Autoreactive T cells induce *in vitro* BM mesenchymal stem cell transdifferentiation to neural stem cells

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**Background**

The degree of post-injury inflammation of the damaged area of a spinal cord is the main difference between the natural successful repair in inferior vertebrates and failure in superior vertebrates. The treatment of rats with anti-myelin lymphocytes after experimental spinal cord injury induces their functional recovery. On the other hand, mesenchymal stem cells (MSC) from adult BM implanted in injured areas recover the morphology and function of spinal cord in mammals. The purpose of this study was to determine whether there is a direct relationship between anti-nervous tissue T cells and MSC reparatory properties.

**Methods**

Circulating autoreactive lymphocytes of patients with spinal cord injuries and amyotrophic lateral sclerosis were isolated and activated in vitro. These cells were cocultured with autologous MSC for 2–15 days. Cocultures of non-selected lymphocytes were used as controls.

**Results**

After 48 h of coculture, MSC adopted a spindle shape with polarization of the cytoplasm that resembled bipolar neurons. Their nuclei diminished the nucleolus number and the chromatin lost its granular appearance. After 15 days of culture the cells developed the typical structure of a neural network. No morphologic changes were observed in control cultures. The differentiated cells reacted positively to tubuline III, GFAP and nestin. No differences were observed between the different patient cell sources.

**Discussion**

We observed that autoreactive cells may induce the transdifferentiation of MSC to neural stem cells. This T-cell–MSC interaction may be a common phenomenon during physiologic nerve tissue repair.

**Keywords**

ALS, MSC, NSC, protective autoimmunity, spinal cord injury, transdifferentiation.

**Introduction**

During the last few years, various laboratories have collected data and observations that have led us to the assumption that BM mesenchymal stem cells (MSC) [1], as well as the autoreactive T cells specific against nervous tissue [2], have a physiologic role in the reparatory process of the central and peripheral nervous systems. MSC received their name because of their ability to differentiate into multiple mesodermal tissues such as bone, cartilage, fat and muscle [3]. In addition, MSC are capable of moving along migration pathways if injected into corpus striatum of rats [4]. It was observed that, after their infusion into the lateral ventricle of neonatal mice, MSC migrated through the forebrain and cerebellum to end up integrated into central nervous system (CNS) cell structures [5]. During this process MSC started to express typical markers of mature astrocytes, oligodendrocytes and neurons. Moreover, it was possible to achieve transdifferentiation of MSC to neural stem cells (NSC) using a concurrence of several neurotrophins [6]. This phenomenon suggests that MSC may overcome germ layer commitment. These neurotrophins are also necessary to induce and sustain the reparatory ability of the CNS in different animal and human models [7].

On the other hand, Moalem *et al.* [2] demonstrated that the exogenous administration of syngenic autoreactive
Autoreactive T cells induce MSC transdifferentiation to NSC

Ventricular T cells in experimental animals for a period of up to 15 days after experimental spinal cord injury led to an almost complete repair of the organ, thus recovering its respective function. This observation was coincidental with findings from other investigators who established that, once activated by neurologic Ag, autoreactive lymphocytes secrete neuro growth and cell-differentiation factors [8].

Because autoreactive anti-nervous tissue CD4 effector and regulator cells secrete a similar spectrum of the above-described neurotrophins [8,9], we envisioned the possibility part of the reparatory function of these lymphocytes may be related to their direct action on the MSC transdifferentiation. The present paper describes a series of in vitro experiments performed to investigate whether there is a direct relationship between anti-nervous tissue T cells and the reparation properties of MSC, based on their transdifferentiation to NSC.

Methods

Autoreactive T lymphocyte purification

Autoreactive lymphocytes from patients suffering from spinal cord lesions and amyotrophic lateral sclerosis (ALS) were isolated from peripheral blood. This procedure is a modification of a previously described technique [10].

Briefly, we performed an apheresis using a Cobe Spectra (Gambro BCT, CO, USA), apheresis machine. We processed about two blood volumes for each patient using the buffy coat program. The obtained buffy coat suspension comprised 85% MNC, 1.8 \times 10^6/\mu L RBC and 6 \times 10^7/\mu L platelets. To purify this MNC sample, we seeded it on a Ficoll–Hypaque gradient (Beckman Coulter Inc, Fullerton, CA, USA) (1.077 density). After this procedure, the composition of the ring was approximately 98% MNC, 0.2 \times 10^6/\mu L RBC and 1 \times 10^6/mL platelets.

This MNC suspension was cultured for 4 days in DMEM enriched with a partial hydrolyzate of bovine brain and a partial hydrolyzate of bovine spinal cord (Laboratorios Villar, Santa Fe, Argentina). After 2 days in cell culture, we added IL-2 (Laboratorio Gautier, Buenos Aires, Argentina) until it reached a final concentration of 100 IU/mL.

At day 5, CD3^+ lymphocytes were isolated by negative selection using a MAb cocktail against the undesired cells (MAb against CD14, CD16, CD19, CD56 and glycoporphin A). This first incubation was followed by a second incubation with a solution of Ab attached to a paramagnetic Teflon bead (Stem Sep Kit; Stem Cell Technology, Vancouver, Canada).

After the immune labeling of the cell suspension, these lymphocytes were passed through a powerful magnetic field, which allowed the passage of CD3^+ cells and retained the rest of the cells. The CD3^+ cells enriched the purity of the purged cell suspension up to 96%.

This CD3^+–enriched suspension was then marked with an anti-CD25 MAb solution (Stem Sep Kit). The cells were then attached to a paramagnetic Teflon bead for a new selection, thus obtaining two different cell population phenotypes, one CD3^+ CD25^- and one CD3^+ CD25^+. The proportions of these two subsets varied depending on the particular condition of each donor.

BM MSC harvest, purification and expansion

A sample of approximately 600/cm^3 BM mononuclear cells was obtained through a needle aspiration of iliac crest BM. This sample presented approximately 8 – 10 \times 10^7 total MNC. Its composition was 20% lymphocytes, 5% MID cells and 75% granulocytes, with 1.6 \times 10^7/\mu L platelets and 4 – 5 \times 10^6/\mu L RBC. This sample showed an immune CD133 labeling of approximately 1.5 – 2 \times 10^6 total cells.

The MSC were purified from the BM cell suspension by negative selection using a MAb cocktail (Rosette Sep Kit; Stem Cell Technology) and a Ficoll–Hypaque gradient. Briefly, the unprocessed BM suspension was concentrated by centrifugation at 300 g. Cells were suspended in a human MSC-enriched cocktail composed of MAb against CD3, CD4, CD19, CD38, CD66b and glycoporphin A. The MAB cocktails produced cross-links between the unwanted cells and the RBC in the suspension, forming immune rosettes. This particular situation increased the density of the unwanted cells so that they were pelleted along with the free RBC when they were centrifuged over a buoyant density medium such as Ficoll–Hypaque (1.077 density). After this incubation, MSC remained unlabelled and unclamped, therefore they could easily be collected as a highly enriched population at the interphase between the plasma and the buoyant density medium. By these means we are able to increase the concentration of MSC by 8-fold. The total amount of cells suspended in the ring ranged from 1.5 to 2 \times 10^6.

The MSC obtained were then cultured in Mesencult medium (Stem Cell Technology) for 4–6 weeks at a
concentration of $1 \times 10^6$ cells/mL, in 25-mm$^2$ plastic Petri dishes at 37°C and an atmosphere of 95% O$_2$, 5% CO$_2$ in a Thermo Forma® CO$_2$ incubator.

The culture was controlled using a phase-control inverted microscope (Nikon Eclipse TS 100). MSC reached a semi-confluent stage at approximately 15 days. At that moment, the selected cells were removed and reseeded on different plastic Petri dishes. We repeated this process twice during a 4–6-week period.

**Coculture of autologous autoreactive T cell and BM MSC**

After 4–6 weeks of culture, when MSC reached the semi-confluent stage, as previously described, for the third time, they were reseeded on 22-mm$^2$ cover slips in two 6-well plates. Fifteen days later, the 12 wells were divided into three different groups of four wells each.

The first group was coincubated with unselected mononuclear cells from the patient and kept as a control. The second group was incubated with the CD3$^+$ CD25$^-$ lymphocytes from the patient. The third group was incubated with the CD3$^+$ CD25$^+$ lymphocytes from the patient.

**Immunohistochemistry**

After 48 h, without removing the autoreactive T cells, the cover slips were removed from the wells and fixed in 4% PBS paraformaldehyde. A Giemsa (Merck) stain was performed for the first cover slip in each group. The second cover slip in each group was immunoperoxidase stained for nestine (R&D Systems Inc.). The third was immunoperoxidase stained for class III beta-tubulin (R&D Systems Inc.). The fourth was immunoperoxidase stained for GFAP (Zymed® Laboratories Inc.).

**Informed consent**

Informed consent was obtained from the participant patients after the nature and possible consequences of the studies were explained.

**Results**

MSC were cultured in a 20-cc Roux bottle at a concentration of $1 \times 10^6$ cells/mL. Every 15 days cells reached a semi-confluent stage and by that time we could observe a flat epithelium sheet that resembled mesothelium cells. In other words, they were polygonal cells with clear cytoplasm, a poor number of organellas and inclusions (Figure 1).

The nucleus was large with a very homogeneous and open chromatin background and the presence of five to seven small nucleoli/cell. Regardless of the MSC or T-cell source, at 48 h of contact with CD3$^+$ CD25$^-$ lymphocytes we observed that almost 80% of the cells present in the tissue culture changed their aspect, adopting a spindle shape. These cells also presented a clear polarization of their cytoplasm and an important nucleus with more intensely stained chromatin and the presence of one or two larger nucleoli only. These morphologic changes occurred only on 30% of the MSC coincubated for same 48-h period with CD3$^+$ CD25$^+$ cells (Figure 2).

The polarization of the cytoplasm was characterized by the accumulation of basophile cytoplasm at one end of the spindle cell. The nucleus was located in the central area of the cells with poor cytoplasm and the opposite end with a
very clear cytoplasm condition (Figure 2). This description was coincidental with the juvenile bipolar cells observed during the development of the lateral ganglia of the spinal cord. None of these changes were observed in the control cocultures (Figure 1).

When we immunostained these cells to mark specific neuron and glia markers, more than 80% of the cells got at least one of the markers (nestin, class III beta-tubulin, GFAP) when the cells were coincubated with CD3+ CD25+ and 30% of the cells when MSC were coincubated with CD3+ CD25+ cells. Only a few cells from the control culture showed a nestin-positive reaction (Figure 3). Fifteen days later we performed a fast contrast observation only, and observed that cells developed multipolar prolongations that attached to one another and resembled a typical neuron or glia network (Figure 4).

**Discussion**

It is well known that the inferior vertebrates, fish, amphibians and reptiles, are capable of completely repairing important traumatic lesions in both the central and peripheral nervous systems. The superior vertebrates, on the other hand, either possess a poor reparation response or lack a response in the case of medium and severe trauma, respectively [2]. The most significant difference Moalem *et al.* [2] found in these biologic examples was related to the extent of inflammation at the site of the lesion. Whereas inflammation was very intense, with substantial presence of macrophages and lymphocytes in the case of inferior vertebrates, the same process was 10 times less significant in superior vertebrates (birds and mammals).

By the second half of the 1990s, the studies carried out by Michal Schwartz's team, together with Irun Cohen and collaborators, all from the Weizmann Institute, Rehovot, Israel, found that the lymphocytes present in the lesion site were autoreactive cells specific against nervous tissue, such as those observed in autoimmune neuritis, multiple sclerosis and experimental allergic encephalitis [2]. Furthermore, both teams demonstrated that the exogenous administration of syngenic autoreactive T cells in experimental animals, for a period of up to 15 days after the lesion was produced, led to an almost complete repair of the organ, thus recovering its respective function [2]. Supporting these observations, Ehrhard *et al.* [10], Kerschensteiner *et al.* [8], Moalem *et al.* [12] and Muhallab *et al.* [13] established that, once activated by neurologic Ag, autoreactive lymphocytes secrete neurotrophins that are growth and differentiation neural cell factors.

Several groups have proved that BM-originated MSC can be induced *in vitro* to transdifferentiate into NSC. To achieve this biologic process, the researchers added a mix of different leukines and neurotrophins [14–17]. Most of them, as we described in the previous paragraph, are produced by the activated specific neurotoxic lymphocytes and its regulators.

![Figure 3. Immunoperoxidase staining of 24-h cocultured MSC-autoreactive T cells.](image3)

![Figure 4. MSC culture after 21 days of coincubation with autologous anti-brain T-cells.](image4)
Nestin [18] is considered an early differentiation marker for NSC, GFAP [18,19] is considered a common differentiation neuroglia maker, and tubuline III [20] is considered a neuron differentiation marker. The poor expression or absence of these markers in the untreated MSC culture as well as their appearance after 48 h of coculture with the anti-neural effector T cells suggest that these T cells induce the transdifferentiation of MSC to NSC. Moreover, the difference between the number of NSC induced by the different T-cell subsets is related to the different role attributed to these two cell populations, CD3\(^+\) CD25\(^−\) and CD3\(^+\) CD25\(^+\) [2,8,9,21].

The CD3\(^+\) CD25\(^−\) cells appear to be not only responsible for the cell recognition and destruction of the damaged nerve cells but also to be powerful inducers of NSC growth and differentiation. The effect observed in the CD3\(^+\) CD25 \(^+\) population indicates that only one-fourth of these cells appears to be related to the differentiation effect. The poor inductor differentiation effect may be attributed to this second population. Apparently between 15% and 30% of the MSC may be pre-differentiated to become NSC [9,15].

We believe that this in vitro phenomenon may be part of the natural reparatory process that takes place in vivo [22]. Therefore the reproduction of the specific effector reaction that happens naturally after an acute nervous system injury followed by the presence of the neo-formed NSC may play a key role in inducing the reparatory process. These cell processes are absent in chronic nervous system injuries as well as in neurodegenerative diseases. Clinical protocols using these concepts for the therapy of acute and chronic nervous system injuries and neurodegenerative disease are currently under development at our institute.

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**References**


