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Immunomodulatory effect of human adipose tissue-derived adult stem cells: comparison with bone marrow mesenchymal stem cells

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Summary

Like mesenchymal stem cells from bone marrow (BM-MSCs), adipose tissue-derived adult stem cells (ADAS cells) can differentiate into several lineages and present therapeutical potential for repairing damaged tissues. The use of allogenic stem cells can enlarge their therapeutical interest, provided that the grafted cells could be tolerated. We investigate here, for the first time, the immunosuppressive properties of ADAS cells compared with the well-characterized immunosuppressive properties of BM-MSCs. ADAS cells did not provoke *in vitro* alloreactivity of incompatible lymphocytes and, moreover, suppressed mixed lymphocyte reaction (MLR) and lymphocyte proliferative response to mitogens. The impairment of inhibition when ADAS cells and BM-MSCs were separated from lymphocytes by a permeable membrane suggests that cell contact is required for a full inhibitory effect. Hepatocyte growth factor is secreted by both stem cells but, similar to interleukin-10 and transforming growth factor-β (TGF-β), the levels of which were undetectable in supernatants of MLR inhibited by ADAS cells or BM-MSCs, it did not seem implicated in the stem cell suppressive effect. These findings support that ADAS cells share immunosuppressive properties with BM-MSCs. Therefore, ADAS cell-based reconstructive therapy could employ allogenic cells and because of their immunosuppressive properties, ADAS cells could be an alternative source to BM-MSCs to treat allogenic conflicts.

Keywords: immunosuppression, bone marrow, adipose tissue, stem cell, cell therapy.

Adipose tissue represents a source of adult stem cells [adipose tissue-derived adult stem (ADAS) cells] that can differentiate into numerous lineages (bone, cartilage, adipose, muscle and endothelium) including neurogenic (Erickson *et al*, 2002; Gimble & Guilak, 2003a,b; Planat-Benard *et al*, 2004a,b; Safford *et al*, 2002; Zuk *et al*, 2002). Thus, ADAS cells have potential clinical application for the repair of damaged tissues and angiogenic therapy. Injection of human ADAS cells was recently shown to improve neovascularization in ischaemic hindlimb and osteoid matrix formation in immunotolerant mice (Hicok *et al*, 2004; Miranville *et al*, 2004). In the same way, ADAS cells increased the functional capacity of damaged skeletal muscle *in vivo* (Bacou *et al*, 2004). Alternatively, ADAS cells can be easily transduced by retroviral vectors and thus they can be engineered to express various molecules and to delivery therapeutic molecules *in vivo* (Gimble & Guilak, 2003a; Morizono *et al*, 2003). Furthermore, ADAS cells were shown to participate to haematopoietic reconstitution in lethally irradiated mice (Cousin *et al*, 2003).

Similar properties were previously reported for bone marrow mesenchymal stem cells (BM-MSCs), which have been extensively studied (Lazarus *et al*, 1995; Pittenger *et al*, 1999; Deans & Moseley, 2000; Jorgensen *et al*, 2003). BM-MSCs have been shown to home to injured tissue in mouse and macaque models, and to improve bone mineral content in patients with osteogenesis imperfecta (Horwitz *et al*, 2001; Chapel *et al*,

2003; Jorgensen *et al*, 2003). Co-transplantation of human BM-MSCs and haematopoietic stem cells seems to improve haematopoiesis after myeloablative therapy in breast cancer patients through reconstitution of the BM stroma (Koc *et al*, 2000).

The therapeutic use of ADAS cells or BM-MSCs requires large quantities of cells for infusion or co-transplantation. A large quantity of ADAS cells can be obtained by lipectomy, which is currently performed with local anaesthesia on healthy people (Aust *et al*, 2004). In contrast, MSCs are present in very low numbers in bone marrow, and bone marrow aspirates are mostly dedicated to transplantation of haematopoietic stem cells. Sufficient BM-MSCs may not be obtained from some patients, particularly from older patients or those with malignant diseases. Furthermore, cultured ADAS cells seem to display an increased *in vitro* proliferative potential compared with BM-MSCs, and could generate a clinically effective cell dose more rapidly than the same number of marrow cells (De Ugarte *et al*, 2003; Fraser *et al*, 2004).

Because of difficulties in obtaining sufficient autologous stem cells, ADAS cells from allogenic donor could constitute a valuable alternative source of stem cells for therapeutic use, and the infusion of allogenic stem cells from a healthy donor, or even from a third party, in case of transplantation, could form the basis for new strategies in cell therapy. A prerequisite when considering allogenic ADAS cells for therapeutic use is the characterization of their immunological properties in allogenic conditions. Several studies reported on the *in vitro* and *in vivo* immunosuppressive properties of BM-MSCs (Bartholomew *et al*, 2002; Djouad *et al*, 2003; Le Blanc *et al*, 2003; Tse *et al*, 2003). These properties strengthen the clinical relevance of BM-MSCs in allogenic transplantation by reducing the incidence and severity of graft-*versus*-host disease (GVHD) (Lazarus *et al*, 2000; Jorgensen *et al*, 2003). Furthermore, BM-MSCs were shown escape to the immune system because they do not express major histocompatibility complex (MHC) class II or co-stimulatory molecule B7, and consequently, they do not induce allospecific T cell proliferative responses (Tse *et al*, 2003). In contrast, despite the increasing interest in the use of ADAS cells in cell therapy, their immunosuppressive properties have not been studied so far.

We investigated, for the first time, the *in vitro* immunosuppressive properties of ADAS cells. We studied their capacity to provoke *in vitro* allogenic reactions, and their inhibitory activity on allogenic mixed lymphocyte reaction (MLR) and on lymphocyte responses to mitogens. We compared these ADAS cell's properties to those obtained with BM-MSCs.

Material and methods

Cell culture

ADAS cells were isolated from human adipose tissue removed from abdominal dermolipectomy in the plastic surgery department of Rangueil University Hospital, Toulouse. Rapidly after surgery, subcutaneous adipose tissue was digested with 2 mg/ml collagenase A (Roche Diagnostic, Mannheim, Germany) in Dulbecco's modified essential medium F12 (DMEM-F12; Gibco, Cergy Pontoise, France) containing 2% bovine serum albumin for 45 min at 37°C. Digested tissue was filtered twice through a 100 μm, then a 25 μm, nylon membrane to eliminate undigested fragments. After centrifugation (600 g for 10 min), cells forming the stroma vascular fraction of the adipose tissue were collected and red cells were lysed in a buffer containing 155 mmol/l NH_4Cl, 20 mmol/l Tris pH 7·6, for 5 min.

Isolated stroma cells were then seeded in plastic plates at the initial density of $3{\cdot}2 \times 10^4$ cells/cm^2 in DMEM-F12 (1:1) medium supplemented with 5% new born calf serum, 100 μg/ml pantothenic acid, 100 μmol/l ascorbic acid, 16 μmol/l biotin, 250 μg/ml amphotericin, 5 μg/ml streptomicyn and 5 U/ml penicillin. After 24 h, non-adherent cells were removed (80% of initial cells seeded) and medium was changed twice a week. Preadipocyte growth was pursued until cells reached confluence (at approximately 8 d with a cell density of about 3×10^4 cells/cm^2) and without any passage before usage.

Human BM-MSCs were isolated either from spongy bone residues obtained during hip surgery or from washed filters used during bone marrow graft processing for allogeneic bone marrow transplantation. Cells were cultivated in 25 cm^2 or 75 cm^2 flasks (TPP; ATGC biotechnologie, Marne la Vallée, France) in minimum essential medium-α (MEM-α) supplemented with 10% fetal calf serum (FCS), ciprofloxacine (10 μg/ml; Bayer, Puteaux, France) at the concentration of 5×10^4 cells/cm^2. After 72 h at 37°C, 5% CO_2, non-adherent cells were removed and medium was changed. Cultures were fed every 3 or 4 d until confluence. At confluence (or after a maximum of 21 d of culture), adherent cells were trypsinized, harvested and cultured at the concentration of 10^3/cm^2 for 1–3 weeks. BM-MSCs were then cryo-preserved before usage.

The differentiation potentials of both ADAS cells and BM-MSCs were checked in specific mediums. For adipocyte differentiation, cells were cultured in 1 μmol/l dexamethazone, 60 μmol/l indomethacine, 450 μl 3-isobutyl-1-methylxanthine. Adipocytes were characterized by microscopic examination and by oil red staining. For endothelial potential, cells were cultured in MethoCult GF H4534 (StemCell Technologies, Meylan, France). Endothelial phenotype was revealed by staining with antibody against Factor VIII. For differentiation into osteoblasts, culture medium was supplemented with 0·1 μmol/l dexamethasone, 10 mmol/l β-glycerophosphate and 60 mmol/l ascorbate. Osteoblasts were characterized by their alkaline phosphatase activity measured using an alkaline phosphatase kit (Sigma, Saint Quentin Fallavier, France) and macroscopic examination. Osteogenesis-induced cells changed from spindle to cuboid-shaped as they differentiated.

Cell surface phenotype

Stem cells (both ADAS cells and BM-MSCs) were stained with various combinations of saturating amounts of monoclonal antibodies conjugated with allophycocyanin (APC), fluorescein

isothiocyanate (FITC) or phycoerythrin (PE): CD13-APC, CD44-FITC, CD73-PE, CD90-APC, HLA-DR PE (BD Biosciences, le Pont de Claix, France), CD45-FITC, CD34-APC, HLA-ABC FITC (Beckman Coulter, Roissy, France). ADAS cells were also stained with CD105, CD144 (Serotec, Cergy Saint Christophe, France) and CD31 (BD Biosciences). At least 15 000 events were analysed by flow cytometry (FACScan, BD Biosciences) with the CELLQUEST software.

MLR and mitogen proliferative assays

Peripheral blood mononuclear cells (PBMCs) were isolated from the peripheral blood of healthy subjects by sucrose gradient centrifugation (Eurobio, Les Ulis, France) and then frozen, for use as responder cells in MLR or in mitogen proliferative assays.

PBMCs and stem cells were resuspended in Roswell Park Memorial Institute (RPMI) 1640 medium containing 20% of pooled human AB serum (Institut J. Boy, Reims, France), 25 mmol/l HEPES, 2 mmol/l L-alanyl-L-glutamine, 1 mmol/l sodium pyruvate, 100 U/ml penicillin and 100 μg/ml streptomycin (InVitrogen, Cergy, France).

For MLR, PBMCs from two HLA-DRB mismatched individuals were seeded in triplicate at the concentration of 5×10^4, cells/50 μl/well in 96-well round-bottom plates (Nunc, Roskilde, Denmark). RPMI 1640 medium (50 μl) or stem cells (5×10^4 cells/50 μl/well, i.e. at a ratio 1 stem cell:1 lymphocyte) were added to the MLR, at the beginning of the experiment unless otherwise stated. In some experiments, stem cells were added in diminishing concentrations ($2{\cdot}5 \times 10^4$; $1{\cdot}25 \times 10^4$; $6{\cdot}25 \times 10^3$; $1{\cdot}56 \times 10^3$; 781; 195; 48·8 cells/50 μl/well). After 5 d of incubation, ^{3}H-thymidine (370 kBq/well; ICN Pharmaceuticals, Orsay, France) was added overnight and thymidine incorporation was measured using a β-scintillation counter (Wallac; Perkin Elmer, Courtaboeuf, France) and expressed as counts per minute (cpm).

For experiments using transwell chambers (BD Biosciences), 24 well plates were used. 5×10^5 PBMCs per well from two HLA-DRB mismatched individuals were seeded in the presence or absence of 5×10^5 stem cells. In that case, stem cells were physically separated from PBMCs by a high density pore membrane and were added in the upper compartment either at the beginning of the MLR or 7, 24 or 48 h after initiation of the MLR.

In mitogen proliferative assays, PBMCs were seeded in triplicate at the concentration of 2×10^5 cells/50 μl/well in 96-well flat-bottom plates with 9 μg/ml of phytohaemagglutinin (PHA, Fisher Scientific, Elancourt, France) or concanavalin A (ConA; Sigma, St Quentin Fallavier, France), 2·3 μg/ml of pokeweed (PWM, Sigma) or 10 μg/ml well-coated OKT3 (Beckman Coulter). RPMI 1640 medium (50 μl/well) or stem cells (2×10^5 cells/50 μl/well, i.e. at a ratio 1 stem cell:1 lymphocyte) were added. After 4 d of incubation, ^{3}H-thymidine (740 kBq/well) was added for 8 h. In the experiments using transwell chambers, 5×10^5 PBMCs per well were stimulated or not with 9 μg/ml of PHA. 5×10^5 stem cells physically separated from lymphocytes were added.

Inhibitory experiments were also performed on MLR with supernatants from stem cell culture or supernatants from MLR. In that case, supernatants from stem cell culture were obtained from stem cells cultivated in flask, in MEM-α 10% FCS (for BM-MSCs) or DMEM 5% new born calf serum (for ADAS cells, as described above). Supernatants referred to as 'conditioned stem cells – MLR supernatant' were obtained from MLR cultivated with stem cells as follows: PBMCs from two HLA-DRB mismatched individuals were seeded in triplicate at the concentration of 5×10^4 cells/50 μl/well in a serum free medium (LP02 medium, MacoPharma, Tourcoing, France) in 96-well round-bottom plates (Nunc). Stem cells (5×10^4 cells/50 μl/well, i.e. at a ratio 1 stem cell:1 lymphocyte) were added to the MLR, at the beginning of the experiment. After 5 d of incubation, conditioned stem cells – MLR supernatants were collected. Supernatants of stem cells cultivated alone as a control in the serum free medium in 96-well round-bottom plates were also collected.

To analyse the impact of both type of supernatants on MLR, PBMCs from two HLA-DRB mismatched individuals were seeded in triplicate at the concentration of 5×10^4 cells/50 μl/well in 96-well round-bottom plates (Nunc) in RPMI 1640 medium 10% FCS in the presence of 50 μl/well of supernatant from stem cells culture or 'conditioned stem cells – MLR supernatant'. In both cases, supernatants from stem cells culture and 'conditioned stem cells – MLR supernatant', the final dilution was one-third.

The percentage of inhibition was calculated as follows: 100 − (cpm MLR + stem cells/cpm MLR) × 100.

MSC production of cytokines

Levels of transforming growth factor-β (TGF-β), hepatocyte growth factor (HGF) and interleukin 10 (IL-10) were measured by enzyme-linked immunoassays (OptEIA ELISA, BD Biosciences for TGF and IL-10, Quantikine human HGF, RD systems, Lille, France for HGF) in supernatants of stem cell culture and 'conditioned stem cells – MLR supernatants'.

Statistical analysis

The Student *t*-test for paired data was used to test the probability of significant differences between samples. Statistical significance ($P < 0{\cdot}05$) is indicated in the figures as follows: $^{***}P < 0{\cdot}0001$; $^{**}P < 0{\cdot}0001$–$0{\cdot}01$; $^{*}P < 0{\cdot}01$–$0{\cdot}05$.

Results

Characterization of isolated stem cells

We carefully checked the antigenic phenotype of all the cell preparations used by flow cytometric analysis (Fig 1). The culture procedure of ADAS cells homogenized cell phenotype

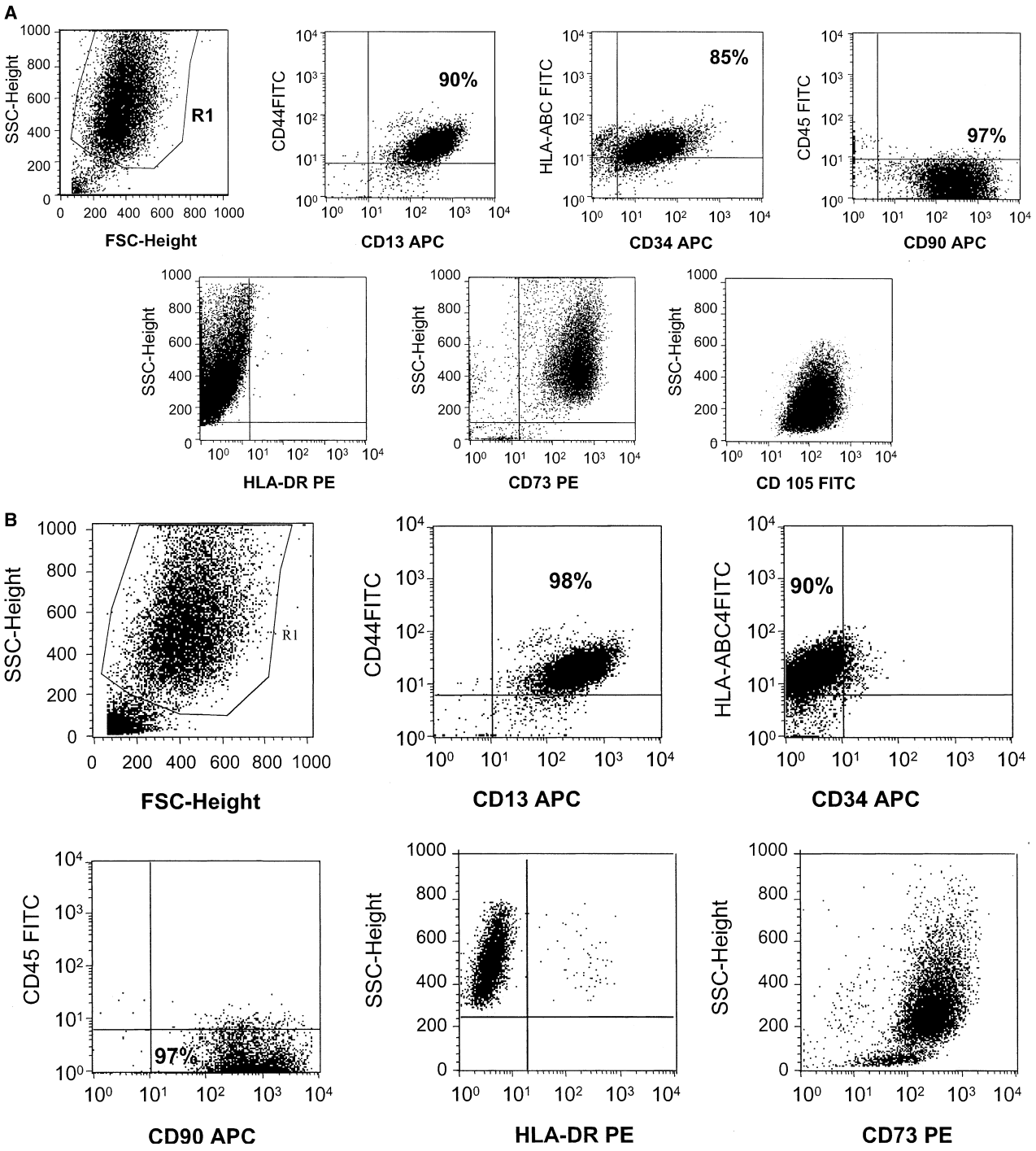

Fig 1. ADAS cells (A) and BM-MSCs (B) surface phenotypes.

(data not shown; Planat-Benard *et al*, 2004b). Among the antigens tested, ADAS cells and BM-MSCs differed only by the expression of the CD34 antigen (83·9 ± 11·5% of $CD34^+$ cells in ADAS cells samples whereas BM-MSCs were negative), in agreement with previous reports (Gronthos *et al*, 2001; Planat-Benard *et al*, 2004b). Notably, $CD34^+$ staining on ADAS cells decreased according to passages (not shown) but, for functional tests, we always used primary cell cultures in which all cells were CD34 positive. Cell preparations were not contaminated by haematopoietic or endothelial cells as flow cytometry analysis demonstrated that cell preparations were always negative for the CD45, CD31 and CD144 antigens (Fig 1 and

data not shown). Both types of stem cells were uniformly positive for CD13, CD44, CD73, CD90, CD105, HLA class I antigens and negative for HLA-DR (Fig 1).

As expected and previously described, culture in the appropriate mediums showed that isolated ADAS cells and BM-MSCs were both capable of *in vitro* differentiation into osteoblasts and adipocytes; ADAS cells were also capable of *in vitro* differentiation into endothelial cells (Fig 2).

ADAS cells do not elicit lymphocyte proliferation

To determine whether ADAS cells and BM-MSCs could function as antigen presenting cells, cells were co-cultured with allogenic PBMCs at a ratio of 1:1. Neither ADAS cells ($n = 7$), nor BM-MSCs ($n = 7$) stimulated lymphocyte proliferation (Fig 3). In parallel control experiments, the PBMCs used as responding cells gave high stimulatory indexes when exposed to allogenic HLA-DRB incompatible lymphocytes (data not shown).

Effect of ADAS cells on MLR

Two-way MLR between two HLA-DRB mismatched lymphocytes were inhibited by ADAS cells from a third party (at a ratio of 1stem cell:1 responding lymphocyte) (83 000 cpm for MLR to- 33 400 cpm for MLR in the presence of ADAS cells; mean inhibition, 60%; range, 0–91%, $P = 0{\cdot}0003$) (Fig 4A). In the same conditions, and as previously demonstrated by others (Di Nicola *et al*, 2002; Tse *et al*, 2003), BM-MSCs suppressed lymphocyte proliferation to the same extent (75 500–30 800 cpm; mean inhibition, 59%; range, 4–98%, $P < 0{\cdot}0001$) (Fig 4A). The inhibitory effect was dependent on the amount of ADAS cells and BM-MSCs added to cultures (Fig 4B). The suppression was still significant with a very low stem cell/lymphocyte ratio (1:1000 i.e. 48·8 BM-MSCs/5×10^4 responding lymphocytes for BM-MSCs, $P = 0{\cdot}03$; 1:8 i.e. $6{\cdot}2 \times 10^3$ stem cells/5×10^4 responding lymphocytes for ADAS cells, $P = 0{\cdot}05$). To characterize the kinetics of ADAS cells and BM-MSCs inhibition on MLR, two-way MLR was established in a transwell chamber device and stem cells (at a ratio of 1 stem cell:1 responding lymphocyte) were added in the upper compartment at various times after MLR initiation: just after MLR initiation, or 7, 24 and 48 h after MLR initiation (conditions giving, respectively, 120, 113, 96, 72 h of co-culture of stem cells with lymphocytes engaged for the MLR) (Fig 4C). For both type of stem cells, the suppression of lymphocyte proliferation was maximum when stem cells were added at the beginning of the culture.

Effect of ADAS cells on mitogen proliferation

ADAS cells strongly inhibited the lymphocyte proliferation in response to PHA (from 372 314 to 27 680 cpm; mean inhibition 90%), ConA (from 118 827 to 35 494 cpm; mean

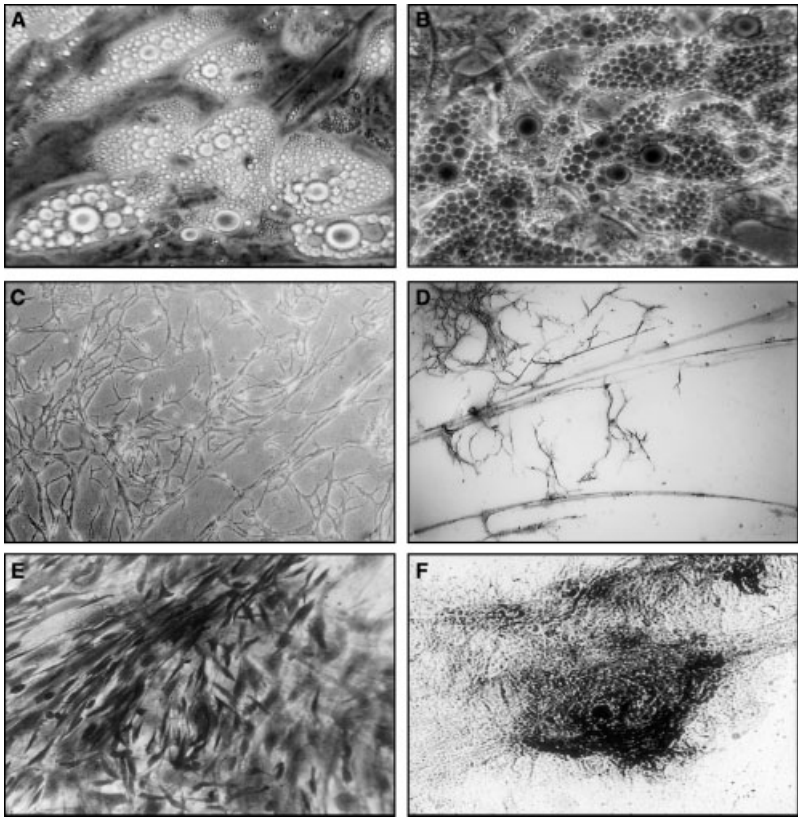

Fig 2. *In vitro* differentiation capacity of ADAS cells. In adipogenic medium, cultured ADAS cells differentiated into adipocytes. (A) phase contrast; (B) oilred staining (magnification ×200). When ADAS cells were seeded in methylcellulose medium, they spontaneously formed branched alignments and tube-like structures; (C) phase contrast; (D) after CD31 immunostaining (magnification ×200). In osteoblastic medium, ADAS cells differentiated into osteoblasts; (E) May–Grunwald–Giemsa staining; (F) alkaline phosphatase (magnification ×40).

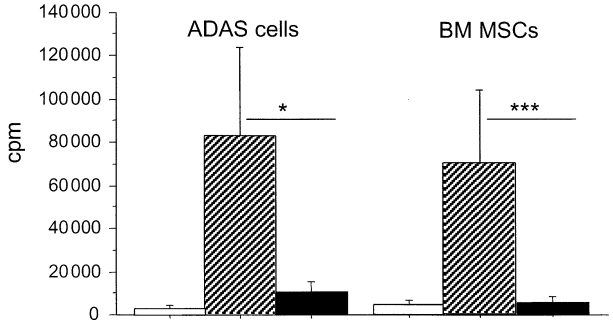

Fig 3. Lack of proliferation of PBMCs in presence of allogenic stem cells. The proliferation of PBMCs alone (white bar), or of PBMCs co-cultured with allogenic PBMCs (hatched bar) or allogenic stem cells (either ADAS cells or BM-MSCs) (black bar) was measured by ^{3}H-thymidine incorporation. Data are expressed as mean ± SD of seven independent experiments each performed in triplicate.

inhibition, 65%) and OKT3 (from 157 341 to 61 019 cpm; mean inhibition, 62%) ($P < 0{\cdot}0001$, $P = 0{\cdot}06$, $P = 0{\cdot}05$ respectively) (Fig 5A–C). BM-MSCs suppressed lymphocyte proliferation to the same extent: from 250 114 to 33 961 cpm, 82% inhibition for PHA ($P < 0{\cdot}0001$); from 128 920 to 34 383 cpm, 65% inhibition for ConA ($P = 0{\cdot}001$); from 223 751 to 65 549 cpm, 61% inhibition for OKT3 ($P = 0{\cdot}009$) (Fig 5A–C). Proliferation in response to PWM was suppressed in only five experiments of seven with BM-MSCs ($P = 0{\cdot}08$), and in all three experiments with ADAS cells ($P = 0{\cdot}02$) (Fig 5D).

Similarly to the inhibitory effect on MLR, suppression by ADAS cells and BM-MSCs of lymphocyte proliferation to PHA was dependent on the amount of stem cells in the well (Fig 5E). Fifty per cent of the inhibitory effect was obtained with only $2{\cdot}5 \times 10^4$ stem cells per well (ratio stem cells/responding lymphocytes: 1:8).

To correlate more precisely the inhibitory effect of ADAS cells and BM-MSCs, we analysed the latter on the same PBMCs samples. When using PBMCs from one given individual, both ADAS cells and BM-MSCs strongly inhibited the lymphocyte proliferation in response to PHA [from 450 747 to 28 535 cpm for ADAS cells ($n = 3$) and from 392 522 to 34 701 cpm for BM-MSCs ($n = 6$)]. Also, ADAS cells and BM-MSCs inhibited the same MLR to the same extent [from 88 356 to 11 013 cpm for ADAS cells ($n = 2$) and from 88 356 to 15 951 cpm for BM-MSCs ($n = 2$)].

Importance of cell contact and soluble mediators for ADAS cells' inhibitory effect

To determine the relative importance of soluble factor(s) or cell contact in the stem cells inhibitory effect, we compared the latter when stem cells were cultured in contact with lymphocytes or separated from them by a transwell membrane. Separation of BM-MSCs and stimulated lymphocytes impaired the strength of inhibition, which, however, persisted (Fig 6).

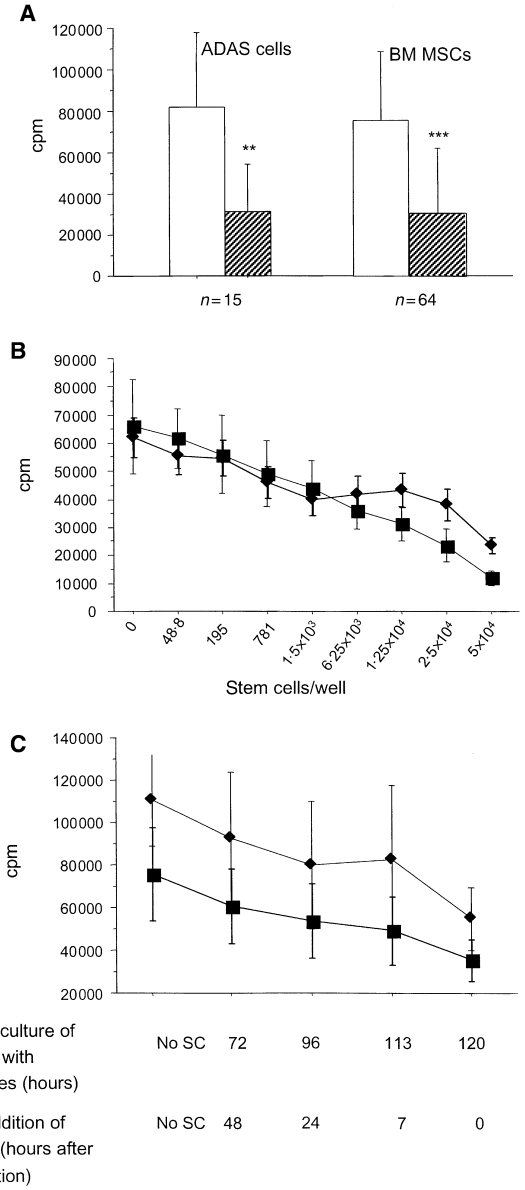

Fig 4. Inhibitory effect of ADAS cells and BM-MSCs on MLR. (A) Five-day mixed cultures were performed with (hatched bar) or without (white bar) allogenic stem cells (either ADAS cells or BM-MSCs). Experiments were performed at a ratio 1stem cell:1 responder lymphocyte. Data are expressed as mean ± SD of the indicated number of experiments, each performed in triplicate. (B) MLR were performed with diminishing concentrations of ADAS cells (■) or BM-MSCs (◆). Data are expressed as mean ± SE of 17 and six experiments respectively. Each experiment was performed in triplicate. (C) MLR were performed with ADAS cells (■) or BM-MSCs (◆) (both at a ratio 1:1) separated by a transwell membrane and added at the initiation of MLR or 7, 24 or 48 h after. Data are expressed as mean ± SE of three and five experiments respectively. Each experiment was performed in triplicate.

The separation of ADAS cells and PHA-stimulated lymphocytes also decreased the power of inhibition (44% vs. 85% inhibition). In contrast to BM-MSCs, the ADAS cells inhibitory

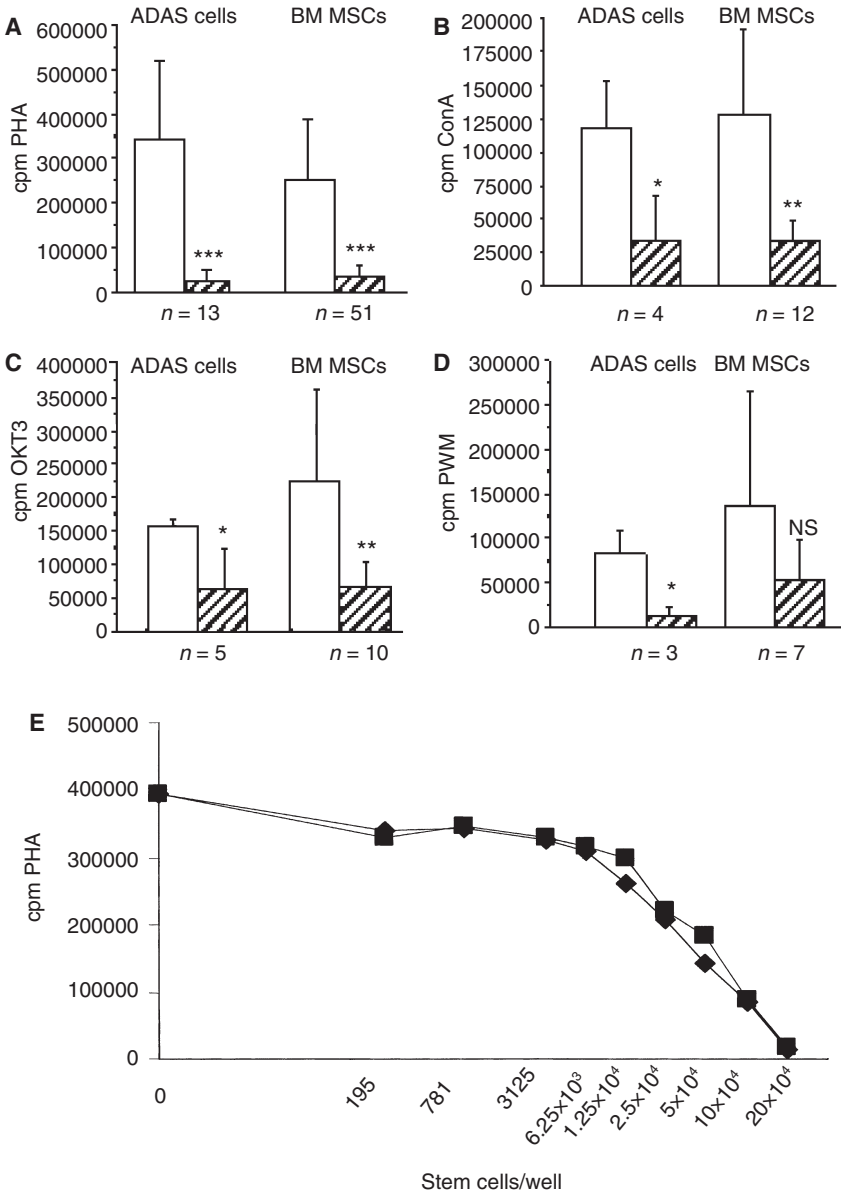

Fig 5. Inhibitory effect of ADAS cells and BM-MSCs on lymphocyte response to mitogens. PBMCs were stimulated with PHA (A), ConA (B), OKT3 (C) or PWM (D) in the presence of stem cells (either ADAS cells or BM-MSCs) (hatched bar) or without stem cells (white bar). All experiments were performed with a ratio 1stem cell:1 responder lymphocyte. Data are expressed as mean ± SD of the indicated number of experiments, each performed in triplicate. (E) ADAS cells (■) or BM-MSCs (◆) were added in diminishing concentration in PBMCs stimulated with PHA. Results of one representative experiment are expressed in cpm.

effect on MLR was not impaired when they were separated from responding lymphocytes (46% vs. 26%). It is important to note that in these five latter experiments, the inhibitory effect of ADAS cells on MLR without the transwell device was low (26% inhibition), which could explain why the inhibitory effect was not impaired when using a transwell membrane.

These results suggest the possible involvement in stem cell inhibitory effect, of soluble mediator(s) secreted by ADAS cells or BM-MSCs. Such soluble mediators can be either produced spontaneously by stem cells or can be secreted only when stem cells are exposed to allogenic lymphocytes. To explore these two hypotheses, we tested first the inhibitory effect of the supernatant of stem cell culture. The BM-MSCs supernatant did not suppressed MLR (65 896 vs. 67 006 cpm, $n = 6$, $P = 0{\cdot}85$), whereas the supernatants of ADAS cell culture did not suppress MLR, but stimulated it slightly (65 896 vs. 78 129 cpm, $n = 6$, $P = 0{\cdot}016$). It has to be noted that the addition of ADAS cell culture medium (used as control of ADAS cell supernatant) slightly increased the MLRs, although this did not reach significance (154 637 vs. 225 323 cpm, $n = 4$, $P = 0{\cdot}19$). These results suggest that stem cells did not spontaneously release the suppressive soluble factors in the supernatant.

Therefore, interaction between lymphocytes and stem cells could be required for the production of inhibitory factors, as evidenced by the transwell experiments. To test the latter hypothesis, we studied the inhibitory effect of 'conditioned stem cells-MLR supernatants'. All conditioned supernatants were collected from highly inhibited MLR (inhibition >75%). MLR was suppressed with only one conditioned supernatant of four with ADAS cells (40% inhibition) and two conditioned

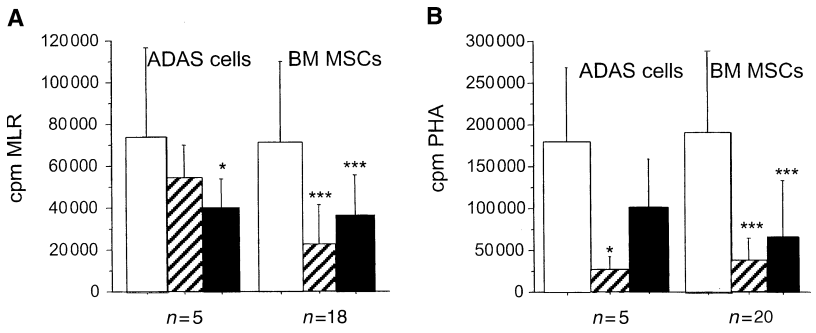

Fig 6. Relative effect of cell contact and soluble factor. (A) Five-day mixed cultures were performed with allogenic ADAS cells or BM-MSCs (hatched bar), ADAS cells or BM-MSCs separated by a transwell membrane (black bar) or without stem cells (white bar). (B) PBMCs were stimulated with PHA in the presence of ADAS cells or BM-MSCs in well (hatched bar), ADAS cells or BM-MSCs separated by a transwell membrane (black bar) or without stem cells (white bar). In all experiments, responder lymphocytes and stem cells were at a ratio of 1:1. Data are expressed as mean ± SD of the indicated number of experiments, each performed in triplicate. Results were compared with those of MLR carried out without stem cells.

supernatants of four with BM-MSCs (31% and 52% inhibition). In contrast, supernatants from regular MLR (performed without ADAS cells) stimulated the MLR (154 617 vs. 227 969 cpm, $n = 12$, $P = 0{\cdot}026$).

ADAS cells production of TGF-β, HGF and IL-10

Two cytokines, TGF-β and IL-10, well known for their immunosuppressive properties, as well as HGF, which could be implicated in stem cell-induced suppression of T cell proliferation, were quantified in ADAS cell and BM-MSC supernatants. The concentrations of IL-10 and TGF-β in supernatant from ADAS cells ($n = 4$) and BM-MSCs culture ($n = 3$) were below the level of detection (8 and 60 pg/ml respectively). Comparable results were obtained in the conditioned ADAS cells-MLR ($n = 4$) and BM-MSC-MLR supernatants ($n = 3$).

Interestingly, in flask-culture supernatants, HGF was detectable in supernatants of BM-MSCs ($n = 3$) (210–1050 pg/ml) but not in supernatants of ADAS cells ($n = 4$). In contrast, when we considered supernatants from stem cells cultivated in 96-well culture plates, HGF was present in both ADAS cells and BM-MSC supernatants, suggesting that culture conditions influence the production of HGF by ADAS cells. HGF was detectable in all conditioned ADAS cells- and BM-MSC-MLR supernatants (233–707 pg/ml). There was no correlation between the HGF level in conditioned ADAS cells- and BM-MSC-MLR supernatants and the ability of these supernatants to suppress MLR. For example, one inhibiting BM-MSC-MLR supernatant and one non-inhibiting BM-MSC-MLR supernatant both displayed a similar level of HGF (240 pg/ml). Supernatants from regular MLR (performed without ADAS cells) were negative for TGF-β, HGF and IL-10 ($n = 7$).

Discussion

We report, for the first time, characterization of the immunosuppressive properties of ADAS cells and compare these results with those obtained with BM-MSCs, used as positive control. We carefully checked the phenotype and the capacity of differentiation of all our cell preparations that were in agreement with previous studies (Pairault & Green, 1979; Laharrague *et al*, 1998; Gronthos *et al*, 2001; Gimble & Guilak, 2003b; Planat-Benard *et al*, 2004b). Although ADAS cells are currently considered as stem cells, it should be noted that their auto-replenishment capacity was not demonstrated *in vivo*.

The functional characterization of ADAS cells can be summarized as follows: (1) ADAS cells are unable to provoke response of allogenic lymphocytes *in vitro*; (2) ADAS cells inhibited lymphocyte proliferation induced by allogenic PBMCs or mitogens, and this inhibition persisted when they were separated from lymphocytes by a permeable membrane; (3) the inhibitory effect was dependent on the number of ADAS cells and the duration of ADAS cells/responder lymphocytes contact; (4) supernatant from ADAS cells culture did not inhibit MLR; (5) conditioned ADAS cells-MLR supernatants only slightly inhibited MLR; (6) ADAS cells differ from BM-MSCs; whereas both BM-MSCs and ADAS cells secrete HGF when cultured in 96-well culture plates, only BM-MSCs secrete HGF when cultured in flasks. ADAS cell culture medium and 'conditioned ADAS cells-MLR supernatants' displayed undetectable levels of IL-10 and TGF-β, but detectable levels of HGF.

We obtained similar results with BM-MSCs, as previously reported by others (Di Nicola *et al*, 2002; Djouad *et al*, 2003; Krampera *et al*, 2003; Tse *et al*, 2003; Le Blanc *et al*, 2004). The inhibitory properties of BM-MSCs have been extensively studied, although the mechanism of suppression is not clearly established at this time. In all studies reported to date in human and murine models, the BM-MSC-induced inhibition persisted, although it was impaired when BM-MSCs were separated from responder lymphocytes by a transwell membrane (Di Nicola *et al*, 2002; Djouad *et al*, 2003; Krampera *et al*, 2003; Tse *et al*, 2003). Our results concerning ADAS cells are concordant with these studies and suggest that ADAS cell-induced inhibition implicates a soluble factor, at least in part.

However, we found that two cytokines that could be implicated in the ADAS cell inhibitory effect (TGF-β and IL-10) were undetectable in ADAS cell culture supernatants and in conditioned ADAS cells – MLR supernatants. Consequently, we did not analyse the impact of monoclonal antibodies directed against these cytokines on MLR in presence of ADAS cells. Our results are in agreement with previous studies concerning BM-MSCs, which reported undetectable levels of IL-10 in BM-MSCs culture supernatants and in conditioned supernatant, and that anti-TGF-β neutralizing monoclonal antibody did not significantly reverse the BM-MSCs suppressive effect (Krampera *et al*, 2003; Tse *et al*, 2003; Le Blanc *et al*, 2004). Only one study reported that the addition of anti-TGF-β1 monoclonal antibody to MLR in the presence of BM-MSCs could restore T lymphocyte proliferation, although TGF-β1 level was not quantified in BM-MSCs supernatant (Di Nicola *et al*, 2002).

ADAS cells and BM-MSCs spontaneously secreted HGF in 96-well culture plates and this secretion was not increased in the presence of two allogenic lymphocytes (i.e. MLR). Furthermore, no correlation was established between the HGF level in conditioned ADAS cells- and BM-MSC-MLR supernatants and the ability of these supernatants to suppress MLR. This leads us to conclude that HGF, if it is implicated in stem cell suppressive effect, is probably not the only factor involved. Two studies reported controversial data about the implication of HGF in the BM-MSC inhibitory effect: one reported that addition of anti-HGF monoclonal antibody to MLR in presence of BM-MSCs restored T lymphocyte proliferation, while in the more recent study, anti-HGF monoclonal antibody failed to restore T lymphocyte proliferation (Di Nicola *et al*, 2002; Le Blanc *et al*, 2004).

The implication of prostoglandin E2 (PGE2) in the BM-MSCs inhibitory effect remains controversial (Tse *et al*, 2003; Aggarwal & Pittenger, 2004). PGE2 secretion by MSCs is time-dependent and decreases after 4–5 d of culture (Aggarwal & Pittenger, 2004). If PGE2, which is secreted early, was responsible for the MSCs inhibitory effect, a short (48 h) contact between stem cells and MLR should be sufficient to observe a maximal inhibitory effect. Our results regarding the kinetic of inhibitory effect of both ADAS cells and BM-MSCs showed that a contact of 5 d with lymphocytes is required to obtain a full inhibitory effect on MLR and thus did not support the implication of PGE2. However, it is possible that the kinetics of PGE2 secretion by stem cells stimulated by MLR is different to that of stem cells alone.

Thus, all the molecules tested here that could be implicated in the stem cell suppressive properties, are probably not involved. Consequently, the soluble factor implicated in ADAS cells remains to be defined. As reported for BM-MSCs in murine models (Djouad *et al*, 2003; Krampera *et al*, 2003), we showed that ADAS cells culture supernatant did not inhibit lymphocyte proliferation. Like BM-MSCs, ADAS cells did not release spontaneously suppressive soluble factors in the supernatant, and the interaction between lymphocytes and ADAS cells may be required for the production of inhibitory factor(s). The conditioned stem cells – MLR supernatants (both from ADAS cells and BM-MSCs) only slightly inhibited lymphocyte proliferation. This could be because of the fact that the supernatants had been frozen and thawed before being tested at the final dilution (1/3). However, one of the two inhibiting 'conditioned BM-MSCs – MLR supernatants' was tested fresh and frozen/thawed without any alteration in the inhibitory effect (30% inhibition of MLR in both conditions). Thus, the most probable hypothesis is that the suppressive factor(s) is fragile and/or not highly concentrated. Thus, even a low dilution (1/3 in our experiments) could severely impair the inhibitory effect of the supernatant. The impairment of inhibitory effect by a transwell membrane could also be explained by the dilution of the inhibitory factor because of the distance between stem cells and lymphocytes. Moreover, if the factor(s) is fragile, it could be partially destroyed before reaching its target. Another hypothesis is that close contact between ADAS cells and responder lymphocytes could be required in order to achieve a full inhibitory effect.

Finally, because an interaction between lymphocytes and stem cells could be required for the production of inhibitory factors, it remains necessary to characterize the stem cell's (both ADAS cells and BM-MSCs) stimulation by lymphocytes. Although our results did not fully characterize this mechanism, the persistence of MLR inhibition by stem cells separated from responder lymphocytes by a transwell membrane suggests again that a soluble factor released by the MLR could be implicated.

The ADAS cell inhibitory effect on MLR was highly variable between ADAS cell samples. Seven of the nine ADAS cell samples displayed inhibitory properties on MLR. As the intensity of inhibition varied slightly between MLR experiments for a given ADAS cell sample, we concluded that the intrinsic inhibitory property of ADAS cells varied between individual samples. On another hand, proliferative responses to the four studied mitogens were inhibited and it has to be noted that 100% of ADAS cells samples inhibited the proliferative response to PHA. We observed that, similarly to ADAS cells, BM-MSCs showed inter-experimental variability regarding the inhibition of MLR. This variability is more pronounced than in the previous studies with BM-MSCs (Le Blanc *et al*, 2003; Tse *et al*, 2003), and is probably due to the larger number of experiments ($n = 64$) carried out with 18 independent samples of BM-MSCs. The inhibition of polyclonal mitogen-induced lymphocyte proliferation also varied with the mitogen. The intensity of inhibitions were the greatest against proliferation induced by PHA, then by ConA and last by OKT3. The lymphocyte PWM-induced proliferation was more regularly sensitive to inhibition by ADAS cells (all three experiments) than to the inhibition by BM-MSCs (five of seven experiments). As all ADAS cells and BM-MSCs samples were processed following a standardized procedure, the cause of the functional variability observed in MLR

remains to be determined. This functional variability requires special attention regarding the selection of tests, which could be proposed for the validation of ADAS cells as therapeutical tools.

Like BM-MSCs, ADAS cells have potential clinical interest. Allogenic ADAS cells have application in damaged tissue repair, as they were shown to differentiate into various cell lineages (Erickson *et al*, 2002; Gimble & Guilak, 2003a,b; Planat-Benard *et al*, 2004a,b; Safford *et al*, 2002; Zuk *et al*, 2002). On the other hand, ADAS cells can be engineered to delivery therapeutic molecules *in vivo* (Gimble & Guilak, 2003a). Furthermore, ADAS cells were shown to participate to haematopoietic reconstitution in lethally-irradiated mice (Cousin *et al*, 2003). We have shown that ADAS cells did not provoke response of allogenic lymphocytes *in vitro*, and consequently, allogenic ADAS cells derived from a donor could be theoretically used for any patient, irrespective of MHC incompatibility.

We also showed that ADAS cells share immunomodulatory properties with BM-MSCs. These inhibitory properties could have particular impact in transplantation and one could expect that, similar to BM-MSCs, the infusion of ADAS cells could favour transplant tolerance and decrease GVHD in allogenic bone marrow transplantation (Lazarus *et al*, 2000; Bartholomew *et al*, 2002; Frassoni *et al*, 2002; Lee *et al*, 2002). However, the mechanism of the inhibitory effect of both ADAS cells and BM MSC *in vivo* is not fully elucidated. The impairment of ADAS cells and BM-MSCs inhibition when cells are separated from responder lymphocytes suggests that full inhibition requires close contact of stem cells with responder lymphocytes. The question remains as to whether stem cells can localize in contact of target lymphocytes *in vivo*? It was previously shown in a baboon model that infused MSCs only poorly migrate in spleen and lymph nodes, where immune responses are initiated and where MSCs could inhibit lymphocyte activation (Devine *et al*, 2003). On another hand, circulating mesenchymal cells can colonize a renal allograft, and infused transgenic MSCs can migrate to a heart allograft undergoing chronic rejection (Grimm *et al*, 2001; Wu *et al*, 2003). Whether MSCs could have a beneficial effect or not in the prevention of allograft rejection remains to be demonstrated.

In conclusion, ADAS cells were known to present, similar to BM-MSCs, a large *in vitro* differentiation capacity. The present study showed, for the first time, that ADAS cells also exhibit similar *in vitro* immunosuppressive properties. This reinforces the therapeutic interest in ADAS cells although further studies are required to fully characterize the immunosuppressive properties of ADAS cells and to evaluate their *in vivo* properties in animal models.

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Note added in proof

Whilst the present article was under revision Aggarwal and Pittenger (2004) reported that PGE2 is one inhibitory factor released by BM-MSCs. We tested the effect of indomethacine on the inhibition by BM-MSCs of lymphocyte stimulation by PHA or MLR. In our experiments, indomethacine partially suppressed (30%) the BM-MSCs inhibition of PHA lymphocyte stimulation but did not influence the inhibition of MLR, which remained intact (three experiments). Our results lead us to conclude that if PGE2 contributes to the suppressive effect of BM-MSCs, it is certainly not the major soluble inhibitory factor released by BM-MSCs.

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