BONE MARROW MESENCHYMAL STEM CELLS INHIBIT THE RESPONSE OF NAÏVE AND MEMORY ANTIGEN-SPECIFIC T CELLS TO THEIR COGNATE PEPTIDE.

Running title: T cell inhibition by mesenchymal stem cells

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Mesenchymal stem cells (MSC) have been recently shown to inhibit T-cell proliferation to polyclonal stimuli. We characterized the effect of MSC of bone marrow origin on the T-cell response of naïve and memory T cells to their cognate antigenic epitopes. The immune response to murine male transplantation antigens, HY, was selected because the peptide identity and MHC restriction of the immunodominant epitopes are known. C57BL/6 female mice immunized with male cells were the source of memory T cells, whereas C6 mice transgenic for HY-specific T-cell receptor provided naïve T cells. Responder cells were stimulated in vitro with male spleen cells or HY peptides in the presence or absence of MSC. MSC inhibited HY-specific naïve and memory T cells in a dose-dependent fashion and affected cell proliferation, cytotoxicity, and the number of IFNγ producing HY peptide-specific T cells. However, MSC inhibitory effect did not selectively targeted antigen-reactive T cells. When MSC were added to the T-cell cultures in a Transwell® system or MSC were replaced by MSC culture supernatant, the inhibitory activity was abrogated. T-cell reactivity was also restored if MSC were removed from the cultures. The expression of MHC molecules, the presence in culture of antigen presenting cells (APC) or of CD4+/CD25+ regulatory T cells were not required for MSC to inhibit. We conclude that MSC inhibit naïve and memory T cell responses to their cognate antigens. Overall our data suggest that MSC physically hinder T cells from the contact with APC in a non-cognate fashion.
Introduction

Bone marrow (BM) stroma contains multipotential non-hematopoietic progenitor cells capable of differentiating into various tissues of mesenchymal origin. First identified for their ability to differentiate into bone and adipocytes\(^1\), further studies have demonstrated that mesenchymal stem cells (MSC) can also differentiate, under appropriate in vitro conditions, to form chondrocytes, tenocytes, skeletal myocytes, neurons, and cells of visceral mesoderm\(^2-5\). MSC have been isolated in different species and are present at a low frequency (1/10\(^5\)) in adult BM but they appear to constitute approximately one-third of the initial adherent bone marrow-derived stromal colonies in vitro. They are characterized by the absence of hematopoietic markers (CD45-/CD34-/CD14-) and the expression of a specific pattern of adhesion molecules (CD106+/CD54+/SH2+/SH3+). Through these molecules, MSC interact with hematopoietic stem cells and influence their homing and differentiation. Evidence for a definite role of BM stroma in T-cell development comes from the observation that the BM microenvironment provides appropriate support for T cells to develop in the absence of the thymus\(^6\) and that the majority of T cells adhering to BM stroma display an immature phenotype\(^7\). Furthermore, after bone marrow transplantation, BM stromal cells appear to migrate to the thymus where they participate in the positive selection of thymocytes\(^8,9\). In addition to providing critical cell-cell contact, MSC produce growth factors for hematopoiesis and may attract infused hematopoietic stem cells to the marrow by inducing homing receptors. In a NOD/SCID system Peled et al. showed that stromal derived factor 1 (SDF-1) and its...
receptor CXCR4 enhance CD34+ cell migration and engraftment in marrow. Therefore the BM microenvironment greatly influences the developmental potential of hematopoietic stem cells. These characteristics have generated substantial interest in the transplantation setting where MSC may be a promising cell type for tissue engineering, cellular therapy, and somatic gene therapy applications. Studies already conducted in both humans and animal models have demonstrated that MSC are capable of long-term engraftment and in vivo differentiation.

Great potential for MSC would be derived from the observation that they can exert an immunoregulatory activity. Their effects, especially on mature T lymphocytes, have not yet been defined. It has recently been reported that MSC can inhibit T-cell proliferation induced in a mixed lymphocyte reaction or by non-specific mitogens. Of particular interest is the observation that in vivo administration of MSC in baboons significantly prolongs the survival of MHC-mismatched skin grafts. Since these properties might open attractive possibilities in the field of hematopoietic as well as solid organ transplantation, better characterization of MSC immunoregulatory activity and the elucidation of its mechanism are crucial. In view of the lack of MHC class I and II on MSC, it is difficult to ascribe specific T-cell receptor/MHC/peptide interactions to their mechanism of immunoregulation. However, MSC might selectively inhibit T cells which have encountered antigen, sparing those which have not been activated by T-cell receptor engagement. The susceptibility of naïve and memory T cells to immunoregulatory stimuli could have profound implications when considering potential clinical applications of MSC. We addressed these questions utilizing an animal model in which
the effect of MSC of bone marrow origin on the immune responses to various peptide epitopes of the transplantation antigen HY was evaluated. In this system the peptide identity and the MHC restriction of the immunodominant epitopes are known\(^{16,17}\). We found that inhibition requires the presence of MSC in culture and MSC-T cell contact. Both naïve and memory cells are subjected to MSC mediated suppression but MSC do not appear to selectively target antigen-reactive T cells. MSC inhibitory effect does not require the presence of antigen-presenting-cells and is not mediated through CD4+/CD25+ regulatory T cells. The implications of these results are discussed.

**MATERIALS AND METHODS**

*Mice*

C57BL/6 (H\(^2^b\)), BALB.B (H\(^2^b\)), and CBA (H\(^2^k\)) mice were purchased form Olac Harlen (Bicester, UK). CBA/Ca, C57BL/6-Thy1.1, and C3H.SW (H\(^2^b\)) mice were bred by CBS at the Hammersmith Hospital. Mice were used between age 7 and 10 weeks of age.

C6 TCR\(^{\text{high}}\) mice (C6) are transgenic for the V\(\alpha 8\) and V\(\beta 11\) T-cell receptor chains from the HY-specific CD8\(^+\) T cell clone, C6, specific for the HY antigen-derived peptide TENSNGKDI presented by H2\(^k\)\(^k\) (HY K\(^k\))\(^{17,18}\). The strain is on a CBA/Ca background. Spleen cells from these mice were used as a source of HY-specific naïve T cells. HY-specific memory T cells were obtained by immunizing C57BL/6 female mice by intraperitoneal (ip) injection of 5x10\(^6\) syngeneic male splenocytes.
**Generation of MSC**

MSC were generated from BALB.B (H2b), C3H.SW (H2b), or CBA (H2b) mice. BM cells, collected by flushing femurs and tibias with medium, were cultivated in 6-well plates or 25 cm² flasks (Costar, Cambridge, MA) at a concentration of 10⁶/ml nucleated cells in Dulbecco's modified Eagle medium (DMEM), with high glucose concentration, GLUTAMAX I™ (GibcoBRL, UK), 10% heat-inactivated adult bovine serum (Labtech International, Sussex, UK), 100 U/ml penicillin and 100 µg/ml streptomycin (GibcoBRL). No cytokines were added at any stage. Cultures were incubated at 37°C in a 5% CO₂ atmosphere. After 72 hours, non-adherent cells were removed. When 70-80% confluent, adherent cells were trypsinized (0.05% trypsin at 37°C for 5 min), harvested, and expanded in larger flasks. A homogenous cell population was obtained after 3 to 5 weeks of culture. Before further expansion and experimental use, MSC were tested for their ability to differentiate into adipocytes and osteoblasts. Adipocytic differentiation was induced by 10⁻⁸ M dexamethasone, whereas 10⁻⁸ M dexamethasone, 50 g/mL ascorbic acid and 10 mM β-glycerophosphate were used for osteoblastic differentiation. Oil-red-O and von Kossa dyes were employed to identify adipocytes and osteoblasts, respectively. More than 90% of the cells differentiated depending on the time left in culture with the differentiating agent. The identity of MSC was also confirmed by immunophenotypic criteria based on the expression of CD106, and the absence of hematopoietic (with anti-CD45, -CD14, -CD11c antibodies) or endothelial cell (with anti-CD31 antibodies) markers. All antibodies were purchased from Pharmingen, Oxford, UK. MSC were maintained in culture for no more than 15 in vitro passages. The
proportion of CD45+ cell in the MSC preparations used in the various experiments never exceeded 3% CD45+ cells.

In selected experiments the 3T3-F442A mesenchymal stem cell line (from a H2d mouse), a kind gift of Dr. H Green (Harvard Medical School, Boston, MA), was used. The cells were cultivated in DMEM containing 10% heat-inactivated adult bovine serum (GibcoBRL) according to the conditions originally described 20.

T-cell cultures

All cultures were carried out in RPMI 1640 supplemented with 5x10^{-5} M 2ME, 10% FCS, GLUTAMAX I™ (GibcoBRL, Life Technologies Ltd, UK), 100 U/ml penicillin and 100 g/ml streptomycin. In some experiments an anti-TGF-β1-β2-β3 monoclonal blocking antibody (Genzyme, Cambridge MA) was added to the cultures at a concentration of 1 and 10 µg/ml21.

Depletion of CD25+ cells

Depletion of CD25+ cells was performed using MiniMacs system (Miltenyi Biotec, Camberley, UK). Biotinylated anti-CD25 antibodies (7D4 clone, BD PharMingen, Germany) were added to spleen cell suspensions at a concentration of 1µl/30x10^6 cells/ml and incubated at 4°C for 20 min. After extensive washing with PBS, cells were incubated with Mini-MACS Streptavidin MicroBeads (Myteni Biotech) for 15 min and subject to passage through selection columns in a magnetic field. FACS analysis of the
eluted fraction (CD25\(^{-}\)) stained with a PE labeled anti-CD25 (PC61 clone) showed CD25\(^{+}\) cells to be less than 1%.

**Antigenic peptides**

Stimulator cells were pulsed with 1000 ng/ml of K\(^{b}\)-restricted (TENSGKDI) HY peptide\(^{17}\) (encoded by the Smcy gene) or with the D\(^{b}\)-restricted Smcy (KCSRNRQYL)\(^{22}\), the Uty (WMHHNMDLI)\(^{23}\) encoded, and the A\(^{b}\)-restricted Dby encoded (NAGFNSNRANSSRSS)\(^{24}\) HY peptides at 37\(^\circ\)C for 2 hours. Unpulsed irradiated CBA or C57BL/6 female splenocytes were used as negative controls.

**T-cell proliferation assay**

T-cell proliferation assays were performed in round-bottom 96-well plates (Costar, Cambridge, MA) in a total volume of 0.2 ml RPMI 1640. A total of 0.5 \(\mu\)Ci of \([^{3}\text{H}]\)-thymidine (ICN, Costa Mesa, CA) was added into each well 2 (naïve transgenic T cells) or 3 (memory T cells) days later as required, and cells were harvested onto glass fiber filters using an LKB 96 well-harvester (Wallac Oy, Turku, Finland) after an additional 24 hours. \([^{3}\text{H}]\) thymidine uptake was measured on an LKB Betaplate counter (Wallac Oy). The results are expressed as mean cpm for triplicate cultures (standard errors were routinely <10%).

**CD3/CD28 stimulation**
CD28/CD3-coated Dynabeads (Dynal, Bromborough, U.K.) were prepared by conjugating 5µg of CD28 and CD3 antibodies (both from Pharmingen) to 10^7 beads, according to the manufacturer’s instructions. For proliferation, 1-5x10^5 responder T cells were incubated with 1-5x10^4 CD3/CD28 coated Dynabeads/well. The cells were cultivated in a total volume of 200µl in flat-bottomed 96 well plates and assessed for proliferation 3 days later.

*T-cell cytotoxicity*

Effector cells were incubated in 96-well plates with ^{51}Cr-labelled peptide-pulsed RMA-S target cells at E:T ratios of 50:1, 17:1, 5:1 and 1.5:1. After 4 h, 100 µl of supernatant was collected and 51Cr release was measured using a gamma counter. Results were calculated from a 12 point regression curve resolved at an E:T ratio of 10:1. The percentage of lysis was calculated from the formula 100 x (E - M)/(T - M), where E is the experimental release, M is the spontaneous release in the presence of medium alone, and T is the maximum release in the presence of 5% Triton X-100.

*Flow cytometric analysis*

In addition to the monoclonal antibodies listed in a previous paragraph, MSC were also stained with CD80 (B7-1), CD86 (B7-2), class I (H2) and class II (H2A) major histocompatibility complex (MHC) molecules, all purchased from Pharmingen. For immunophenotype analysis, MSC were detached using trypsin/EDTA, washed and resuspended at 10^6/ml. One hundred microlitres of cell suspension were incubated at +4°C for 15 min with mouse unconjugated immunoglobulins (Sigma Immunochemicals,
Poole, UK), followed by incubation with the specific antibody at +4°C for 30 minutes. Cells were washed with PBS containing 1% bovine serum albumin and 0.1% sodium azide (PBSAS). At least 10,000 events were analyzed by flow cytometry (FACScan, Becton Dickinson) using Cell Quest software.

Antigen specific T-cell responses were measured by enumerating IFNγ producing T cells in response to HY peptides. Irradiated syngeneic splenocytes, pulsed with 10 nM of HY peptides at 37°C for 2 hours, and then washed, were added to responder T cells at a 1:1 ratio. Prior to peptide pulsing, stimulator cells were T-cell-depleted with anti-mouse pan-T Dynabeads (Dynal, Bromborough, U.K.). Non peptide-pulsed, irradiated syngeneic splenocytes were used as the negative control. During the last 4 hours of stimulation, Brefeldin A (Sigma) was added at a concentration of 10 µg/ml to block cytokine secretion. At the end of the cultures, the cells were stained with anti-CD4 and -CD8 (Pharmingen). Subsequently, the cells were washed with PBS and fixed/permeabilized with a commercial solution (Permeabilizing solution, Becton Dickinson) for 10 minutes. After further washing, the cells were stained with anti-IFNγ antibody (Pharmingen) for 30 minutes at R/T. At least 100,000 events in the lymphocyte gate were analyzed at FACScan.

CD8+ T cells expressing H2b restricted HY-specific T-cell receptors were analyzed by using soluble/MHC peptide tetramers. Tetramers were produced by refolding H2-Db heavy chain with β2microglobulin and HY peptides WMHHNMDLI (encoded by the Uty gene) or KCSRNRQYL (encoded by the Smcy gene)22,23, as previously described16.
Tetramers were conjugated with PE-labeled ExtrAvidin-R-PE complex (Sigma). Before staining, cells were depleted of the B-cell fraction by negative selection with anti-mouse pan-B Dynabeads (Dynal).

**Transwell cultures**

Splenocytes (15x10^6/ml) from HY-immunized C57BL/6 female mice were cultivated with irradiated syngeneic male splenocytes (15x10^6/ml) in the lower chamber of a 24 mm diameter Transwell® plate with a 0.3 μm pore size membrane (Costar, Cambridge, MA). Autologous or allogeneic MSC (2x10^5) were seeded onto the transwell membrane of the inner chamber 1-2 hours before the beginning of the culture. Control cultures did not contain MSC or MSC were added directly to the secondary MLR. After 7 days, viable cells were evaluated for antigen-specific intracellular IFNγ and frequency of peptide: MHC tetramer+/CD8+ T cells.

In selected experiments, MSC were replaced with supernatant from MSC cell cultures. A day before being split, MSC culture supernatant was harvested, centrifuged, and filtered through a 0.2μm Millipore filter.

**RESULTS**

*MSC fail to stimulate T cells in secondary H2-mismatched MLR*

Initial experiments showed MSC to be poor stimulators of secondary mixed leukocyte
reactions (MLR). Their ability to function as antigen presenting cells (APC) was tested by using MSC as stimulators of secondary allogeneic mixed leukocyte reaction (MLR). Spleen cells (2x10^5/well) from CBA (H-2^k) mice were used as responder cells in primary cultures stimulated with irradiated BALB.B spleen cells (5x10^5/well). Cells harvested at day 7 from these primary cultures were then seeded at 2x10^5/well and re-stimulated with graded numbers (10^2, 10^3, 10^4 per well) of irradiated (60cGy) MSC of BALB.B origin. BALB.B splenocytes (2x10^5/well) were used as stimulators in the control cultures. The limit of the maximum number of MSC (10^4/well) was chosen because at this concentration MSC occupy on a 96-well plate the approximately same surface as 2x10^5 splenocytes, the number producing the best stimulation of secondary MLR (not shown). No proliferation was detected at any of the MSC doses employed (Figure 1). No difference was seen whether the MSC were irradiated or not. This lack of stimulator cell activity was consistent with their phenotype: MSC expressed neither MHC class I nor class II molecules. The analysis for co-stimulatory molecules showed that they did not express CD86, although they were positive for CD80 (Figure 2). To assess whether the lack of MHC class I expression was the major factor for the inability of MSC to present alloantigens, MSC were pre-treated with IFNγ prior to their use as stimulators of secondary MLR. Despite the induction of MHC class I expression (Figure 2) no proliferation was observed (Figure 1). MHC class II was not expressed following IFNγ pretreatment.

MSC inhibit naïve and memory antigen-specific T cells

Since, despite the expression of MHC class I and some co-stimulatory molecules, MSC
failed to induce T-cell stimulation, we tested whether this could be attributed to an inhibitory effect on T-cell activation. The effect of addition of MSC to cultures of both naïve and memory HY-specific T cells in the presence of their cognate peptides was tested. Splenocytes from C6 mice transgenic for a H2-K\(^k\)-restricted HY-specific T-cell receptor were used as a source of naïve T cells and cultivated in the presence of irradiated syngeneic (CBA, H2\(^b\)) spleen cells pulsed with the cognate K\(^k\) restricted HY peptide (HY K\(^k\)Smcy) as APC. Graded doses of MSC were added as third party cells for the duration of the in vitro stimulation. After 24 hours CD8+ cells were analyzed for IFN\(\gamma\) by intracellular staining (Figure 3B). Parallel cultures were assessed for T-cell proliferation after 48 hours (Figure 3A). Both HY peptide specific IFN\(\gamma\) production and T cell proliferation were inhibited. Inhibition was dependent on the number of MSC in culture. The proliferative activity was significantly inhibited at a MSC/responder (M/R) ratio as low as 1:10\(^4\). At the highest ratio (1:10\(^1\)), T-cell proliferation and the percentage of IFN\(\gamma\) producing CD8+ T cells were reduced by 85% and 53%, respectively.

Splenocytes from wild type female C57BL/6 (H2\(^b\)) mice immunized with syngeneic male spleen cells were used as a source of HY specific memory T cells. They were restimulated in vitro with syngeneic male spleen cells. Graded doses of MSC were added as a third party to the MLR. After 7 days, the cells were harvested and restimulated with H2\(^b\)-restricted HY peptide- (HY D\(^b\)Smcy, HY D\(^b\)Uty, HY A\(^b\)Dby) pulsed female spleen cells and assessed for T-cell proliferation (Figure 4A), number of HY peptide specific IFN\(\gamma\)-positive (Figure 4B) and HY D\(^b\)Smcy and HY D\(^b\)Uty tetramer-positive CD8\(^+\) T cells (Figure 4C). To measure HY-specific cytotoxic activity, cells were harvested after
5 days and tested against HY peptide (D<sup>b</sup>Smcy and D<sup>b</sup>Uty) pulsed RMA-S cell line in a 51Cr-release assay (Figure 4D). Proliferation, number of IFNγ producing and tetramer-positive CD8<sup>+</sup> cells, and the cytotoxic activity of these HY specific memory T cells were inhibited by MSC in a dose dependent fashion. It is interesting to note that the effector functions (IFN-γ production and cytotoxicity) of memory T cells appeared to be more susceptible to inhibition than antigen-specific T-cell expansion (proliferation and expansion of tetramer-positive T cells). Furthermore, the percentage of inhibition observed on IFNγ production is greater than that observed on cytotoxic activity, thus suggesting that MSC could selectively interfere with certain pathways of T-cell activation/function.

**MSC do not specifically target antigen-reactive T cells**

Although MSC can inhibit antigen-specific immune responses, it is unlikely that this results from the recognition of the cognate peptide presented by MSC because they lack expression of MHC molecules. Nevertheless, MSC might specifically interact with and inhibit only the T cells which become activated after encountering antigen. To address this question we utilized as responder cells a population containing different proportions of HY-specific T cells but we kept the number of MSC fixed. Memory spleen cells from HY-immune female C57BL/6 mice were stimulated in vitro with male cells. After 7 days, the cultures, which contained an average of 50% of D<sup>b</sup>Uty tetramer-positive CD8<sup>+</sup> T cells (Figure 5A) were used for restimulation either directly or diluted 1:2 or 1:10 with splenocytes from naïve female mice to reduce the concentration of HY-specific T cells to 25 or 5%, respectively. Naïve and memory cells were from mice polymorphic for the
Thy1 allele (Thy1.1\textsuperscript{+} and Thy1.2\textsuperscript{+}, respectively) in order to be able to confine the analysis to the memory T-cell population. Responder cells were restimulated with H2\textsuperscript{b}-restricted HY peptide- (HY D\textsuperscript{b}Smcy, HY D\textsuperscript{b}Uty, HY A\textsuperscript{b}Dby) pulsed female spleen cells and assessed for number of HY peptide specific IFN\textgamma- positive Thy1.2\textsuperscript{+} cells generated in the presence or absence of a fixed number of MSC. In this system the ratio between MSC and T cells remained the same but the ratio between MSC and HY-specific T cells varied. The inhibitory effect of MSC on HY-specific memory T cells after in vitro expansion is significant but not complete. By reducing the ratio between antigen-specific and non-antigen-specific T cells of up to 10 fold, we would expect a higher inhibition if MSC exclusively targeted antigen-specific T cells. However, no difference in the magnitude of inhibition was observed at different ratios between MSC and HY-specific T cells (Figure 5B).

**MSC inhibitory effect is not MHC-dependent**

MSC do not constitutively express MHC molecules, but they can do so after IFN\textgamma treatment. In the cultures in which MSC were added to the MLR as third party, we observed that after 7 days they expressed MHC class I molecules, probably as a result of IFN\textgamma production in the culture (data not shown). Although the presence of MHC class I on MSC does not appear to induce the proliferation of allogeneic T cells in MHC mismatched cultures (Figure 1), we tested whether MHC class I molecule expression was required for the inhibitory effects on naïve and memory HY specific T cells to be exerted. For these experiments, we used the 3T3-F442A MSC line of BALB/c origin, which does not express MHC molecules even after IFN\textgamma treatment (data not shown). No
difference in the inhibitory activity was detected in comparison with H2<sup>b</sup> matched BALB.B MSC in terms of proliferation and IFNγ production by naive (Figure 6A) or memory (Figure 6B) responder cells. These findings were consistent with the observation that C6 splenocytes (H2<sup>k</sup>) can be inhibited by H2<sup>b</sup> MSC (Figure 3).

**MSC inhibitory effect is transient**

The fact that MHC expression on MSC does not influence the MSC mediated inhibition suggests that it also does not require the T cells to recognize antigen on MSC. Moreover, our data shows that MSC do not exclusively target antigen-reactive T cells. It remained to be determined whether the MSC induced persistent non-responsiveness or whether their inhibitory effect was transient. To address this question, splenocytes from C6 mice were stimulated with the K<sup>k</sup>-restricted HY peptide in the presence or absence of MSC as a third party. Twenty-four hours later, non-adherent cells were harvested and depleted of CD106<sup>+</sup> cells to exclude the interference of any residual MSC. The CD106<sup>-</sup>/non-adherent C6 cells were re-stimulated with the HY peptide without MSC. IFNγ producing CD8<sup>+</sup> T cells were enumerated 24 hours later. The results are shown in Figure 7. Although the presence of MSC in the first 24hr culture inhibited the first antigenic stimulation, when MSC were removed the response to the HY peptide was restored. These findings demonstrate that MSC inhibit T cells only when they are present in culture, but the effect is reversed when MSC are removed.

**MSC inhibitory effect requires cell contact**

The MSC inhibitory effect could be mediated by soluble factors or could require cell
contact to be exerted. The fact that the inhibitory activity was dose-dependent and transient (ie entirely dependent on the presence of MSC) was in support of the latter hypothesis. MSC culture supernatant was added (1:1 dilution) to the cultures in which memory HY-specific T cells were stimulated with syngeneic male spleen cells. After 7 days the numbers of HY Db Smcy tetramer-positive CD8+ T cells were evaluated. In parallel, cells were stimulated with HY peptide pulsed APC for 6 hrs to assess the numbers of IFNγ+/CD8+ T cells. The results are reported in Figure 8. No difference in the number of tetramer positive T cells was detected (Figure 8A) and the number of IFNγ+/CD8+ T cells was not reduced in the presence of MSC supernatant (Figure 8B). The necessity of cell contact for the inhibitory effect was confirmed by experiments in a transwell system. MSC were added to the inner chamber and the MLR was cultivated separately in the lower chamber. When the MSC were not in contact with the MLR, the inhibition of antigen-specific T cells was much reduced (Figure 8A and 8B). Consistent with a need for cell-cell contact and the absence of an effect of soluble factors, we did not find any IL-10 in the MSC culture supernatant as evaluated by ELISA; further, addition of anti-TGFβ blocking antibody had no influence on inhibitory activity even when used at concentrations 10 fold higher (10µg/ml) than those described by others13 (data not shown).

**MSC inhibitory effect is neither dependent on antigen-presenting cells nor on CD4+/CD25+ regulatory T cells**

MSC inhibitory effect could be exerted directly on effector T cells or mediated via different splenocyte types present in culture. To address this question, we asked whether
antigen presenting cells (APC) were required for the inhibitory effect to be exerted. We stimulated T cell proliferation with CD3/CD28 coated beads. This type of stimulation circumvents the need for APC, as antibodies interact directly with T cells to activate them. When MSC were added to the cultures they potently inhibited spleen cell proliferation in response to the antibody coated beads (Figure 9A). The results were confirmed using a HY D<sup>b</sup>Uty-specific CD8<sup>+</sup> T-cell clone (CTL-10)<sup>25</sup> as responder cells (Figure 9B). Alternatively, MSC might exert their inhibitory effect via regulatory T cells. Much attention has recently been paid to a distinct CD4<sup>+</sup> T cell subset expressing the CD25 molecule which appears to actively suppress T cell activation<sup>26</sup>. In order to ascertain whether CD4<sup>+</sup>/CD25<sup>+</sup> regulatory T cells are required for the MSC mediated inhibition, spleen cells from C6 mice were depleted of CD25<sup>+</sup> cells and stimulated with H2<sup>k</sup>-restricted HY peptide- (HY K<sup>k</sup>Smy) pulsed female spleen cells in the presence or absence of MSC and 24 hours later assessed for the number of HY peptide specific IFN<sub>γ</sub>-positive CD8<sup>+</sup> cells. CD25-depleted and CD25-replete C6 T cells were equally inhibited by MSC (50% vs 44% = not statistically significant) (Fig 10).

**Discussion**

Transplantation has been one of the major advances in medicine during the last few decades. BM transplantation in particular, can cure a variety of malignancies by exploiting the graft-versus-tumor effect exerted by the lymphocytes contained in the
donor BM preparation. Major problems remain with the lack of suitable BM donors. The option of increasing donor-recipient histoincompatibility is associated with a high risk of both graft rejection and/or graft-versus-host disease, a situation in which more powerful and selective strategies to diminish immune responses following transplantation would be desirable. There has recently been enormous interest in the stem cells contained in the BM since it appears that they can differentiate into lineages other than hematopoietic \(^{27-32}\). BM also contains rare stem cells which differentiate into mesenchymal lineage cells which not only have multipotential differentiation ability \(^{33}\) but they also appear to modulate immune responses in vitro. This property could have a substantial impact in transplantation since infusion of MSC in conjunction with the donor organ or bone marrow might favor engraftment. Administration of MSC has been reported to prolong donor skin graft survival in non-human primates\(^ {14}\). The ability of MSC to suppress immune responses could also be harnessed to reduce GvHD, the idea underlying a multicentric clinical trial in which patients with advanced hematologic malignancies receive donor MSC at the time of haemopoietic stem cell infusion as a prophylaxis for GvHD. So far, the reported overall incidence of acute and chronic GvHD appears to be significantly lower in the group infused with MSC as compared to the controls (p=0.002 and p=0.02, respectively)\(^ {34}\). However, the characteristics and the mechanisms of this inhibition are entirely unknown.

Here we show that BM derived MSC have a profound inhibitory effect on activation of T cells by their cognate peptides in vitro. This inhibition, which affects both naïve and memory T cells, is manifest in antigen-specific proliferation, IFN\(\gamma\) production and
cytotoxic activity. Physiologic inhibition of T cells is considered to occur by deletion, anergy, or suppression\textsuperscript{35}. Whereas in the first case T cells die, in the other situations T cells remain alive but are unable to respond to antigen. Here we show that the inhibitory effect exerted by MSC on naïve T cells is already detectable at 24 hours but it is relieved once MSC are removed from the culture, so they are not deleted (Fig 7). However, in the cultures in which MSC are incubated with memory T cells for 7 days which are then harvested and re-stimulated with antigen, T cells remain unable to respond (Fig 4). In the case of naïve antigen reactive T cells, which recover responsiveness when separated after 24 hours with MSC, it seems unlikely that their non-responsiveness in the continued presence of MSC is due to anergy, since 24 hours is a reasonable time-frame for anergy induction\textsuperscript{36}. The alternative hypotheses are that MSC induce T-cell suppression either directly by soluble factors or indirectly by preventing T-cell activation. The requirement of cell contact for MSC to display their inhibition suggests they may operate by preventing T cell activation. The absence of IL-10 in MSC culture supernatants and the inability of anti-TGFβ blocking antibodies to inhibit the MSC effect (data not shown) are in accord with such a hypothesis. It is not yet clear whether naïve are more difficult to anergize than memory T cells\textsuperscript{15}. Although the inhibition of IFNγ production seems more pronounced on memory T cells than in naïve T cells, the precursor frequency of responder T cells differ in the two populations. At the time of culture with MSC the numbers of antigen-specific T cells were higher in the naïve transgenic splenocytes than in the memory HY primed splenocytes. The proportion of HY-specific T cells in spleens of immunized female mice is 10\%\textsuperscript{16} compared with greater than 90\% in naïve C6 transgenic mice\textsuperscript{18}. 
A crucial question is whether the inhibitory effect depends on the presence of cognate antigen. The evidence that MSC do not require MHC molecules to inhibit T-cell responses (Fig 6) strongly argues against a cognate mechanism but does not rule out the possibility that MSC inhibit specifically the cells which are activated by interaction with antigen. However, it is the ratio between MSC and total T cells rather than with antigen-specific T cells which influences the inhibition (Fig 5). These results favor the notion that MSC do not specifically target T cells which encounter antigen. Since neither APC (Fig 9) nor CD4+CD25+ regulatory T cells (Fig 10) are required for the inhibition to occur, overall our data suggest that MSC physically hinder T cells from the contact with APC in a non-cognate fashion.

Two papers have recently reported that MSC inhibit T-cell proliferation induced by non-specific mitogens or in polyclonal, polyepitope MLR13,14. Ours is the first demonstration of the inhibitory effect of MSC on T-cell response to cognate peptide. It is noteworthy that our findings differ from those reported by Di Nicola et al13 since in their work the effect of MSC appears to be mediated by soluble factors, and in particular by TGF-β and/or HGF, and does not require cell contact. Although this discrepancy might merely be explained by the fact that experiments were carried out in different species, our MSC have been generated in a different way. It is possible that BM stromal cells are different from MSC in this respect. These differences may also explain the much higher inhibitory efficiency of our MSC: an effect is clearly visible when the ratio between MSC and responder T cells is as low as 1:10^3 as compared to ratio of 1:1 required to see an effect
with stromal cells of Di Nicola et al. However, it is possible that soluble factors may have a small role also in our system (Fig 8).

Regardless of the mechanisms underlying the MSC inhibitory effect, the physiological role of this is unclear. It is well known that mesenchymal elements play a major role in T-cell ontogeny in the thymus. Since we observed that cell contact is required, MSC might provide a niche in the bone marrow in which lymphocytes are suppressed or deleted. However, the effect of MSC may not be specific to the immune system but have a more general “inhibitory” activity on protein synthesis and/or the cell cycle in a variety of cell types.

Our findings clearly have possible therapeutic implications. Because of their differentiation ability and susceptibility to stable gene transduction, MSC represent an attractive target to develop gene therapy. The evidence that MSC do not present alloantigen and that they do not require MHC expression to exert their inhibitory effect, suggests that they can be derived from a donor irrespective of their MHC haplotype and prepared as an “off-the-shelf” reagent for any patient. Here we show that MSC have a profound inhibitory effect on T-cell responses to transplantation antigens and may thus be used to control host-versus-graft (HvG) and GvHD, especially in those situations in which bone marrow transplantation is performed following reduced intensity conditioning. More importantly, we have demonstrated that not only naïve but also antigen-experienced (memory) T cells can be inhibited. The current ongoing clinical trials involve the infusion of MSC at the time of bone marrow transplant\textsuperscript{34}. When this
procedure is used for patients with leukemia, it is possible that the inhibitory effect may also jeopardize the graft-versus-leukemia effect which is crucial for a successful outcome. This potential complication needs to be considered against the probability that, according to our data, MSC have the potential ability to inhibit even ongoing GvHD.

References

1. Fridenstein A. Stromal bone marrow cells and the hematopoietic microenvironment. Arkh Patol. 1982;44:3-11


Legends to Figures

**Figure 1. MSC do not stimulate allogeneic T cells.**

The ability of MSC to function as antigen presenting cells was tested by using MSC as stimulators of secondary allogeneic MLR. Spleen cells from CBA (H2k) mice, stimulated in primary cultures with BALB.B (H2b) spleen cells (2x10^5/well), were re-stimulated with (A): BALB.B or (B): autologous CBA splenocytes as stimulators in the control cultures and with (C): graded numbers (10^2, 10^3, 10^4 per well) of irradiated (60cGy) (black bars) or non irradiated (white bars) MSC of BALB.B origin. (D): In a separate experiment BALB.B MSC were pre-treated with IFNγ (100U/ml for 48 hrs) before being used in the cultures. (E): Proliferation of MSC alone not irradiated. Cultures were performed in triplicate and the results reported are the average of 3 experiments of identical design. The bars show the standard deviation.

*: statistically significant (at least p<0.01) vs control cultures (stimulation with syngeneic spleen cells) in all experiments

**Figure 2. Immunophenotype of BM MSC**

MSC were analysed for the expression of CD106, CD80, and MHC class I molecules in standard conditions (A) or after 48h-culture with 100 U/ml IFNγ (B).

**Figure 3. MSC inhibit the response of naïve antigen-specific T cells in a dose-dependent fashion.**

Splenocytes from C6 mice transgenic for a K^k-restricted HY-specific T-cell receptor were
stimulated with irradiated syngeneic (CBA, H-2\(^k\)) spleen cells pulsed with the cognate HY K\(^k\)Smcy(TENSGKDI) peptide. Graded doses of MSC were added as third party cells for the duration of the in vitro stimulation. After 24 hours CD8\(^+\) cells were analyzed for IFN\(\gamma\) by intracellular staining (B). T-cell proliferation was assessed after 48 hours (A). Results are shown for cells cultured in the presence (solid lines) or absence (dotted lines) of the HY peptide. The results reported are the average of 3 experiments of identical design. T-cell proliferation cultures were performed in triplicate and results averaged. The bars show the standard deviation.

MSC/R: MSC to responder T cells ratio.

*: statistically significant (at least p<0.01) vs control cultures without MSC in all experiments

**Figure 4. MSC inhibit the response of antigen-specific memory T cells in a dose-dependent fashion.**

Splenocytes from normal, non transgenic female C57BL/6 (H-2\(^b\)) mice immunized with syngeneic male spleen cells were restimulated in vitro with syngeneic male spleen cells. Graded doses of MSC were added as a third party to the MLR. After 7 days, the cells were harvested and restimulated with female spleen cells pulsed with the HY peptides HY D\(^b\)Smcy (KCSRNQRQYL), HY D\(^b\)Uty (WMHHNMDLI), and HY A\(^b\)Dby (NAGFNSNRANSSRSS) and then assessed for T-cell proliferation (A), numbers of HY-specific IFN\(\gamma^+\) cells (B) and HY D\(^b\)Smcy and HY D\(^b\)Uty tetramer-positive CD8\(^+\) T cells (C). HY-specific cytotoxic activity was tested against HY peptide (HY D\(^b\)Smcy and HY D\(^b\)Uty) pulsed RMA-S cell line in a \(^{51}\)Cr-release assay (D). The results refer to the
cultures in the presence (solid lines) or absence (dotted lines) of the HY peptide. The results reported are the average of 5 experiments of identical design. T-cell proliferation (2 experiments) were performed in triplicate and results averaged. The bars show the standard deviation.

MSC/R: MSC to responder T cells ratio.

*: statistically significant (at least p<0.01) vs control cultures without MSC in all experiments

**Figure 5. MSC do not specifically target antigen-reactive T cells**

Spleen cells from normal Thy1.2 C57BL/6 HY-immune female mice were stimulated with syngeneic male cells. After 7 days the proportion of HY-TCR$^+$ T cells were 50% (A). These responder cells were used as such or mixed with splenocytes from Thy1.1 C57BL/6 naïve female mice at a 1:2 or 1:10 ratio. 5x10$^6$ cells of the different mixtures were stimulated with equal number of HY peptide ($D^b$Smcy, $D^b$Uty, $A^b$Dby) pulsed syngeneic female cells in the presence or absence of a fixed number ($10^5$) of MSC cells. After 24 hours the Thy1.2$^+$ cell population was assessed for numbers of peptide-specific IFN$^+$ cells. The figure (B) reports the percentages of inhibition exerted by MSC on the different mixtures of responder cells.

**Figure 6. MSC inhibitory effect is MHC-independent.**

Spleen cells from C6 Tg (naïve) (A) or normal C57BL/6 HY-immune (memory) (B) mice were stimulated with HY K$^b$Smcy peptide or syngeneic male cells respectively, as described previously, in the presence or absence of the 3T3-F442A MSC line which does
not express MHC class I molecules even after IFNγ treatment. T-cell proliferation (cpm) and percentage of IFNγ producing CD8+ T cells were evaluated. The results refer to the cultures in the presence (white bars) or absence (black bars) of HY antigen. The results reported are the average of 3 experiments of identical design. T-cell proliferation cultures were performed in triplicate and results averaged. The bars show the standard deviation.

*: statistically significant (at least \( p<0.01 \)) vs control cultures without MSC in all experiments.

Figure 7. The MSC inhibitory effect is transient

(A): Spleen cells from C6 Tg ( naïve) mice were stimulated for 24 hrs with HY K^kSmcy (TENSGKDI) peptide-pulsed APC (black bar). Cells were then harvested, washed and restimulated with the same peptide for a further 24 hours (white bar). (B): The same responder cells (C6 Tg) were stimulated for 24 hrs with HY K^kSmcy peptide-pulsed APC in the presence of MSC (MSC/R ratio = 1:10) (black bar). Cells were then harvested, depleted of MSC by CD106 antibodies and Dynabeads before restimulation with the same peptide (white bar). The Y-axis reports the percentage of IFNγ producing CD8+ T cells. The results reported are the average of 3 experiments of identical design. The bars show the standard deviation.

*: Statistically significant (at least \( p<0.01 \)) vs. control cultures containing MSC (A, black bar) in all experiments. The comparison between the restimulation of C6 cells with HY K^kSmcy peptide (white bars) previously stimulated with the same peptide in the presence (B) or absence (A) of MSC was not statistically significant. The bars show the standard deviation.
**Figure 8. MSC inhibitory effect requires cell contact**

HY-specific memory T cells from immunized B6 female were stimulated in vitro with syngeneic male spleen cells (B6) in the presence of MSC (B6+MSC) or supernatant from MSC cultures (B6+MSC-SN). In parallel, responder T cells were also stimulated with syngeneic male spleen cells in the presence of MSC in a Transwell® system (B6+MSC-T/W). The percentage of HYD<sup>b</sup>Sm<sub>cycl</sub> and HYD<sup>b</sup>Uty tetramer-positive CD8<sup>+</sup> T cells (A) and/or HY-specific IFN<sub>γ</sub>-positive CD8<sup>+</sup> T cells (B) are shown for each culture. The results reported are the average of 4 identical experiments. The bars show the standard deviation.

*:* Statistically significant (at least p<0.01) vs. control cultures (B6)

**Figure 9. MSC inhibitory effect does not require antigen presenting cells**

Proliferative response of B6 splenocytes (A) or HY-specific T-cell clone CTL-10<sup>25</sup> (B) to CD3/CD28-coated beads in the presence or absence of MSC. One representative experiment is shown of three performed. The background counts for unstimulated spleen and T-cell clone was less than 1% and less than 5%, respectively. The scale of the y-axis on the left and on the right is different.

*:* statistically significant (at least p<0.01) vs control cultures without MSC in all experiments.

**Figure 10. MSC inhibitory effect does not require CD4<sup>+</sup>/CD25<sup>+</sup> regulatory T cells**
5x10^6 CD25-depleted or CD25-replete C6 splenocytes were stimulated in vitro with HY K^KSmcy peptide pulsed female cells in the presence or absence of MSC (10^5). After 24 hours HY-specific IFNγ-positive CD8^+ T cells were enumerated. MSC inhibition on CD25-negative responders was 50% as compared to 44% in the control CD25-replete cultures (not statistically significant). The bars show the standard deviation.
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Bone marrow mesenchymal stem cells inhibit the response of naive and memory antigen-specific T cells to their cognate peptide

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