Bone marrow mesenchymal stromal cells (BM-MSCs) from healthy donors and auto-immune disease patients reduce the proliferation of autologous- and allogeneic-stimulated lymphocytes \textit{in vitro}

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\textbf{Objectives.} To investigate the ability of bone marrow (BM)-derived mesenchymal stromal cells (BM-MSCs) in suppressing the proliferation of stimulated lymphocytes across a range of conditions including autologous BM-MSCs derived from autoimmune disease (AD) patients.

\textbf{Methods.} \textit{In vitro} cultures of BM-MSCs from healthy donors and AD patients were established and characterized by their differentiation potential into adipocytes and osteoblasts, and their fibroblast-colony-forming unit (CFU-F) ability and phenotype by flow cytometry.

BM-MSCs (irradiated and non-irradiated) from healthy and AD patients were tested for their ability to suppress the \textit{in vitro} proliferation of autologous and allogeneic peripheral blood mononuclear cells (PBMC) (from healthy donors and patients suffering from various ADs) stimulated with anti-CD3 \kappa antibody alone or in combination with anti-CD28 antibody. The anti-proliferative effect of the BM-MSCs from healthy donors was tested also on transformed B-cell lines as a model of non-antigen-stimulated lymphocytes.

\textbf{Results.} BM-MSCs from healthy donors and AD patients reduced the proliferation of autologous and allogeneic PBMCs by up to 90\% in a cell dose-dependent fashion. The immunosuppression was independent of the proliferation of the BM-MSCs and was also effective on already proliferating cells. It was independent also of the clinical activity of AD. An MSC dose-dependent pattern of suppression of proliferation was observed also with transformed B-cell lines, similar to that observed with proliferating PBMC.

\textbf{Conclusions.} The BM-MSCs exhibit extensive anti-proliferative properties against lymphocytes under different conditions. This property might offer a form of immunomodulatory cellular therapy for AD patients if further confirmed in animal models.

\textbf{KEY WORDS:} Stem cell, Mesenchymal, Autoimmune disease, Immunomodulation, Proliferation.

\textbf{Introduction}

Bone-marrow-derived mesenchymal stromal cells (BM-MSCs) are multipotent mesenchymal precursor cells that are often referred to as mesenchymal stem cells. As a true stem property has not yet been established, a recent consensus statement recommended the term multipotent mesenchymal stromal cells, still using the acronym MSC \textsuperscript{[1]}. MSCs originate in the bone marrow (BM) but may also be isolated from adipose tissue, fetal liver, cord blood or the synovial membrane \textsuperscript{[2, 3]}. They can be expanded \textit{in vitro} as monolayers of plastic-adherent cells typically with a so-called ‘fibroblast’-like morphology. Although consisting of a heterogeneous population of cells, they are conventionally identified by their ability to form fibroblast colonies, the so-called fibroblast-colony-forming unit (CFU-F) and their almost homogeneous expression of a set of non-haematopoietic surface markers, including adhesion molecules \textsuperscript{[4, 5]}. The physiological role of these cells in adults is not yet clear. The cells appear to have a supportive function for haematopoietic stem cells in the BM niche \textsuperscript{[6]} and as such, are being used in human transplantation protocols to enhance haematopoietic stem cell engraftment \textsuperscript{[7]}. BM-MSCs are better known, however, for their \textit{in vitro} differentiation into various tissues, mainly bone, fat and cartilage, properties that are being exploited for \textit{in vitro} tissue regeneration \textsuperscript{[8]}. Another \textit{in vitro} property of BM-MSCs is their ability to reduce the proliferation of lymphocytes of various types \textsuperscript{[9–11]} while enjoying immune privilege \textit{in vitro} and \textit{in vivo} \textsuperscript{[12, 13]}. The \textit{in vitro} proliferation of activated T- and B-cells has been shown to be impaired by BM-MSCs in both mouse and humans. Lymphocytes whose proliferation is inhibited by BM-MSCs are driven either to a state of anergy \textsuperscript{[11]} or to an irreversible state of cell-cycle arrest \textsuperscript{[14]}. Recently, third-party BM-MSCs expanded \textit{in vitro} have been successfully infused in patients to treat acute graft vs host disease (GvHD) following allogeneic transplantation for leukaemia \textsuperscript{[15]}, suggesting that BM-MSCs may possess \textit{in vivo} immunosuppressant activity.
BM-MSCs have chemotactic ability and appear to migrate to sites of inflammation and injury [16]. Among animal models of autoimmune diseases, mouse experimental autoimmune encephalomyelitis (EAE) has been successfully treated with mouse in vitro-expanded MSC [17, 18], whereas in a mouse model of collagen-induced arthritis (CIA), the disease was exacerbated following BM-MSCs infusion [19].

There are published reports and current abstracts suggesting that BM stromal cells from autoimmune disease patients have defects in various critical functions including defective haematopoietic support in systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) [20], abnormal induction of a destructive synovial phenotype in RA [21] and defective endothelial precursor production in scleroderma [22].

We are currently investigating the use of BM-MSCs as a potential cellular therapy for autoimmune diseases (AD), such as SLE, systemic sclerosis (SSc), RA, vasculitis and other disorders which manifest both inflammation and tissue injury, often resulting in fibrosis. Although such changes are orchestrated through a complex cytokine and cellular network, the resulting dysregulated lymphocyte proliferation is a key element and a potential target for intervention.

This study confirms that BM-MSCs derived from healthy donors suppress alloimmune and autologous lymphocyte proliferation. In addition, we show that they can suppress already actively proliferating lymphocytes whether antigen driven or constitutively proliferating, such as transformed B-cells. Finally, we show for the first time that BM-MSCs from AD patients actively suppress autologous and alloimmune lymphocyte proliferation.

### Materials and methods

**BM-MSCs from healthy donors and AD patients**

BM-MSC cultures were derived from BM aspirates from seven healthy donors and three AD patients as described previously [23]. Ethics committee approval and signed patient consents were obtained from both healthy donors and AD patients.

PBMCs were isolated from healthy donors and AD patients by Ficoll–Hystopaque density gradient (density 1.077 g/l, Sigma). The clinical status of the AD patients is described in Table 1.

### BM-MSCs culture conditions

Cells were cultured either in low-glucose Dulbecco’s Modified Eagle’s Medium (DMEM) or alpha-modified Minimum Essential Medium (MEM) medium supplemented with 10% fetal bovine serum (FBS) as already described [24], without any additional growth factors. All culture media were from GibcoBRL.

BM-MSCs were passaged up to a maximum of four times, and were not stored frozen longer than 3–4 months before use in culture.

### Characterization of BM-MSCs

The median number of CFU-F per 10^6 nucleated cells was calculated by plating Ficoll gradient purified mononucleated cells from healthy and AD patients at a density of 4500 cells/cm². Colonies were counted 2 weeks later. Osteogenic and adipogenic in vitro differentiation of MSCs cells was performed according to published protocols [4] on cells at their first or second passage. The surface phenotype of cultured MSC was determined by four-colour flow cytometry. Cells were labelled in phosphate-buffered saline (PBS), 1% FBS, 0.01 M sodium azide (FACSWash) for 30 min at 4°C in the wells of round-bottom microtitre plates containing saturating concentrations of mixtures of monoclonal antibodies (mAb) directly conjugated with either fluorescein isothiocyanate (FITC), phycoerythrin (PE) or allophycocyanin (APC). Fluorescence intensity of positively-stained samples was calculated by combining negative control values with the combination of anti-CD3e monoclonal antibody (anti-CD3e mAb) previously described [25]. MSC are very large cells with considerable side scatter signals. Viable cells were gated according to a combination of forward Scatter (FSC), Side Scatter (SSC) and absence of PI fluorescence.

**In vitro PBMC proliferation assay with anti-CD3e monoclonal antibody (anti-CD3e mAb).** The proliferation of PBMCs from healthy donors and AD patients in the presence of BM-MSCs was performed in 96-well microtitre plates. BM-MSCs cells, irradiated (30 Gy) or non-irradiated as indicated, were seeded at dilutions (each in triplicate) of: 0.5, 2, 10 or 50 × 10^4 cells per well and allowed to attach for at least 1 h at 37°C before adding PBMCs.

PBMCs (10⁵ per well) in RPMI 1640 medium, supplemented with 5% pooled human serum, were added with or without anti-CD3e mAb (0.5 μg/ml, a gift from Antonio Lanzavecchia, Bellinzona, Switzerland). The plates were incubated at 37°C for 48 h, then pulsed for 18 h with 1 μCi/well ³H-thymidine (Amersham), harvested and the ³H counts per minute (cpm) counted. In some supplementary experiments and in those with the AD BM-MSCs, the combination of anti-CD3e mAb (0.5 μg/ml) and anti-CD28 monoclonal antibody (aCD28 mAb) (1 μg/ml, Becton, Dickinson and Company (BD), San Jose, CA) was used and showed no significant differences (data not shown). All assays with the AD BM-MSCs have been performed with both anti-CD3e mAb alone or together with aCD28 mAb. In some experiments,

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<th>Disease activity</th>
<th>Prednisone (mg/day)</th>
<th>Immunosuppressive drugs</th>
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*Median 46, range 35–81.

bAccording to EULAR scleroderma activity index.

SLEDAI, Systemic Lupus Erythematosus Disease Activity Score; DAS, Disease Activity Score; MTX, methotrexate; Nk, not known.

Bold type face indicates AD patients whose BM-MSCs were also available and tested.
the BM-MSCs were either added directly to the anti-CD3ε mAb-activated (for 48 h) lymphocytes, or activated (always for 48 h) lymphocytes were added to pre-seeded BM-MSCs. After 4 or 5 h, cultures were pulsed for 18 h with 1 μCi/well ³H-thymidine before harvesting. In some experiments, PBMCs were pre-labelled with 2μM 5,6 carboxyfluorescein diacetate succinimidyl ester (CFSE) for 15 min at room temperature and washed in 10% FBS containing RPMI 1640 before stimulation and later with the addition of the BM-MSCs. Their proliferation was analysed using flow cytometry.

Transformed and malignant B-cell lines

Epstein Barr Virus (EBV) transformation was performed by incubating peripheral blood lymphocytes (PBL) in 96-well plates at a concentration of 1 x 10⁶/ml in the presence of virus-containing supernatant of the cell line B95.8 (a gift of G. DeLibero, Basel, Switzerland) and cyclosporin A in RPMI 1640, 10% heat-inactivated FBS, 101U/ml penicillin, 10µ/ml streptomycin and 1% L-glutamine (RPMI-FCS) as previously described [26]. U-266 is an established human multiple-myeloma B-cell line [27] and NALM-6 a human B-cell precursor leukaemia cell line [28]. The cells were maintained in culture in RPMI-1640 supplemented with 10% FBS.

B-cells of each type were added in the amount of 2 x 10⁴ or 5 x 10⁴ or 10⁶ to BM-MSCs from healthy donors with the same protocol as that used for PBMC. The B-cells were incubated with the BM-MSCs for 24–48 h and then pulsed for 18 h with ³H-thymidine before harvesting.

Results

BM-MSC cultures: characterization and properties

No difference was observed in the proliferation of BM-MSCs obtained from seven healthy donors (median age 43, range 29–73) and that of the three AD patients used in this study [age: 42 (RA), 40 (SSc) and 71 (primary Sjögren Syndrome), patient numbers 9, 10 and 11 in Table 1].

No difference in CFU-F ability was noticed between the two types of BM-MSCs donors. The median colony number was 110 (range 18–150) for the healthy donors used in this study. Higher CFU-F numbers have also been counted with other BM-MSCs (data not shown). The CFU-F number for the AD BM-MSCs were: 144 (RA), 180 (SSc) and 52 (Sjögren Syndrome).

No significant difference could be established between the two groups of BM-MSCs in their adipogenic and osteogenic differentiation capacity. Each AD BM-MSCs differentiated to the osteogenic and adipogenic lineages. Mineralization and adipocyte formation was variable, but in the range of those found in the healthy donor BM-MSCs. BM-MSCs from more AD patients will be necessary to ascertain whether any preferred differentiation pattern is associated to any disease.

The absence of contaminating haematopoietic stem cells was assessed by cytofluorimetric analysis with antibodies against CD14, CD34 and CD45 antigens. Contamination was negligible at P0 (first expanded cell monolayer) and always negative at the second and third passage at which they were used in proliferative assays. Phenotypically, BM-MSC were negative for surface human leukocyte antigen DR (HLA-DR) expression but, as expected for these cells, positive for CD29, CD44, CD73, CD90, CD105, CD166 and HLA-ABC expression (Fig. 1). No phenotypic differences have so far been seen between healthy and AD BM-MSCs.

In vitro anti-CD3ε mAb-stimulated PBMCs from healthy donors are immunomodulated by allogeneic and autologous BM-MSCs

The effect of BM-MSCs (irradiated and non-irradiated) on the proliferation of anti-CD3ε mAb-activated PBMC is shown in Fig. 2. The result is representative of at least three such experiments. Anti-CD3ε mAb-stimulated proliferation of PBMCs was reduced by up to 90% (P<0.01) by the presence of BM-MSCs in a cell dose-dependent fashion. PBMCs cultured in the presence of allogeneic BM-MSCs but without anti-CD3ε mAB
simulation showed no proliferation (data not shown), indicating that BM-MSC were not by themselves responsible for the measured proliferation. In addition, non-irradiated BM-MSCs did not proliferate upon addition of anti-CD3ε mAb alone. Cultures using autologous PBMCs from two healthy BM-MSC donors were also included, and in accordance with previously published reports [24], a reduction in proliferation similar to that seen with allogeneic PBMC and BM-MSC was obtained.

Stimulation with anti-CD3ε mAb/anti-CD28 mAb was also inhibited and the results are included in Fig 2. As expected, higher PBMC proliferation (~30% more cpm as compared with anti-CD3ε mAb alone) was achieved. Similar levels of inhibition were observed in the presence of 2000 to 50 000 BM-MSC cells per well as with anti-CD3ε mAb alone (data not shown).

No difference in anti-proliferative activity was detected between irradiated and non-irradiated BM-MSCs (Fig. 2) suggesting that their active proliferation is not necessary in order to exert their anti-proliferative effect. In addition, in the time frame of these experiments, the non-irradiated cells did not proliferate to a detectable degree alone or with unstimulated lymphocytes at any cell concentration used.

**BM-MSCs reduce in vitro the proliferation of previously stimulated and actively proliferating PBMCs**

Addition of BM-MSCs (irradiated or non-irradiated) to allogeneic PBMCs that had been stimulated with anti-CD3ε mAb 48 h previously, or the addition of anti-CD3ε mAb 48 h activated PBMCs to pre-seeded BM-MSCs, resulted in a significant reduction of PBMC proliferation in a dose-dependent manner as previously observed (Fig. 2). When the PBMCs were pre-labelled with CFSE and the course of their proliferation followed by fluorescence activated cell sorting (FACS) analysis, they appeared already activated and proliferating at the time of addition of the BM-MSCs (at 48 h, BM-MSC to PBMC ratio 1 to 10) as evidenced also by strong ongoing DNA synthesis through 3H-thymidine incorporation, increase in cell size as measured by a shift in SSC and FSC parameters and the appearance of a shoulder of proliferating cells on the main peak of undivided PBMCs.

Although at 60 h proliferation and division of PBMC was still ongoing, at 72 h proliferation of PBMC was significantly reduced in the presence of the BM-MSCs but not when cultured alone (Fig. 3). Therefore, BM-MSCs appear, even at the ratio of 1:10 PBMC, effective in arresting already proliferating lymphocytes, albeit in a delayed fashion.

**BM-MSCs exert an anti-proliferative effect on transformed and malignant B-cells**

Proliferating B-cells have also been shown to be inhibited by BM-MSCs, both from mouse [14] and man [17]. In order to test if the anti-proliferative effect of the MSCs was effective also on constitutively proliferating B-cells, we tested their activity on EBV-transformed B-cells, one human B-cell precursor leukaemia cell line, NALM-6, and one human multiple-myeloma cell line, U-266. BM-MSCs were added to 10⁶, 5 × 10⁵ or 2 × 10⁵ B-cells at different ratios. The same BM-MSC dose-dependent effect on their proliferation was observed as with the PBMCs (Fig. 4).
PBMCs were isolated from 11 patients with different AD (Table 1). The patients (median age 46, range 35–80) represented a spectrum of AD, disease activity and current immunosuppressive drugs exposure. The PBMCs from the AD patients were stimulated with anti-CD3e mAb (or combined with anti-CD28 mAb in some cases), and added to irradiated BM-MSCs from two different healthy donors and tested as described for a reduction of their proliferation. All anti-CD3e mAb-stimulated PBMCs derived from AD patients showed a reduced proliferation in a cell dose-dependent fashion as seen previously with PBMCs from healthy donors (Fig. 5). In some cases, the PBMC proliferation was enhanced rather than reduced at very low BM-MSC numbers (at 1:250 and also 1:50 BM-MSC to PBMC ratio when stimulation was with anti-CD3e mAb/anti-CD28 mAb), an event already noticed in experiments also with healthy donor PBMCs by us and by others (data not shown) [29]. Residual PBMC proliferation in the presence of $5 \times 10^5$ BM-MSCs averaged $24 \pm 12\%$ (mean $\pm$ S.D.) of that seen in the absence of BM-MSCs. Non-irradiated BM-MSCs have been used also with three AD PBMCs without any detectable difference in the pattern of reduced proliferation compared with those with irradiated BM-MSC and have been included in the group analysed in Fig. 5.

BM-MSCs from three AD patients, RA, SSc and Sjögren (patients 9, 10 and 11, Table 1), irradiated or non-irradiated, also reduced the proliferation of autologous PBMCs (PBMCs 9, 10 and 11, Table 1) as effectively as they did with allogeneic PBMC from healthy donors (mean residual proliferation in the presence of $50 \times 10^5$ BM-MSCs: $26 \pm 6\%$), and therefore are included in Fig. 5.

**Discussion**

In this report, and in agreement with others, we show that in vitro-expanded BM-MSC inhibit in vitro the proliferation of activated lymphocytes from healthy donors, PBMC proliferation being induced by stimulation with an anti-CD3e monoclonal antibody in most cases. Combined stimulation with anti-CD3e and an anti-CD28 mAbs showed similar results. In addition, we show that the reduction in T-cell proliferation does not differ using irradiated or non-irradiated BM-MSCs (as they are currently used in in vivo clinical applications) and does not therefore depend on the active proliferation of the BM-MSCs. All healthy-donor-derived BM-MSCs showed such anti-proliferative activity and were not immunostimulatory when added to allogeneic PBMCs, as shown also by others (they were HLA-DR-negative as shown in Fig. 1). Importantly, a significant reduction of PBMC proliferation was measured also when the BM-MSCs, irradiated or not, were added 48 h after anti-CD3e mAb stimulation, when the PBMCs are fully activated and already proliferating. A suppression of lymphocyte proliferation by BM-MSCs added to mixed lymphocyte cultures (MLC) at late times after starting of MLCs has been reported also by others [9, 12]. In our experiments, presumably those cells that have already entered the S-phase continue their cell cycle but there is an arrest in the G0 phase, as suggested by the experiments of others [14]. The arrest is significant albeit delayed, considering also the low ratio of BM-MSCs to PBMCs.

This result shows also that the measurement of DNA synthesis by the uptake of $^3$H-thymidine provides complementary information to that of CFSE staining, which instead shows the distribution of cell division number that the population has undergone. We detected at 3 and 4 days after PBMC stimulation, a similar reduction in DNA synthesis by $^3$H-thymidine uptake (data not shown), but only at 4 days a significant reduction in cell divisions as judged by CFSE labelling was detected. For the first time, we report that the proliferation of B-cells, either transformed by EBV or derived from patients with B-cell malignancies, is reduced in a BM-MSC cell dose-dependent fashion. We do not know if the anti-proliferative effect of BM-MSCs on these transformed cells is operating through the same mechanisms as that on stimulated PBMCs. Further experiments are warranted to answer this question.

We report also for the first time that the anti-CD3e mAb (alone or combined with a CD28 mAb) triggered proliferation of PBMCs from AD patients is also significantly reduced on addition of healthy donor BM-MSCs. No discriminating features could be determined in any AD tested, although a higher number of cases per disease would be required for this purpose. The BM-MSCs from three AD patients exhibited also a similar anti-proliferative effect on allogeneic and autologous PBMCs. These results with such a small number of samples do not allow one to draw definitive conclusions, but they mean that at least a defective anti-proliferative effect is not a general property of the AD BM-MSCs. We are currently studying additional BM-MSCs from a variety of AD patients. In any eventual clinical application the advantages and disadvantages of autologous vs allogeneic MSCs infusion will need to be more extensively evaluated. This issue has been recently highlighted by the finding that allogeneic MSCs in a non-myeloablative murine model do not exhibit immune privilege [30].

It is encouraging that with the assays used so far no differences between BM-MSCs from healthy or AD patients have been detected, or at least, if any exists, it does not result in an altered anti-proliferative effect in vitro against activated lymphocytes. Further discriminating assays must be devised to assess their existence and their relevance to the AD pathophysiology.

The anti-proliferative effect of BM-MSCs on already proliferating cells, activated PBMCs or transformed B-cell lines implies a more fundamental event than simple immunosuppression and underlines again the ability of BM-MSCs to act on actively proliferating cells. Whether this phenomenon can have a clinical application is not known, but it could support the safety aspect of the reinfusion of BM-MSCs for GvHD, a condition in which...
reactivation of EBV may occur. In addition, the general anti-proliferative effect could be of particular relevance to AD in which polyclonal activation of bystander lymphocytes plays an important role in the pathogenesis and in many of the clinical manifestations.

We observed a maximum inhibition of proliferation at a 1:2 BM-MSC/PBMC ratio, although a similar reduction can also be seen at a 1:10 ratio. However, it is not known how and if such ratios have a physiological in vivo significance or whether they simply reflect an in vitro requirement.

Whether BM-MSCs themselves proliferate in the presence of activated PBMC in vitro (in order to reach a high concentration) is not known. The non-irradiated MSC behaviour in vitro does not resolve that issue. This is an important question, since in a potential clinical setting it remains to be seen if MSCs could reach their target tissue or proliferate in situ in sufficient numbers to execute their anti-proliferative effect. On the other hand, it is also possible that a different temporal and spatial in vivo situation might not have the same requirements.

Therefore, despite being able to show anti-proliferative activity of healthy donor and AD patient BM-MSCs in vitro and the evident success of infusing BM-MSCs in a limited number of clinical settings, several questions remain concerning the mechanism of the BM-MSCs immuno-modulatory action in vivo. Such questions could be addressed in a rigorous way by using mouse and human BM-MSCs in animal models of AD.

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