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Stem cells ameliorate EAE via an indoleamine 2,3-dioxygenase (IDO) mechanism

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Abstract

Syngeneic, pluripotent Lin[−]Sca1⁺ bone marrow stem cells (SC), transferred to mice with experimental autoimmune encephalomyelitis, a model of multiple sclerosis, enhanced recovery, prevented relapses and promoted myelin repair. SC-treated mice showed elevated interferon- γ production and induction of indoleamine 2,3-dioxygenase (IDO) in CD11c⁺ dendritic cells (DC). IDO induction was specific since in the presence of IDO-producing CD11c⁺ DC, PLP stimulated T-cell proliferation was inhibited and the IDO-inhibitor, 1-MT, abrogated the SC effect. Relapse prevention during chronic disease correlated with decreased responsiveness to PLP_{178–191} and MBP_{85–99}. Thus, pluripotent SC induce IDO in DC leading to inhibition of antigen reactivity and spreading in EAE.

Keywords

Stem cells; Experimental Autoimmune Encephalomyelitis; Indoleamine 2,3-dioxygenase; immunomodulation

INTRODUCTION

Multiple sclerosis (MS) is a chronic inflammatory demyelinating condition of the human central nervous system (CNS), characterized by recurrent episodes of immunemediated demyelination and axonal loss (Frohman et al., 2006). Of recently proposed therapeutic strategies, transplantation of myelin-forming precursor cells or stem cells (SC) to the site of injury has generated both interest and reservations (Lassman, 2005; Pluchino and Martino, 2005). In this regard, it is recognized that limitations like the multifocal nature of CNS lesions in MS and the restriction of cell migration within demyelinated plaques pose serious challenges to the SC approach.

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Stem cells, a potential source of cells for all tissues due to their ability to differentiate into almost any cell type, develop through a series of stages which finally give rise to lineage specific cell types. In mice, lineage negative (Lin⁻) cells expressing the Sca1 molecule (Lin⁻Sca1⁺) represent a pluripotent population of bone marrow SC (BMSC) depleted of mature hematopoietic precursors and enriched in mesenchymal SC (Kucia et al., 2005; Vogel et al., 2003). With further development, expression of Sca1 is lost and SC acquire tissue specific markers (Zuba-Surma et al., 2006). Mesenchymal (or stromal) SC are capable of differentiating into myogenic, osteogenic, chondrogenic and adipogenic cell lineages (Seshi et al., 2000; Pittenger et al., 1999). In murine transplant models, mesenchymal SC can also give rise to non-mesenchymal elements, including liver (Petersen et al., 1999), neuronal (Brazelton et al., 2000) and glial cells (Kopen et al. 1999). It has been suggested that mesenchymal bone marrow stem cells might provide a non-embryonic or non-fetal source of SC suitable for cell replacement in treatment of CNS disorders (Alhadlaq and Mao, 2004).

Although several recent reports have described that administration of neural and mesenchymal SC is capable of ameliorating the course of experimental autoimmune encephalomyelitis (EAE), a model of MS (Pluchino et al., 2003; Zappia et al., 2005; Einstein et al., 2007; Gerdoni et al., 2007; Pluchino et al., 2005), and have proposed that SC induce changes in the immunological milieu within the CNS that result in reduction of immune reactivity leading to tissue injury (Gerdoni et al., 2007; Pluchino et al., 2005), no definitive mechanism has been identified. Indoleamine 2,3-dioxygenase (IDO) is a rate-limiting enzyme in the catabolism of tryptophan (Mellor and Munn, 1999) which is expressed in many human and animal tissues, particularly in lymphoid organs and placenta. In healthy individuals, expression of IDO is low but increases markedly during infection or inflammation. IDO activation can result from lipopolysaccharide and cytokine stimulation, particularly interferon- γ (IFN- γ) (Byrne et al., 1986). Low tryptophan concentrations induced by IDO are associated with inhibited proliferation of viruses, protozoan parasites and other pathogens and also with decreased proliferation of tumor cells (Gupta et al., 1994; Aune and Pogue, 1989). Recent studies have suggested a role for IDO in the regulation of T cell responses, either by lymphocyte deprivation of tryptophan or by induction of the tryptophan metabolites, 3-OH-kynurenine and 3-OH-anthranilic acid which inhibit T-cell reactivity (Terness et al., 2006; Terness et al., 2002; Frumento et al., 2002).

In this study, we have assessed the role of IDO during pluripotent Lin⁻Sca1⁺BMSC-induced down-regulation of EAE. Our results suggest that these cells induce IDO which leads to T cell unresponsiveness (Meisel et al., 2004), events manifested at the level of the CNS by decreased autoimmune demyelination and increased myelin repair.

MATERIALS AND METHODS

Isolation of enriched populations of Lin⁻Sca1⁺ BMSC

Normal SJL mice, 6 to 8 weeks old, were used for the isolation of BMSC. Bone marrow cells were obtained from femurs and tibias of euthanized mice by flushing with PBS. Cells were washed twice in sorting medium (PBS supplemented with 0.5% BSA, Sigma-Aldrich, St. Louis, MO), and subjected to negative magnetic sorting using the Lineage Cell Depletion Kit (Mitenyi Biotech., Bergisch Gladbach, Germany). Depletion of cells expressing lineage antigens by monoclonal antibodies and magnetic beads resulted in a pure fraction of Lin⁻ cells. Purity of the Lin⁻ fraction was assessed by flow cytometry using Lineage Cocktail (anti-CD3e, anti-CD11b, anti-CD45R/B220, anti-Ly6G and Ly-6C, and anti-TER-119) (BD Biosciences, San José, CA), and was invariably >98%.

Induction and treatment of EAE

Female SJL mice, 6 to 8 weeks old, were obtained from the Animal Care Department, Medical University of Lodz. All animals were housed in pathogen-free conditions and were treated according to the guidelines of the local Animal Ethics Committee. Mice were immunized i.v. according to a previously published protocol (Tuohy et al., 1989) using 0.15mg PLP_{139–151} in incomplete Freund's adjuvant (Difco Laboratories), supplemented with 4mg/ml *Mycobacterium tuberculosis*. Immunization with PLP peptide was followed by i.v. administration of 400 ng of Pertussis toxin (Sigma-Aldrich), on days 0 and 2.

Lin[−]Sca1⁺ BMSC or Lin[−]Sca1[−] BMSC (2×10^6 in 200 μ l PBS), were transferred i.v. at the peak of disease. Control mice with EAE received a sham injection of the same volume (200 μ l) of medium. In some experiments, prior to transplantation, BMSC were stained with PKH26 (red fluorescence; Sigma-Aldrich), according to the manufacturer's protocol. PKH26 is the fluorescent dye that binds irreversibly to cell membranes, and is used as a cell tracer in transplantation experiments. In a separate set of experiments, 1-methyl-DL-tryptophan (1-MT)- tryptophan analog, was administered orally using an intrapharyngeal needle (5mg/mouse/day), to control and BMSC-transplanted animals. The clinical course of EAE was evaluated daily by blinded observer on a 0 to 5 scale (0-healthy; 1-limp tail; 2-ataxia and/or paresis of hind limbs; 3-paralysis of hind limbs and/or paresis of forelimbs; 4-tetraparalysis; 5-moribund or dead). Mice were observed for 90 days following immunization.

Histopathology of the CNS

At different timepoints after BMSC transplantation (2, 4 and 6 weeks), mice were deeply anesthetized and perfused intracardially with cold 2.5% glutaraldehyde in phosphate buffer (pH 7.2). Brains and spinal cords were removed and thin slices made from 10 levels of the neuraxis, postfixed in cold 1% osmium tetroxide for 1 h, dehydrated, and embedded in epoxy resin (Epon 812). One-micrometer sections of epoxy-embedded tissue were cut, stained with toluidine blue and examined by light microscopy under code by a blinded observer.

Flow cytometry

Localization of Lin[−]Sca1⁺ BMSC stained with PKH26 was analyzed 1, 2 and 6 weeks after transfer. Mice were perfused transcardially with PBS and immunofluorescent cells were assessed by flow cytometry in cell suspensions prepared from cerebral hemispheres, cerebellum, upper and lower spinal cord, bone marrow, spleen and liver, using a FACSCalibur® cytometer and CELLQuest® software (BD Biosciences, San José, CA). Presence of regulatory T cells was analyzed *ex vivo* in spleens obtained 2 weeks after BMSC transplantation. Spleen cells were washed three times in PBS, counted and suspended in PBS for three-color flow cytometry analysis. For this, monoclonal antibodies specific for CD4, CD25, CD152 (CTLA-4), and appropriate isotype controls (BD Biosciences), were used.

Immunocytochemistry

Mice were perfused transcardially with ice-cold 0.9% sodium chloride. Brains and spinal cords were removed immediately and frozen in liquid nitrogen. Frozen sections (25 μ m) were prepared and analyzed by fluorescence microscopy for localization of PKH26-labeled cells. Sections showing PKH26⁺ cells were double-stained with anti-O1, anti-O4 and anti-CNPase for oligodendrocytes. All primary and biotin-conjugated secondary antibodies were obtained from Chemicon.

Proliferation assay

Spleen cells were obtained 2, 4 or 12 weeks after immunization for EAE and cultured (2×10^5 cells/well) in triplicate for 72 h in medium supplemented with different myelin

determinants (10 µg/ml PLP_{139–151}, PLP_{178–191} or MBP_{85–99}, respectively) (Polygen, Poland). For the next 16 h, 1 µCi [³H]thymidine (TdR, Amersham Biosciences, United Kingdom), was added to each well. At the end of the culture period, incorporation of [³H]-thymidine was determined in a Wallac Betaplate liquid scintillation counter (Perkin Elmer Life Sciences, Wellesley, MA). Results were expressed as cpm.

Assessment of cell death

Spleen cells from Lin[−]Sca1⁺ BMSC-transplanted and control EAE mice were cultured with or without PLP_{139–151} (10 µg/ml). After 24 and 72 h of culture, cell death was assessed based on the fractional DNA content. Briefly, cells were collected, spun down, fixed in 70% ethanol, washed, resuspended in DNA staining solution containing propidium iodide - PI (20 µg/ml) and DNase-free RNase (0.2 mg/ml; Sigma Aldrich), and incubated for 30 min at room temperature. DNA content was assessed by flow cytometry.

ELISA

Quantitative analysis of IFN-γ, IL-4 and IL-10 was performed by enzyme-linked immunosorbent assay (ELISA), using commercially-available kits (R&D Systems). Supernates were derived from 3-day cultures of spleen cells obtained from mice with EAE transplanted with BMSC or control mice with EAE. Cells were stimulated with PLP_{139–151} peptide or left unstimulated. Supernates were frozen and analyzed according to the manufacturer's instructions. Standard, control and test samples were added to each well and incubated for 2 h at room temperature. After washing four times, mouse IFN-γ, IL-4 or IL-10 conjugate was added to each well and incubated for 2 h. After repeating the washing procedure, substrate solution was added to each well for 30 min at room temperature in the dark. Finally, Stop Solution was added and the optical density of each well was determined within 30 min using a microplate reader.

Western blotting

Cell lysates were obtained from control mice with EAE and Lin[−]Sca1⁺BMSC transplanted mice with EAE, 2 weeks after transfer. Presence of IDO protein was analyzed in spleen cells, magnetically-sorted CD11c⁺ dendritic cells (DC), and CD11c[−] cells, as well as in Lin[−]Sca1⁺ BMSC. In some cases, prior to lysis, cells were incubated with IFN-γ for 24 h in culture. Cells were pelleted and lysed in lysing buffer (20mM Tris-HCl [pH 7.4], 0.15 M NaCl, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM Na₃VO₄, 1mMPMSF, 1 µg/ml aprotinin and leupeptin). Lysates were centrifuged at 14,000 rpm for 5 min. An equal amount of protein from each cell lysate was separated by SDS-PAGE electrophoresis and transferred to PVDF membranes (Immobilon, Millipore). Membranes were blocked with 5% dried milk in Tris-buffered saline-TBS (Sigma) overnight, followed by incubation for 1–2 h with 1 µg/ml of antibody specifically recognizing IDO (Chemicon). After washing with TBS, blots were incubated with peroxidase-coupled secondary anti-mouse antibody (1:20,000; Sigma-Aldrich). Proteins were detected by enhanced chemiluminescence-ECL (Amersham). Densitometry was performed for comparison of Western blot data (Alpha Innotech, San Leandro, CA), results were presented as IDV – Integrated Density Value.

Statistical analysis

All data are expressed as the mean ± S.D. and differences between groups were determined using the Student *t*-test; probability values of <0.05 were considered significant.

RESULTS

Transplantation of Lin⁺Sca1⁺ BMSC promotes recovery from EAE and prevents relapses

Mice receiving 2×10^6 Lin⁺Sca1⁺ BMSC showed significantly lower clinical scores and greater improvement compared to control mice with EAE - Fig.1. Seven days post-transfer, animals had a mean clinical score lower by one grade (1.0 ± 0.35). Furthermore, the residual EAE score 30 days after peak of disease in Lin⁺Sca1⁺BMSC-transferred mice was still significantly lower (by a grade of 1.5 ± 0.46) than in control animals ($p = 0.00095$). Over a 90 day period, Lin⁺Sca1⁺BMSC-transplanted animals showed no exacerbations whereas control mice experienced at least one relapse at about 30 days post-induction of EAE - Fig. 1A. Histopathologically, control animals with EAE displayed extensive meningeal inflammation and broad zones of subpial involvement displaying demyelination and nerve fiber damage (Wallerian degeneration) – Fig. 1B. On the other hand, Lin⁺Sca1⁺BMSC-treated mice at the same timepoint showed a moderate reduction in inflammatory cell infiltration and demyelination, areas of CNS damage that were more restricted, and a striking decrease in the amount of Wallerian degeneration - Fig. 1C. However, in contrast to control mice with EAE, the CNS transferred mice displayed diffuse remyelination in the spinal cord which increased with time post-treatment - Fig. 1D,E. Evidence of prior nerve fiber damage was invariably present in treated animals but the extent was never as widespread as that seen in controls.

Lin⁺Sca1⁺ BMSC transfer inhibits PLP_{139–151} - specific proliferation of spleen lymphocytes

Two weeks following transfer of Lin⁺Sca1⁺ BMSC, proliferation of spleen-derived T cells in response to PLP_{139–151} was assessed and found to be strongly inhibited in comparison to control mice with EAE (SI: 1.8 versus 8.9, respectively; $p = 0.0019$) - Fig.2A. At 10 weeks post-transfer, PLP_{139–151} -induced proliferation of T cells continued to be lower than in T cells from control EAE mice (SI: 1.8 versus 5.5 respectively; $p = 0.0025$).

Lin⁺Sca1⁺BMSC-induced loss of PLP reactivity correlates with high secretion of IFN- γ

Inhibition of T cell proliferation to PLP_{139–151} in Lin⁺Sca1⁺BMSC-transferred mice showed strong correlation with IFN- γ secretion. There was a 15 fold increase in PLP_{139–151}-induced IFN- γ secretion in mice two weeks post-transplantation compared to levels in untreated EAE mice - Fig.2B. Interestingly, increase in IFN- γ by PLP-reactive cells was still present 10 weeks post-transfer - Fig.2B. Thus, transplantation of these cells induced an increase in IFN- γ secretion which persisted for a prolonged period. This was associated with lower clinical severity and prevention of relapses. In addition, in transferred mice, no increase in IL-4 or IL-10 was observed in response to PLP_{139–151} stimulation, compared to control mice with EAE, and levels of IL-4 and IL-10 remained low (data not shown).

Transfer of unsorted bone marrow cells or Lin⁺Sca1⁺BMSC does not affect EAE

To address the specificity of Lin⁺Sca1⁺BMSC-induced amelioration of EAE, we then tested the effect of unsorted, whole bone marrow cell populations as well as Lin⁺Sca1⁺BMSC upon the course of EAE. Unsorted bone marrow cells and Lin⁺Sca1⁺BMSC were injected i.v. at a concentration of 2×10^6 at the peak of EAE, in parallel with the injection of Lin⁺Sca1⁺BMSC into a matching group of mice. In contrast to the beneficial effect of Lin⁺Sca1⁺BMSC, in mice transferred with unsorted whole bone marrow cells or Lin⁺Sca1⁺BMSC, no effect was seen over a 30 day period - Fig.3.

Transplanted Lin⁺Sca1⁺ BMSC do not specifically accumulate in the CNS

Flow cytometry performed at 7, 14 and 42 days after i.v. transplantation of Lin⁺Sca1⁺ BMSC stained with the fluorescent dye, PKH26, showed accumulation of transplanted cells in many organs (spleen, liver and bone marrow – 3%, 1% and 1% of the whole cell population,

respectively) - Fig. 4A. There was no clear difference in organ distribution of Lin⁻Sca1⁺ BMSC between naïve mice and mice immunized with PLP for EAE. In mice with EAE, we found that Lin⁻Sca1⁺BMSC had entered the CNS by day 7 after iv transplantation and localized predominantly to brainstem and upper and lower spinal cord. However, frequencies of Lin⁻Sca1⁺ BMSC in these regions were low compared to other organs (0.3%, 0.8%, 0.8%, respectively) – Fig. 4B, and in cerebral hemispheres, Lin⁻Sca1⁺ cells were rare (0.05%). Immunocytochemistry of frozen CNS tissue obtained from transplanted animals at timepoints corresponding to flow cytometry analysis confirmed the presence of low levels of Lin⁻Sca1⁺BMSC, predominantly in meningeal regions and only occasionally in CNS parenchyma - Fig.4C.

Lin⁻Sca1⁺ BMSC inhibit proliferation in response to spread of myelin antigen determinants

Exacerbations of clinical signs occurring after the initial acute phase of EAE have been attributed to immune responsiveness to new myelin epitopes – “antigen spreading” (McRae et al., 1995). In PLP_{139–151}-induced EAE, the immune response typically spreads to PLP_{178–191} and then to MBP_{85–99} (Yu et al., 1996). Since transfer of Lin⁻Sca1⁺ BMSC prevented clinical relapses, we examined proliferation of T cells to these “spread” myelin determinants during the relapsing phase of the disease. As anticipated, control mice with EAE displayed exacerbations that correlated with proliferative responses of T cells to both PLP_{178–191} and to a lesser extent, MBP_{85–99}. However, mice transplanted with Lin⁻Sca1⁺BMSC displayed no detectable reactivity to these new epitopes - Fig.5A.

Lin⁻Sca1⁺ BMSC do not induce death of PLP-reactive cells

Stem cell-induced immune regulation involves increased apoptotic death of antigen-activated T cells (Pluchino et al., 2005). To examine this, we assessed death of PLP-reactive cells two weeks after Lin⁻Sca1⁺ BMSC. Spleen cells tested at 24 and 72 h post-stimulation with PLP showed no increase in PI staining compared to control mice with EAE - Fig. 5B. These results indicated that Lin⁻Sca1⁺ BMSC-mediated inhibition of PLP-induced proliferation was not dependent on the selective death of antigen-specific cells.

Lack of induction of CD4⁺CD25⁺ regulatory cells in response to transfer of Lin⁻Sca1⁺ BMSC

In order to further elucidate mechanisms involved in the inhibition of the proliferative response to PLP_{139–151} in Lin⁻Sca1⁺BMSC-transferred mice, we assessed the generation of CD4⁺CD25⁺ regulatory T cells and expression of the co-inhibitory molecule, CTLA-4. Flow cytometry of spleen cells obtained 2 weeks after transfer showed no difference in frequency of CD4⁺CD25⁺ T cells compared to control mice with EAE, and no difference in fluorescent intensity of CD25- Fig. 5C. Also, expression of CTLA-4 on CD4⁺ T cells was not affected by Lin⁻Sca1⁺BMSC transfer - Fig.5C.

Increased indoleamine 2,3-dioxygenase in CD11c⁺ cells from Lin⁻Sca1⁺ BMSC mice

For elucidation of the inhibition of PLP_{139–151}-induced proliferation and the amelioration of EAE, we turned to regulatory mechanisms associated with high IFN- γ secretion. For this, we investigated IDO expression in mice transferred with Lin⁻Sca1⁺BMSC. In spleens obtained two weeks after transfer, we noticed a greater than 2-fold increase in IDO expression compared to untreated EAE - Fig 6A. In addition, spleen cells from transferred mice had a greater potential to express IDO after IFN- γ stimulation than spleen cells from control mice - Fig.6A. Increased IDO expression in Lin⁻Sca1⁺BMSC transfer mice correlated with increased secretion of IFN- γ , inhibition of PLP_{139–151}-specific proliferation of T cells and clinical improvement.

Lin⁻Sca1⁺ BMSC alone expressed very low levels of IDO (data not shown). In order to elucidate which cell population was responsible for increased IDO, spleen cells, isolated 2

weeks after Lin⁺Sca1⁺ BMSC transplantation, were divided into CD11c⁺ and CD11c[−] populations. This revealed that IDO was present predominantly in the CD11c⁺ dendritic cell (DC) population, whereas the CD11c[−] population showed very little expression of IDO - Fig. 6B. These findings suggest that inhibition of EAE by Lin⁺Sca1⁺BMSC involved regulation by IDO-positive DC.

Inhibition of PLP reactivity by CD11c⁺ dendritic cells expressing IDO

To demonstrate the instrumental role of CD11c⁺ DC and increased IDO expression with enhanced recovery from EAE and inhibition of relapses, we performed co-culture experiments using CD11c⁺ cells isolated from mice treated with Lin⁺Sca1⁺ BMSC and PLP-reactive spleen-derived T lymphocytes. PLP-induced proliferation was significantly reduced ($p=0.008$) in the presence of CD11c⁺ cells with high IDO expression - Fig. 6C. Dependence of inhibition of PLP reactivity on CD11c⁺ cells and IDO was confirmed by the demonstration that CD11c⁺ DC isolated from mice transferred with Lin[−]Sca1[−] BMSC which did not show enhanced IDO expression, had no effect on PLP-induced proliferation.

Inhibition of IDO reverses tolerance induced by Lin⁺Sca1⁺BMSC

To prove that IDO was involved in Lin⁺Sca1⁺BMSC-induced amelioration of EAE, we then treated control EAE and Lin⁺Sca1⁺BMSC-transplanted mice with the specific competitive inhibitor of IDO, 1-methyl-DL-tryptophan (1-MT). This showed that in mice treated with 1-MT beginning one day prior to transfer of Lin⁺Sca1⁺BMSC continuing every second day for the entire period of observation, recovery from EAE was not affected. The mean clinical score for this group of mice was significantly higher – grade 3.5, ($p=0.000912$), than that of mice not treated with 1-MT but transferred with Lin⁺Sca1⁺BMSC, grade 2.35 Fig. 6D.

Taken together, these results strongly suggest that induction of IDO was a critical factor underlying Lin⁺Sca1⁺BMSC-induced amelioration of EAE, a conclusion further supported by the demonstration that blocking IDO led to loss of the immunoregulatory effects of Lin⁺Sca1⁺BMSC.

DISCUSSION

In this communication, we focused on the mechanistic effects of pluripotent Lin⁺Sca1⁺BMSC on EAE in a paradigm where SC were given at peak of disease. While confirming a number of recent reports (Pluchino et al., 2003; Zappia et al., 2005; Einstein et al., 2007; Gerdoni et al., 2007; Pluchino et al., 2005) showing that transfer of both mesenchymal and neural SC into animals sensitized for EAE diminishes disease severity, we also extend the field by showing that the ability of pluripotent Lin⁺Sca1⁺BMSC to ameliorate EAE is mechanistically related to the induction of indoleamine 2,3-dioxygenase (IDO) in CD11c⁺ DC. Although SC-induced clinical improvement has been found to correlate with reduced reactivity of T cells to the encephalitogenic antigen employed (Zappia et al., 2005), the precise tolerogenic mechanism remained unknown. In accord with previously published findings (Zappia et al., 2005), when SC were administered to mice with clinical signs, a faster rate of recovery from EAE was observed that was statistically significant ($p=0.00095$). In addition, mice given Lin⁺Sca1⁺BMSC displayed no relapses after the first bout of disease. During relapses of untreated EAE in SJL/J mice, animals usually develop T cell responsiveness to new antigenic determinants, a feature also evidenced in our study. This phenomenon is known as antigen spreading (McRae et al., 1995; Yu et al., 1996). However, mice with EAE treated with Lin⁺Sca1⁺BMSC, showed no development of responsiveness (T cell proliferation) to new myelin antigenic determinants, (PLP_{178–191} and MBP_{85–99}), in contrast to untreated mice sensitized for EAE. Thus, Lin⁺Sca1⁺BMSC-transfer provided a mechanism which not only facilitated recovery from EAE but also protected against development of subsequent relapses

by preventing antigen spreading. At the level of the CNS, animals treated with Lin⁻Sca1⁺BMSC showed a considerable reduction in inflammation and demyelination that corresponded to the improved clinical signs. Interestingly, amelioration of EAE by Lin⁻Sca1⁺BMSC correlated also with a substantial degree of remyelination within the CNS that increased with time post-transfer.

The reduction of inflammation within the CNS might suggest that Lin⁻Sca1⁺BMSC were immunoregulatory predominantly in the peripheral compartment of the immune system. Accordingly, transplanted PKH-labeled Lin⁻Sca1⁺BMSC were seen only occasionally within the CNS and were restricted to submeningeal areas when present, being exceedingly rare in CNS parenchyma. In contrast, transplanted Lin⁻Sca1⁺BMSC were easily located in other organs, including spleen, liver and bone marrow. In agreement with previous reports (Meisel et al., 2004; Beyth et al., 2005), we encountered a substantial effect of SC on antigen-induced T cell proliferation. However, unlike Zappia et al, we found that stimulation of spleen lymphocytes with antigen (PLP) led to significant levels of IFN- γ secretion. The difference in effect of SC on IFN- γ production between this previous study and the present, might be related to the use of different stem cell populations. Zappia et al used mesenchymal SC derived from cultured bone marrow cells exposed to a series of passages in selection media whereas we used freshly-isolated pluripotent BMSC. Also, Zappia et al co-cultured mesenchymal SC with lymphocytes from animals with EAE, whereas we assessed PLP-induced proliferation with spleen lymphocytes obtained *ex vivo* from mice transferred with Lin⁻Sca1⁺BMSC with ameliorated EAE. This might indicate that the environment created by BMSC *in vivo* is required for induction of IFN- γ . Although IFN- γ is a Th1-type cytokine with strong proinflammatory properties, it has also been convincingly implicated in several immunoregulatory processes (Willenborg et al., 1996; Willenborg et al., 1999). IFN- γ deficient mice display enhanced manifestations of EAE (Chu et al., 2000), and IFN- γ inhibits T cell reactivity to antigen (Liu and Janeway, 1990).

IFN- γ immunoregulation involves two possible mechanisms, namely, activation-induced cell death (AICD) (Rafaeli et al., 2002), and induction of IDO (Terness et al., 2006). AICD depends on antigen- induced apoptotic death of effector T cells (Rafaeli et al., 2002). However, in EAE mice treated with Lin⁻Sca1⁺BMSC, we observed no enhancement of T cell death in response to PLP - therefore, it was unlikely that AICD was responsible for the amelioration of disease. A similar observation was made in the recent report by Einstein et al (Einstein et al., 2007). Furthermore, we saw no increase in CD4⁺CD25⁺ regulatory cells in the same mice and expression of the co-inhibitory molecule, CTLA-4, was not changed.

Distinguishing the present study from its recent predecessors (Pluchino et al., 2003; Zappia et al., 2005; Einstein et al., 2007; Gerdoni et al., 2007; Pluchino et al., 2005) is the mechanistic bent of our approach and the novel demonstration of a role for IDO. IDO is a tryptophan catabolizing enzyme that converts tryptophan to N-formylkynurenine, and its expression and activation have been shown to correlate with T cell unresponsiveness (Terness et al., 2002; Frumento et al., 2002). The mechanism of IDO-dependent immunoregulation depends either on immune cell deprivation of tryptophan or generation of tryptophan derivatives, such as kynurenine, 3-OH-kynurenine and 3-OH-anthranilic acid (Terness et al., 2006). IDO has been shown to be selectively induced by IFN- γ in many cell types (Byrne et al., 1986). Upon transfer of Lin⁻Sca1⁺BMSC into EAE mice, we observed increased IDO in CD11c⁺ DC which correlated with decreased T cell reactivity to PLP. In addition, IFN- γ displayed a greater potential to induce IDO in DC from SC-transferred mice. BMSC alone showed only marginal expression of IDO, indicating a primary role in induction of IDO in professional antigen presenting cells. The selectivity of the induction of IDO in DC by Lin⁻Sca1⁺BMSC in mice with ameliorated EAE was confirmed by the demonstration that IDO was not up-regulated in mice transferred with unsorted whole bone marrow cells. Accordingly, mice transferred with

whole bone marrow cells or Lin[−]Sca1[−]BMSC showed no modification of EAE. The definitive proof of a functional role for IDO in BMSC-induced amelioration of EAE came from the demonstration that in the presence of CD11c⁺ DC with high IDO expression, PLP-induced proliferation was reduced. Moreover, application of a specific and competitive IDO inhibitor, 1-methyl-DL-tryptophan-(1MT), led to loss of the ameliorating effect. This gives added currency to our conclusion that IDO expressed by DC played a critical role in Lin[−]Sca1⁺BMSC-mediated improvement of EAE. Of particular relevance to our findings is the observation that human bone marrow stromal cells blocked allogeneic T cell responses elicited by IDO-mediated tryptophan degradation (Meisel et al., 2004). In support of this is recent work showing that synthetic tryptophan metabolites are capable of suppressing proliferation of myelin-specific T cells and reversing paralysis in mice with EAE (Platten et al., 2005). It is also of interest that in estrogen induced EAE tolerance expression of IDO in dendritic cells was observed (Xiao et al., 2004). Similarly the reduced exacerbation and progression in MS during pregnancy was also linked with IDO (Zhu et al, 2007). Thus, induction of IDO in dendritic cells might be a common pathway leading to immune regulation in EAE and MS.

Taken in concert, we have not only shown that SC (in this case, pluripotent Lin[−]Sca1⁺BMSC) ameliorate EAE, thus confirming recent observation from several laboratories (Pluchino et al., 2003; Zappia et al., 2005; Einstein et al., 2007; Gerdoni et al., 2007; Pluchino et al., 2005), but also that the underlying molecular mechanism depended on the induction of IDO within DC, which in turn led to decreased antigen-induced reactivity of T cells and a lack of antigen spreading. Since these mechanisms were operating in a model in which the matching histopathologic read-out was decreased CNS damage and enhanced repair of myelin, this approach bodes well for future strategies to treat multiple sclerosis.

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Figure 1.

Transfer of $\text{Lin}^- \text{Sca1}^+$ BMSC into EAE mice at peak of disease. **A)** At peak of disease, mice with EAE were injected i.v. with 2×10^6 $\text{Lin}^- \text{Sca1}^+$ BMSC (arrow) and were assessed clinically according to the grading system given in Materials and Methods. The results show the average scores from 12 mice (3 experiments). SD remained within 15%. Shortly after cell transfer, clinical severity decreased on average by a grade of 1.0 ± 0.35 within 19 days post-cell transfer. Note that transferred mice were also protected from subsequent exacerbations after day 30; the average difference in EAE score between $\text{Lin}^- \text{Sca1}^+$ BMSC-treated (-▲-) and control mice with EAE (-■-) within 60 days after day 30, was 1.5 ± 0.46 . **B–E)** Histopathology of CNS from normal and $\text{Lin}^- \text{Sca1}^+$ BMSC- transferred mice. Toluidine blue staining of 1 μm epoxy sections

of L6 spinal cord; left mag. x300; right mag x750. **B)** Control mouse with EAE. Note the large lesion along meningeal surface of the spinal cord, comprising inflammation, demyelination and nerve fiber damage 2 wk after peak of disease. To the right, widespread Wallerian degeneration and inflammation is shown at higher magnification. **C)** Lin⁻Sca1⁺BMSC-treated EAE, 2 wk post-transfer with Lin⁻Sca1⁺BMSC. Note less extensive involvement of the spinal cord with damage being restricted to a narrow, subpial rim. On the right, higher magnification shows the margin of the cord to contain a narrow zone of fibers undergoing Wallerian degeneration. **D)** 4 wk post Lin⁻Sca1⁺BMSC-transfer. The subpial zone of damage shows some ongoing nerve fiber damage and gliosis. At higher magnification (right), a narrow zone of thinly remyelinated fibers (outlined), can be seen. Nerve fibers undergoing Wallerian degeneration are also present. **E)** 6 wk post Lin⁻Sca1⁺BMSC-transfer. The subpial zone of spinal cord at low magnification (left) shows some myelin pallor which at higher magnification, corresponds to a zone of remyelinated nerve fibers (outlined). A few fibers undergoing Wallerian degeneration are also apparent.

Figure 2.

Lin⁻Sca1⁺ BMSC transfer inhibits PLP₁₃₉₋₁₅₁ - specific proliferation of spleen lymphocytes.

A) PLP-induced proliferation of spleen cells from Lin⁻Sca1⁺BMSC -transferred (white) and control EAE mice (black). Left panel shows inhibited proliferation 2 weeks post Lin⁻Sca1⁺BMSC-transfer (*p=0,0019), and the right panel, 10 weeks post-cell transfer (**p=0,0025); and **B)** IFN-γ secretion by PLP-stimulated spleen cells from Lin⁻Sca1⁺BMSC - transferred and control EAE mice. Left panel shows a 15 fold increase at 2 weeks post-Lin⁻Sca1⁺BMSC transfer; Right panel, a 10 fold increase at 10 weeks post-cell transfer. The results show the average scores from 12 mice (3 experiments).

A.

B.

Figure 3.

Transfers of whole bone marrow cell or Lin⁻Sca1⁻BMSC population do not affect the course of EAE. **A)** 2×10^6 unsorted bone marrow cells were injected i.v. and EAE was assessed according to the clinical scale given in Materials and Methods. No difference between whole (unsorted) bone marrow- transferred(-▲-) and control (untreated) mice with EAE (-■-) were noted. The results show the average scores from 10 mice (2 experiments). **B)** 2×10^6 Lin⁻Sca1⁻BMSC freshly isolated from bone marrow were injected i.v. and EAE was assessed as above. No differences in clinical course between Lin⁻Sca1⁻BMSC (-▲-) and control EAE mice (-■-) were observed. The results show the average scores from 10 mice (2 experiments).

Figure 4.

Flow cytometry of Lin⁻Sca1⁺ BMSC homing to the CNS and other organs. Immediately after isolation, Lin⁻Sca1⁺BMSC were stained with red fluorescent dye, PKH26, and transferred, to mice with EAE, 1 and 2 weeks after transplantation, localization of Lin⁻Sca1⁺BMSC was examined by flow cytometry in peripheral organs (A) and different compartments of the CNS (B). The presence of Lin⁻Sca1⁺BMSC in CNS was confirmed by fluorescence microscopy only in submeningeal areas (C).

Figure 5.

Lin⁺Sca1⁺ BMSC-induced inhibition of antigen spreading is not dependent on apoptosis of PLP-reactive cells and does not involve regulatory cells. **A)** Spleen cells from control mice with EAE and Lin⁺Sca1⁺ BMSC-transplanted mice with PLP₁₃₉₋₁₅₁-induced EAE were isolated 2 and 10 wk after transplantation and stimulated with other myelin antigens. Bars show the proliferative responses (cpm) of unstimulated spleen cells (dotted bars), and spleen cells stimulated with PLP₁₇₈₋₁₉₁ (striped bars) and with MBP₈₅₋₉₉ (black bars). Note the inhibition of antigen spreading in Lin⁺Sca1⁺BMSC-transferred mice ($p=0.009$) which was present in control EAE at time of relapses (10 wk after transplantation); **B)** Mice with EAE were injected i.v. with Lin⁺Sca1⁺ cells. At 2 wk post-cell transfer, cell death was assessed by flow cytometry

of PI staining. No difference in number of dead cells was noted between PLP-stimulated spleen T cells in Lin⁻Sca1⁺BMSC-transferred and control mice with EAE ($p=0,059$). C) At 2 wk post- Lin⁻Sca1⁺BMSC transfer, spleen T cells were stained for CD25 and CTLA-4 and the frequency of positive cells was measured by flow cytometry. No differences were noted between Lin⁻Sca1⁺BMSC-transferred and control mice with EAE.

Figure 6.

Lin $^{-}$ Sca1 $^{+}$ BMSC-induced improvement of EAE depends on IDO expression by CD11c $^{+}$ dendritic cells. **A)** At 2 weeks post-BMSC transfer, spleen cells or CD11c $^{+}$ DC were obtained and processed for Western blotting for IDO expression. Note the significantly higher levels of IDO in spleen cells from Lin $^{-}$ Sca1 $^{+}$ BMSC-transferred mice ($p=0,019$). Spleen cells exposed to IFN- γ showed increased expression of IDO. The results show the average scores from 12 mice (3 experiments) **B)** IDO was specifically expressed at high levels in CD11c $^{+}$ dendritic cells but not in CD11c $^{-}$ cells. **C)** CD11c $^{+}$ cells were isolated from mice 2 wk after Lin $^{-}$ Sca1 $^{+}$ transfer and were incubated at the ratio of 1:30 with PLP-reactive spleen T lymphocytes. The bars show proliferation in response to PLP₁₃₉₋₁₅₁, 10 μ g/ml. Note inhibition of PLP-specific

proliferation of T lymphocytes co-cultured with CD11c⁺ with high IDO expression (*p=0.008) but not with CD11c⁺ cells with low IDO. **D**) Lin⁻Sca1⁺BMSC-transferred mice were given 1-MT every second day for a period of 5 weeks and assessed clinically according to the scale given in Methods. Mice treated with 1-MT (-□-) had higher clinical scores than untreated mice (-■-). There were no obvious differences in clinical scores between 1-MT treated and control EAE mice (-▲-). The results show average scores from 8 mice (2 experiments). SD remained within 15%.