing EAE, the main mechanism was related to the immune-suppression exerted by murine BM-MSC on autoreactive B and T cells (Puissant et al.,'05; McIntosh et al.,'06; Gerdoni et al.,'07), while their effects within the inflamed CNS are still a matter of debate. In this regard, it has been demonstrated that murine and human BM-MSC showed different homing into CNS, related to distinct panels of adhesion molecules expressed. The lack of VLA-4 on murine BM-MSC prevented migration through BBB which can exert their biological effects only in peripheral target. On the other hand, it has been shown that human VLA-4^{+} BM-MSC (Brooke et al.,'08) could exert, in addition to immunomodulation, a direct neuroregenerative effect due to the capacity to migrate into the CNS (Zhang et al.,'04). In line with this evidence, human BM-MSC have been recently shown to be effective on chronic EAE: clinical improvement was accompanied by changes in glial cell responses with increased

oligodendrocytes and decreased astrocytes in lesion areas as well as changes in spleen-cell responses. In addition, in animals that received BM-MSC, there was a significant reduction in pro-inflammatory cytokines including IL-17, IFN-γ and TNF-α and a significant increase in anti-inflammatory cytokines including IL-4 and IL-5, thus BM-MSC could regulate the balance of T lymphocytes between Th1/Th17 to Th2-polarized immune response and modify the cytokines released during EAE (Bai et al.,'09). Interestingly, recent data show that BM-MSC specifically inhibit key mechanisms responsible for EAE induction by a paracrine conversion of CCL2 chemokine from agonist to antagonist of the Th17 cell activation and migration (Rafei et al.,'09).

However, although promising results have been obtained with BM-MSC in the experimental model of MS, several aspects dampen the initial enthusiasm for their application in humans. First of all, the lack of therapeutic effect exerted in EAE when murine BM-MSC are injected after the onset of disease. Translating the results obtained in EAE to MS patients, we should expect a good preventive effect on relapsing MS by BM-MSC, but little or no expectation once the patient enters the progressive phase. These limitations will greatly reduce the impact of stem cell-based therapy in MS, since the same effects can be nowadays obtained by a number of immune modulating or immune suppressive compounds. In addition, the invasive nature of BM biopsies may further limit their use for wider clinical applications. For these reasons, alternative sources to isolate MSC have been exploited in recent years; in this regard, much attention has been paid to adipose tissue because it represents an abundant and accessible source of adult stem cells satisfying the criteria of MSC (Dominici et al.,'06). It has been estimated that the frequency of ASC in collagenase-digested adipose tissue is about 500-fold higher than in freshly isolated BM cells (Zuk et al.,'02; Fraser et al.,'06).

Moreover, the most important features of adipose tissue as a cell source might be the relative expandability of this tissue and the consequent ease with which it can be obtained

in relatively large quantities with minimal risks (Lin et al.,'08). In the present study, we show that ASC have a significant beneficial effect on chronic EAE not only when administered in the pre-clinical phase of disease, but also when injected after disease entered an irreversible clinical course, thus displaying a true therapeutic effect. In both cases, the amelioration of clinical scores was accompanied by a strong reduction of spinal cord inflammation as well as of demyelination and axonal damage in EAE lesions. Our results show that these cells exerted their beneficial effects by acting simultaneously in two distinct sites: lymphoid organs and inflamed CNS. Similarly to BM-MSC, ASC induced dramatic changes on antigen-specific T cells in vitro, with dose-dependent inhibition of their proliferation and modulation of cytokine secretion. The comparison of the influence of ASC on the immune system in the preventive and therapeutic protocol provided relevant information regarding the timing of immune activation and ASC administration in vivo. We show that the migration to lymph nodes of ASC was particularly high when administered shortly after the induction of the autoimmune response, whereas it was significantly lower when injected in mice with established disease. This preferential lymphoid migration resulted in a strong anti-proliferative effect and broad inhibition of both pro- and anti-inflammatory cytokine production by ASC injected only in the pre-clinical phase of disease. Translating these results in human patients, we may hypothesize that ASC-induced immune suppression will be effective only immediately after the immune activation occurs, i.e. in a pre-clinical phase. Interestingly, we provided evidence that ASC injected in mice with established disease induced a Th2-type shift of antigen-specific CD4 T cells in lymph nodes supporting recent results obtained with human BM-MSC in chronic EAE (Bai et al.,'09).

The knowledge of the molecular mechanisms controlling ASC-based therapy is of critical importance in the prospective of potential future applications of ASC in humans. The characterization of the molecular mechanisms involved in the immune modulation and

those sustaining ASC homing into the CNS during EAE/MS represent an important contribution to the understanding of ASC tissue-specific delivery. However, the mechanisms involved in the migration of MSC (of any origin) into the brain are largely unknown.

In addition to their immune modulatory activity shared with BM-MSC, our results suggest that the beneficial effect of ASC on chronic EAE relies also on the ability to penetrate into the inflamed CNS, due to the expression on a relevant ASC subset of activated α4β1 integrin, a key adhesion molecule involved in leukocyte and stem cell migration into the inflamed CNS (Pluchino et al.,'05). By flow cytometry, we demonstrated the presence on a subset of ASC of α4 integrin, which forms with β1 integrin, constitutively present on ASC, the very late activation antigen 4 (VLA-4) which is the counter-ligand of VCAM-1, highly expressed on brain endothelium. Intravital microscopy experiments confirmed that the interaction of ASC with inflamed brain endothelium was through a VLA-4-dependent mechanism. Moreover, bioluminescence in vivo assay definitely demonstrated that ASC display α4 integrin-dependent migration in inflamed CNS, suggesting that adipose tissue may represent a valuable source of stem cells able to cross BBB and exert their action into the inflamed CNS.

The analysis of distribution of GFP^+ ASC in EAE mice injected either before or after disease onset indicates that these cells, in addition to migrate into lymphoid organs, were able to penetrate in the inflamed spinal cord and persist there up to three months, thus suggesting long-lasting effects in target tissues. The persistence of ASC up to 75 days after i.v. injection has been already described in previous studies (Meyerrose et al.,'07). In our model, chronic inflammation and expression of VCAM-1 on brain endothelium in MOG-induced EAE may help to continuously recruit ASC expressing α4 integrin from the blood.

We then asked whether and how ASC could have a neuro-regenerative effect in EAE spinal cord. In this regard, ASC may exert a beneficial effect participating to remyelination by either differentiating into mature oligodendrocytes able to form new myelin or indirectly by promoting the survival and proliferation of endogenous precursors cells. A direct participation of ASC to remyelination seems unlikely, since the proportion of GFP^+ cells expressing PDGFαR in EAE lesions of both protocols was comparable to that observed in ASC cultures before injection. Another evidence pledging against a direct neuro-regenerative effect of ASC came from the analysis of the markers of mature glia expressed by ASC *in situ*, which revealed that only a very limited proportion of GFP^+ ASC acquired the phenotype of mature oligodendroglial cells. In particular, less than 2% of $O4^+$ oligodendrocytes derived from ASC at all time points investigated, a proportion that probably does not significantly contribute to the process of remyelination. Although we did not observe any binucleated GFP^+ cells expressing markers of mature glia in our samples, we were not able to establish whether the expression of neural markers on such limited ASC sub-population in EAE spinal cord reflected a process of true differentiation towards a glial phenotype or rather derived from a process of cell fusion, as seen in other experimental conditions (Spees et al.,'03).

Thus, the levels of engraftment of ASC and the lack of relevant neural differentiation of ASC do not explain the clinical and histological effects observed. In this regard, it is conceivable that ASC may mediate their beneficial effects into target tissues by complex paracrine and autocrine mechanisms. Several observations support the hypothesis that stem cells may display a valuable influence on host tissues by releasing a large number of trophic factors in response to injury and that the secretion of these bioactive factors can profoundly influence local cellular dynamics (Caplan et al.,'09). Neurotrophic factors constitute a family of polypeptides supporting neuronal maturation and specification during development as well as modulation of axonal growth, plasticity, and

neurotransmission throughout adulthood (Crisostomo et al.,'08). Several factors are known to be involved in neurogenesis: NGF, BDNF, neurotrophin-3, CNTF, VEGF, bFGF, PDGF-AB, erythropoietin and BMI-1. It has been demonstrated that indeed the implantation of MSC in the CNS induced a generalized increase in expression of all these chemokines, resulting in the stimulation, migration and proliferation of the endogenous NSC (Munoz et al.,'05). In line with these observations, Zhang and colleagues have observed that BM-MSC treatment increased in the CNS of EAE mice the expression of BDNF, which supports neuronal survival, regulates neurotransmitter release and remyelination (Zhang et al.,'05). Experimental evidence indicates that other factors may be directly involved in remyelination pathways: FGF-2 and IGF-I are mitogens for oligodendrocytes precursors and regulate their differentiation and maturation (Goddard et al.,'99); PDGF-AB mediates glial cells proliferation and differentiation, in addition to sustain neuronal survival and regeneration (Lachapelle et al.,'02); finally, VEGF, first characterized as an angiogenic factor, is known to increase axonal outgrowth, block neuronal apoptosis and promote neurogenesis (Lunn et al.,'09).

In addition to the effects exerted to local cellular dynamics, growth factors could orchestrate migration, survival, self-renewal and differentiation of exogenous stem cells (Boucherie et al.,'09; Zaragosi et al.,'06). Here we found that in basal condition ASC were able to secrete large amount of IGF-I and VEGF and considerable quantities of BDNF, PDGF-AB and FGF-2, all factors well known to activate endogenous neural progenitors.

In addition to promote the homing of ASC, we speculate that chronic inflammation might be responsible also for the prolonged survival of ASC in inflamed spinal cord. Interestingly, we found that these cells after stimulation with TNF-α produce increased amount of VEGF, FGF-2 and PDGF-AB as compared to basal condition, all factors known to promote MSC self-renewal (Zaragosi et al.,'06; Wang et al.,'06). In addition to

ASC, activated microglia present in active EAE lesions are also known to produce bFGF (Liu et al.,'98). From these observations, it is conceivable that the local inflammatory environment supports the prolonged survival of ASC within EAE lesions. These results indicate that a cross-talk between neural cells and ASC occurs in inflamed CNS, resulting in a positive loop resulting in a positive effect on both inflammation and neuro-regeneration.

Overall, our results indicate that the main mechanism responsible for the neuroregenerative effect in chronic EAE is a robust activation of endogenous progenitors in EAE lesions, which probably accounted for the process of remyelination. Indeed, the number of endogenous oligodendrocyte precursors (GFP-/PDGFαR^{+}) was significantly higher in ASC treated animals in comparison to control EAE.

Regarding the molecular mechanism involved in the cross-talk between ASC and oligodendroglial precursors, MSC are known to produce a variety of neurotrophic factors with relevant effects on NPC proliferation, migration and differentiation (Caplan et al.,'06; Rivera et al.,'06; Crisostomo et al.,'06). Here we show that ASC are able to secrete VEGF, IGF-I, bFGF, PDGF-AB and BDNF, all factors strongly supporting the process of oligodendrogenic differentiation. In fact, a key role for these factors in the amelioration of EAE observed in our experiments is also suggested by two studies in which the delivery of bFGF or BDNF induced a beneficial effect of clinical and pathological scores in EAE together with increase of mature oligodendrocyte and their progenitors in EAE model (Ruffini et al.,'01; Lachapelle et al.,'02; Zhang et al.,'04).

In conclusion, our results show that ASC produce a beneficial effect in chronic EAE by a bimodal mechanism, through suppression of the autoimmune response in early phases of disease and promotion of Th2-polarized immune response as well as through the activation of local neuro-regeneration by endogenous progenitors in animals with established disease. Our data show that ASC have relevant therapeutic potential in an

animal model of chronic MS and might represent a valuable tool for stem cell-based therapy in chronic inflammatory diseases of the CNS. The persistence of ASC in EAE lesions, together with the beneficial effect displayed in the therapeutic protocol in chronic EAE, when clinical and pathological signs are irreversible, may have important implications for the future therapeutic use of ASC also in other chronic, non-inflammatory CNS diseases, where the recruitment of local progenitors is warranted.

Prompted by these results, we decided to evaluate the beneficial effect of ASC in the experimental model of an adult-onset and progressive degenerative disorder of the CNS, the amyotrophic lateral sclerosis (ALS). ALS is a chronic neurological disease characterized by diffuse neurodegeneration which affects upper and lower motor neurons, resulting in paralysis and death. The vast majority of ALS are sporadic, only 10% are familial (FALS) and about 20% of FALS are due to the mutation in the gene encoding Cu/Zn superoxide dismutase (SOD)1. Transgenic mice over-expressing human SOD1 carrying a Gly93-Ala mutation provide a widely used model for FALS. Preliminary results showed that, although no survival improvement was observed, animals injected with ASC at the onset of disease sustained significant higher behavioral performance as compared to controls for almost 7 weeks after injection. Moreover, ASC penetrated and survived into spinal cord up to 2 months but did not expressed neural and glial markers. These results induce us to speculate on the possible neuroprotective role of ASC through the release of trophic factors like VEGF, IGF-I, BDNF and FGF-2, rather than a direct trans-differentiation of injected cells.

The results obtained in EAE model and the preliminary data in ALS model demonstrated that ASC could penetrated into damaged CNS due to the expression of VLA-4, secrete a vast panel of neurotrophic factors, resulting in the proliferation of endogenous oligodendrocyte precursors in the EAE model, in the sustainance of motorneurons in the ALS model as well as in the self-renewal of exogenous stem cells (Figure 32).

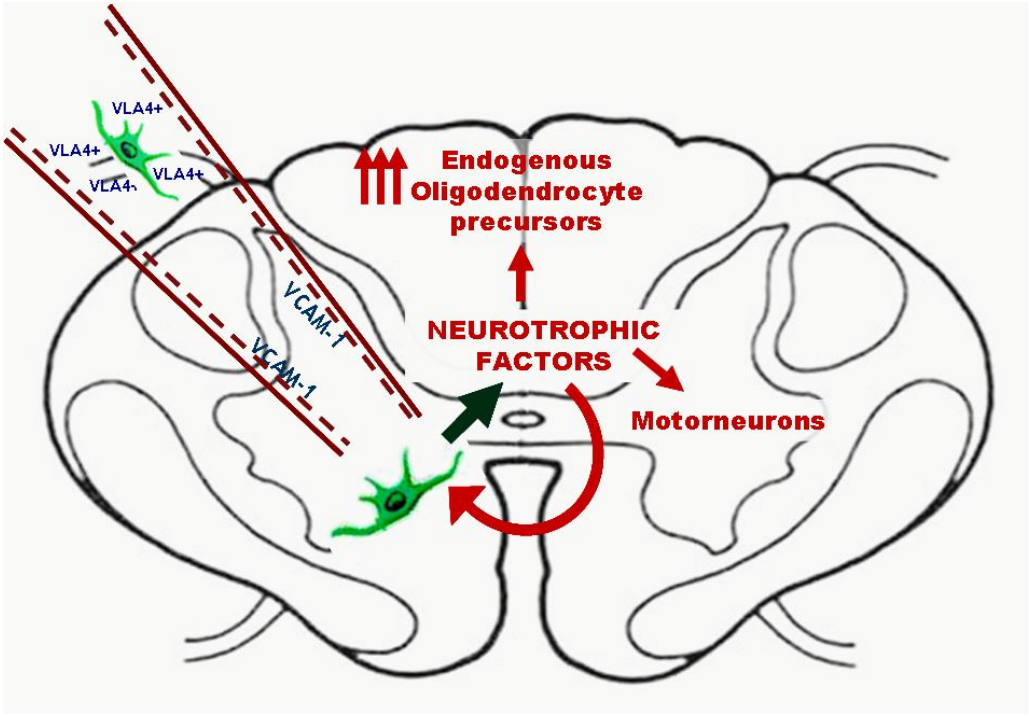

Figure 32: Molecular mechanisms underlying the beneficial effect of ASC

The convincing results obtained in EAE and the encouraging preliminary data from ALS models strongly indicate that, before considering their use in human patients, adult stem cells need to be well characterized for their adhesion molecule profile and for their capacity to secrete a broad range of neurotrophins. On this basis, we believe that ASC may represent a valuable therapeutic approach not only for autoimmune but also for progressive neurodegenerative disorders of CNS and could be considered an elective strategy for future clinical trials in human patients affected with a wide range of neurological disorders.

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