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Clinical-grade mesenchymal stromal cells produced under various good manufacturing practice processes differ in their immunomodulatory properties: standardization of immune quality controls

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Abstract

Clinical-grade mesenchymal stromal cells (MSC) are usually expanded from bone marrow (BMMSC) or adipose tissue (ADSC) using processes mainly differing in the use of fetal calf serum (FCS) or human platelet lysate (PL). We aimed to compare immune modulatory properties of clinical-grade MSC using a combination of fully standardized *in vitro* assays. BMMSC expanded with FCS (BMMSC-FCS) or PL (BMMSC-PL), and ADSC-PL were analyzed in quantitative phenotypic and functional experiments including their capacity to inhibit the proliferation of T, B, and NK cells. The molecular mechanisms supporting T-cell inhibition were investigated. These parameters were also evaluated after pre-stimulation of MSC with inflammatory cytokines. BMMSC-FCS, BMMSC-PL, and ADSC-PL displayed significant differences in expression of immunosuppressive and adhesion molecules. Standardized functional assays revealed that resting MSC inhibited proliferation of T and NK cells, but not B cells. ADSC-PL were the most potent in inhibiting T-cell growth, a property ascribed to IFN- γ -dependent indoleamine 2,3-dioxygenase activity. MSC did not stimulate allogeneic T cell proliferation but were efficiently lysed by activated NK cells. The systematic use of quantitative and reproducible validation techniques highlights differences in immunological properties of MSC produced using various clinical-grade processes. ADSC-PL emerge as a promising candidate for future clinical trials.

MESH Keywords Adipose Tissue ; cytology ; drug effects ; immunology ; Animals ; B-Lymphocytes ; cytology ; immunology ; Blood Platelets ; chemistry ; immunology ; Bone Marrow Cells ; cytology ; drug effects ; immunology ; Cattle ; Cell Extracts ; chemistry ; pharmacology ; Cell Proliferation ; Coculture Techniques ; Cytokines ; biosynthesis ; immunology ; Humans ; Immunomodulation ; Indoleamine-Pyrrole 2,3-Dioxygenase ; immunology ; metabolism ; Killer Cells, Natural ; cytology ; immunology ; Mesenchymal Stromal Cells ; cytology ; drug effects ; immunology ; Organ Specificity ; Quality Control ; Serum ; chemistry ; immunology ; T-Lymphocytes ; cytology ; immunology

Introduction

Adult mesenchymal stromal cells (MSC) are considered a promising tool for cell therapy in regenerative medicine, and for prevention or treatment of severe inflammatory and autoimmune diseases[1]. Indeed, preliminary encouraging results have been recently reported in steroid-resistant graft-versus-host disease, fistulating Crohn's disease, progressive multiple sclerosis, or kidney transplant rejection[2–5]. Despite intensive efforts, no specific MSC marker has been identified. The widely adopted MSC definition according to the International Society for Cellular Therapy (ISCT) relies on three main criteria: (i) their adhesion to plastic; (ii) their expression of a set of membrane molecules (CD73, CD90, CD105), together with a lack of expression of HLA-DR and the hematopoietic and endothelial markers CD11b, CD14, CD34, CD31, and CD45; and (iii) their ability to differentiate along adipogenic, osteogenic, and chondrogenic pathways[6]. However, even these minimal criteria designed to harmonize the identification of cultured MSC are not definitive, and differences may exist depending on tissue sources, culture conditions, and species. In agreement, several important issues should be taken into account to delineate efficient and safe clinical-grade cell culture conditions, including starting material, cell density, number of population doubling, and culture media. First, the most reliable sources of MSC for clinical application are bone marrow and adipose tissue that are widely available, easy to collect under standardized procedures, and give rise to high numbers of MSC upon various *ex vivo* culture processes[7]. Several differences have been already reported between MSC obtained from bone marrow (BMMSC) and adipose tissue (ADSC). In particular, ADSC express CD34, especially in early stages of culture, and display a CD49^{hi} CD54^{hi} CD106^{lo} phenotype when compared to BMMSC[8, 9]. Moreover, even if *ex vivo* expanded MSC share many biological features, some specific discrepancies have been reported

between ADSC and BMMSC in their differentiation potential, gene expression and proteomic profiles, or immunological properties[9–13]. Finally, expression of HLA-DR is modulated depending on starting material, *i.e.* use of unprocessed BM *versus* BM mononuclear cells obtained by density gradient centrifugation, and the presence of fibroblast growth factor-2 (FGF-2)[14–16]. Concerning culture conditions, even if a consensus on the best medium for MSC culture is lacking, both fetal calf serum (FCS) and human platelet lysate (PL) contain the essential growth factors to sustain MSC expansion, whereas FGF-2 is the most common growth supplement capable of increasing MSC growth rate and life span[17, 18].

Although MSC initially attracted interest for their ability to differentiate into multiple cellular phenotypes, it is now widely accepted that their paracrine production of trophic factors together with their broad immune modulatory and anti-inflammatory functions are the most likely mechanisms for their therapeutic activity. MSC profoundly affect the function of a large panel of effector cells of adaptive and innate immunity, including T cells, B cells, NK cells, monocytes/macrophages, dendritic cells, neutrophils, and mast cells[1, 19]. Inhibition of immune cells relies on a combination of factors that are not constitutively expressed by MSC but are induced following MSC priming by inflammatory stimuli[20]. Interferon (IFN)- γ is the pivotal licensing agent for MSC suppressive function[21], whereas tumor necrosis factor (TNF)- α or interleukin (IL)-1 α/β cooperate with IFN- γ to reinforce MSC-mediated inhibition of T-cell proliferation[22]. The specific molecular mechanisms involved in the immune regulatory properties of MSC are still under evaluation and involve both cell contact-dependent mechanisms, such as the Jagged/Notch and PD-1/PD-L1 pathways[23, 24], and soluble inducible factors, including indoleamine-2,3-dioxygenase (IDO), prostaglandin-E2 (PGE2), nitric oxide (NO), heme oxygenase (HO-1), galectins, HLA-G5, transforming growth factor (TGF)- β 1, and TNF- α -induced protein 6 (TSG-6)[21, 25–29]. Interestingly, besides the general concerns about validity of mouse models, major interspecies differences amongst the molecular pathways supporting immune regulating activity of MSC have been reported. In particular, murine MSC preferentially use inducible NO synthase (iNOS), whereas IDO is the most important T cell inhibitory system in human MSC[30]. Therefore, it is crucial to design fully standardized and reproducible *in vitro* assays, including phenotypic and functional experiments, to compare qualitatively and quantitatively the immunological properties of clinical-grade MSC. So far, such effort of standardization has not been undertaken, leading to inconstant, not comparable, and sometimes contradictory results.

In this study, we developed reproducible immunological assays to quantify the differences in immune modulatory properties of MSC produced according to Good Manufacturing Practice (GMP) following three procedures: BMMSC expanded in medium supplemented with clinical-grade PL (BMMSC-PL), BMMSC expanded with FCS and FGF-2 (BMMSC-FCS), and ADSC-PL. This comprehensive work led to the identification of significant differences among these various GMP-grade MSC subsets that could be relevant for their further clinical use.

Material and methods

GMP-grade mesenchymal stromal cells production

Healthy donor recruitment followed institutional review board approval and written informed consent process according to the Declaration of Helsinki. Cryopreserved *ex vivo*-expanded clinical-grade human MSC at passage 1 (P1) were provided by the French Blood Bank of Toulouse (France) for ADSC-PL and Tours (France) for BMMSC-FCS, and by the Institute of Clinical Transfusion Medicine and Immunogenetics in Ulm (Germany) for BMMSC-PL. ADSC-PL were obtained from lipoaspirates after digestion with 0.4 U/mL NB6 collagenase (Roche Diagnostics, Mannheim, Germany) for 45 min at 37°C, filtration, and centrifugation to obtain the stromal vascular fraction (SVF)[31]. SVF was seeded at 4×10^3 cells/cm² onto CellSTACK closed cell culture chambers (Corning, Lowell, MA) in α MEM (Macopharma, Tourcoing, France) supplemented with 2% apheresis-derived clinical-grade PL produced as previously described[32], 1 IU/mL heparin, and 10 μ g/mL ciprofloxacin (Ciflox, B. Braun, Boulogne, France). BMMSC-FCS were obtained as previously reported from unprocessed BM seeded at 5×10^4 cells/cm² onto CellSTACK in α MEM supplemented with 10% screened FCS (Hyclone, Logan, UT), 1 ng/mL FGF-2 (R&D Systems, Abington, UK), and 10 μ g/mL ciprofloxacin[14]. Finally, BMMSC-PL were produced from unprocessed BM seeded at 1.5×10^4 cells/cm² onto CellSTACK in α MEM supplemented with 8% whole blood-derived pooled clinical-grade PL, 2 IU/mL heparin (Braun, Melsungen, Germany) and 12 μ g/mL ciprofloxacin (Fresenius Kabi, Bad Homburg, Germany)[33]. For all MSC cultures, the medium was renewed twice a week until cells reached confluence (end of P0). Cells were then detached using trypsin (Trypzean, Lonza, Verviers, Belgium), reseeded at 1000/cm² (2000/cm² for ADSC-PL) until near confluence (end of P1) and then frozen until use.

MSC were thawed, seeded at 1000 cells/cm² in the same kind of culture medium used during the expansion step, and cultured until almost confluence to avoid any bias associated with the use of freshly thawed MSC[34]. All phenotypic and functional experiments were then performed at the end of P2.

Immunophenotypic study

Thawed MSC were stimulated or not for the last 40 hours of culture by 100 IU/mL (10 ng/mL) IFN- γ and 15 ng/mL TNF- α (R&D Systems). The lack of cell cytotoxicity of this inflammatory stimulus was also checked[35]. Resting and primed MSC (pMSC) were then assessed for the expression of a panel of markers (Supplementary Table 1). For each staining, 10^5 MSC were incubated with the

appropriate monoclonal antibody or appropriate isotypic control in PBS-30% human serum for 30 min at 4°C. For ULBP-3 expression, cells were incubated with specific primary unconjugated antibody, followed by staining with conjugated goat anti-mouse IgG secondary antibody. According to manufacturer's instruction, ULBP-1 expression was validated by intracellular staining using the Cytotfix/Cytoperm kit (Becton-Dickinson, Le Pont de Claix, France). Data were expressed as the ratio of geometric mean fluorescence intensity (rMFI) obtained for each marker and its isotype-matched negative control.

Real time quantitative PCR (Q-PCR) analysis

RNA was extracted from resting and primed MSC using RNeasy Micro kit (Qiagen, Valencia, CA) and cDNA was generated using Superscript II reverse-transcriptase (Invitrogen). For quantitative PCR, assay-on-demand primers and probes, Taqman Universal MasterMix, and ABI Prism 7000 apparatus were used (Applied Biosystems, Courtaboeuf, France). Gene expression was quantified based on the $\Delta\Delta C_T$ calculation method. We identified *CDKN1B* and *EIF2B1* as appropriate internal control genes with low variability among 3 MSC and 3 pMSC samples using the TaqMan Express endogenous control plate (Applied Biosystems) and the geNorm software (<http://medgen.ugent.be/~jvdesomp/genorm/>). PCR data were normalized to the geometric mean of the two housekeeping genes. Results were then standardized by comparison to gene expression of a pool of 5 peripheral blood mononuclear cells (PBMC).

Evaluation of immunosuppressive properties of mesenchymal stromal cells

CD3^{pos} T cells, CD19^{pos} B cells, and CD56^{pos} NK cells were purified from peripheral blood using appropriate negative selection kits (Miltenyi Biotec, Bergisch Gladbach, Germany) with at least 95% cell purity as evaluated by flow cytometry. Inhibition of immune cell proliferation by resting and primed MSC is described in details in the *Online Supplementary Design and Methods*.

Each immune cell batch was initially validated for its capacity to proliferate strongly without cell death in response to their specific stimuli: for T cells, proliferation > 70% and DAPI^{neg} > 60%; for B cells, proliferation > 50% and DAPI^{neg} > 80%; for NK cells, proliferation > 70% and DAPI^{neg} > 50%. The same controls were repeated in all individual experiments.

Mesenchymal stromal cell immunogenicity

Allogeneic purified T cells were cultured in round-bottomed 96-well plates at 10⁵ cells/well in RPMI-10% human AB serum with either γ -irradiated resting MSC at ratios ranging from 1/1 to 729/1 T/MS, 10⁵ γ -irradiated autologous PBMC (auto-MLR), 10⁵ γ -irradiated allogeneic PBMC (allo-MLR), or 0.5 μ g/mL anti-CD3/anti-CD28 antibodies. Each experiment was performed in quadruplicate culture wells. Proliferation was assessed after 6 days of culture by incorporation of tritiated thymidine (³H-TdR) during the last 18 hours and quantification of the radioactivity on a scintillation counter. The relative response index (RRI) was calculated as followed: $RRI(\%) = [cpm(T + MSC) - cpm(auto - MLR)] / [cpm(allo - MLR) - cpm(auto - MLR)] \times 100$. Any RRI > 20% was considered positive.

Resting and primed MSC were also used as NK target cells in a non radioactive cytotoxicity assay (Delfia Cytotoxicity kit, Perkin Elmer, Monza, Italia). Briefly, MSC were loaded with a fluorescent dye and incubated for 3h at various ratios of allogeneic NK cells preactivated during 48 hours by 100 IU/mL rhIL-2. Cytotoxicity was quantified by assessing fluorescence release in coculture supernatants by a time-resolved fluorimeter (Victor™ X4, Perkin Elmer).

Indoleamine 2,3-Dioxygenase activity

Tryptophan and its catabolite kynurenine were measured in supernatants of resting and primed MSC by high-performance liquid chromatography (HPLC) using added 3-nitro-L-tyrosine as an internal standard, as previously described[36]. IDO activity was expressed as the kynurenine/tryptophan ratio.

Statistical analysis

Data are expressed as median and ranges. Differences between groups were analyzed with Prism software (GraphPad, La Jolla, CA) using the Wilcoxon test for matched pairs for comparison of MSC versus pMSC or the Mann-Whitney non-parametric *U* test for comparison of ADSC-PL, BMMSC-PL, and BMMSC-FCS. Correlation between inhibition of T cell proliferation and IDO activity was done using two-tailed non-parametric Spearman's rank correlation test.

Results

Modulation of GMP-grade mesenchymal stromal cell phenotype depending on culture procedures

MSC were produced and validated following GMP procedures in three different Cell Therapy Units (5 batches/production process), cryopreserved to fit in with clinical applications that generally require repeated MSC infusions, and centralized for immunological evaluation. MSC were then compared using standardized methods, including selected batches of antibodies, PCR reagents, culture supplements, standard operating procedures, and inclusion of internal controls for all functional experiments. Several markers were commonly expressed by ADSC-PL, BMMSC-PL, and BMMSC-FCS (Figure 1A and Supplementary Table S2). In particular, all resting

MSC were negative for costimulatory molecules CD40, CD80 and CD86, and expressed similar level of each NK activating ligand. Among them, ULBP-2 was always detected at intermediate to high levels independently of the production process (4.6 [2.1–9.4]), whereas ULBP-1 and ULBP-3 were poorly expressed (ULBP1: 1.4 [1–2.1]; ULBP3: 1.4 [1–4.5]). Overall, the global expression of NK activating ligands was similar in all MSC subsets (Supplementary Figure S1B). The balance between triggering of activating and inhibitory NK receptors determines NK cell functions. Resting MSC displayed differential levels of HLA-ABC molecules with BMMSC-PL exhibiting the highest (31.8 [16.3–53.7]) and BMMSC-FCS the lowest (16.9 [10–30.6]) but this difference was not significant (Figure 1B). Conversely, HLA-DR was expressed on 3/5 BMMSC-FCS batches, as previously described[14], whereas ADSC-PL and BMMSC-PL never expressed this marker. Of note, the 3 HLA-DR-expressing BMMSC-FCS batches also expressed high levels of the CD200 immunosuppressive molecule, which remained very low on 9/10 batches of MSC produced in PL (Figure 1B and Supplementary Figure S1A and Table S2). Interestingly, ADSC-PL exhibited lower expression of CD274/PD-L1 than BMMSC. Finally, we confirmed the overexpression of CD54/ICAM-1 and the low expression of CD106/VCAM-1 in ADSC as compared to BMMSC.

Overall, these results obtained on GMP-grade MSC analyzed simultaneously using standardized methods confirmed and extended some previous data showing that ADSC-PL, BMMSC-PL, and BMMSC-FCS displayed reliable differences in their phenotypic profile.

Effect of inflammatory cytokines depending on mesenchymal stromal cell culture processes

Since tissue injury is usually associated with inflammation, we evaluated the modifications of MSC immunophenotype and gene expression after stimulation with IFN- γ and TNF- α . Among the 15 molecules tested, 8 were reproducibly induced by inflammatory stimuli (Figure 2A and Supplementary Figure S2 and Table S3). The expression of these molecules was increased in the 3 MSC subsets, except for CD155 which was upregulated in BMMSC but slightly reduced in ADSC. Concerning costimulatory molecules, whereas CD80 and CD86 expression remained negative, CD40 was strongly upregulated especially in primed MSC expanded in PL (pADSC-PL: 8 [4.5–8.8]; pBMMSC-PL: 5 [2.7–8.7]), which significantly overexpressed CD40 compared to pBMMSC-FCS. Adhesion molecules were also upregulated in primed MSC, as expected. Interestingly, CD54 and CD106 induction inversely correlated with their level in resting MSC (CD54: 120-fold for ADSC-PL, 200-fold for BMMSC-PL, 400-fold for BMMSC-FCS; CD106: 10-fold for ADSC-PL, 5-fold for BMMSC-PL, 3-fold for BMMSC-FCS); therefore, primed MSC reached similar expression of CD54 and CD106 regardless of their production process. HLA-DR induction was highly variable depending on pMSC batches, without any link with tissue origin or culture medium. Conversely, HLA-ABC was slightly less induced on pBMMSC-FCS and displayed a significantly lower expression on pBMMSC-FCS than on pMSC obtained in PL, in agreement with its lower level in this MSC subset in unprimed conditions (Figure 1). CD112 and CD155, unlike ULBP and MICA/B, could be induced by inflammatory cytokines, except for pADSC-PL. This lack of induction of CD155 led to a global lower expression of NK activating ligands by pADSC-PL as compared to pBMMSC-FCS (Figure 2B). In relation to their lower HLA class I expression, pBMMSC-FCS exhibited a significantly higher ratio of activating/inhibitory NK ligands than the two other MSC subsets. Finally, pBMMSC-FCS also overexpressed CD274 as compared to pMSC produced in PL.

We next sought to evaluate the differences in the main molecular pathways that are supposed to be used by human MSC to inhibit immune response. Particular attention was focused on four factors reproducibly involved in the suppression of T cells, NK cells, and cells of innate immunity, *i.e.* IDO, PGE2, NO, and TSG-6[20, 27, 29, 30]. To this aim, we quantified in both resting and primed MSC the expression of genes encoding for IDO (*INDO*), the PGE2-producing enzyme Cox-2 (*PTSG2*), the NO-producing enzyme iNOS (*NOS2*) and TSG-6 (*TNFAIP6*). The four immunosuppressive molecules were upregulated by inflammatory cytokines. As previously described, *INDO* and *NOS2* were not expressed by unstimulated MSC (data not shown), but were strongly induced by IFN- γ and TNF- α exposure (Figure 3). Notably, pBMMSC-PL significantly expressed *INDO* at lower levels, while pADSC-PL significantly expressed more *NOS2*. *PTGS2* was constitutively expressed by MSC, but with important differences among the different MSC subtypes: for instance, ADSC-PL expressed 3.6-fold less Cox-2 mRNA transcripts than BMMSC-PL and 3.2-fold less than BMMSC-FCS. However, Cox-2 expression was strongly upregulated after stimulation and all primed MSC displayed similar expression of this immunosuppressive enzyme. More strikingly, *TNFAIP6* was significantly overexpressed by both resting and primed ADSC-PL. Altogether, two opposite MSC subsets were evident, as pADSC-PL exhibited a CD40^{hi} iNOS^{2hi} TSG-6^{hi} phenotype, whereas pBMMSC-FCS was HLA-ABC^{lo} PD-L1^{hi} CD200^{dim} and overexpressed NK activating ligands.

These results underline some major differences between the immunological profiles of clinical-grade primed MSC depending on their production processes, thus suggesting that they could display some differences in immunosuppressive functions.

Standardized functional analyses reveal differences between ADSC-PL, BMMSC-PL, and BMMSC-FCS

Several culture conditions have been reported to evaluate immune properties of MSC. To get reproducible and quantitative results, we decided to use purified immune cells obtained by negative selection and stimulated with fully standardized signals. In addition, considering crucial the anti-apoptotic activity of MSC, we evaluated the viability of responding cells and defined the minimal percentage of both viable cells and proliferating cells for each immune cell subset.

On the basis of these methods and criteria, we studied the capability of modulating T cell, B cell, and NK cell proliferation of the 15 MSC batches. Interestingly, whereas resting ADSC always inhibited T cell proliferation by more than 60%, the results were more heterogeneous for both BMMSC subsets, with 2 out of 5 batches inhibiting T cell proliferation by less than 50% (Figure 4A, left). Prestimulation of MSC by inflammatory cytokines enhanced their T cell suppressive capacity, as expected, and leveled the variations among MSC production processes. Regarding interactions between B cells and MSC, several studies reported confusing results claiming that MSC favor or restrain B cell growth and differentiation[37–39]. We confirmed here that resting MSC did not inhibit B cell proliferation and that ADSC-PL and BMMSC-FCS, but not BMMSC-PL, could even increase the percentage of B cells that have undergone more than one division (Figure 4A, middle). However, priming by IFN- γ and TNF- α drove MSC towards a B cell inhibitory phenotype. Finally, the three MSC subsets similarly inhibited NK cell proliferation, although less efficiently than T cell proliferation. MSC priming slightly but significantly increased their NK inhibitory potential. A trend for a better NK inhibitory potential by BMMSC-FCS was observed (Figure 4A, right).

Despite their immunosuppressive properties, MSC should not be considered as intrinsically immunoprivileged cells, in agreement with their capacity to behave as antigen-presenting cells under specific conditions and to be recognized and killed by NK cells[21, 40]. We first explored their capacity to activate purified allogeneic T cells. Regardless of the production processes, MSC were poorly immunogenic *in vitro* (Figure 4B), as confirmed by the evaluation of the relative response index at 1/1 ratio (3.6% [-0.3–10.4]).

Activated NK cells may efficiently lyse resting and not IFN- γ -exposed BMMSC, but this kind of information is currently lacking for ADSC[41]. Thus, classical cytotoxicity assay was performed using ADSC-PL, BMMSC-PL and BMMSC-FCS as target cells (Figure 4C). These experiments revealed a significantly lower susceptibility of pMSC to NK-mediated lysis; in particular, even if the low number of samples precluded any statistical analysis, our data suggested that resting BMMSC-PL were more resistant than the two other cell subsets, in agreement with their higher expression of HLA class I (Supplementary Figure 1). Among pMSC, pBMMSC-FCS were killed more efficiently by NK cells, consistently with their higher expression of NK activating ligands.

Indoleamine 2,3-dioxygenase activity correlates with the T cell inhibitory potential of mesenchymal stromal cells

As functional analyses revealed some differences in T cell immunosuppressive potential between MSC expanded in various culture systems, we next focused our attention on the mechanisms involved in the inhibition of T cell proliferation that could explain these differences. On the basis of the QPCR results, we evaluated specific inhibitors of iNOS (L-NMMA), IDO (L-1MT) and Cox-2 (NS398) as well as IFN- γ blockade in the proliferation assay (Figure 5A). L-1MT, unlike iNOS and Cox-2 inhibitors, abolished the T cell inhibitory activity of resting BMMSC-PL, BMMSC-FCS, and ADSC-PL and IFN- γ blockade partially restored T cell proliferation, as previously described[20]. Similar results were obtained with primed MSC (Supplementary Figure 3). To further confirm the role of IDO-1 we compared the effect of L-1MT and its inactive enantiomer D-1MT. Both ADSC-PL and BMMSC-FCS activity was completely inhibited by L-1MT, whereas D-1MT was inefficient (Figure 5B). These data clearly confirmed that IDO was the central effector of T cell suppressive function for all MSC and pMSC subsets.

A recent study reported a correlation between the amount of IDO evaluated by Western Blot and the inhibition of T cell proliferation by BMMSC[42]. To extend these data to ADSC and since IDO activity is regulated by transcriptional, translational and post-translational mechanisms[43], we decided to determine IDO activity through the quantification of tryptophan and kynurenine concentrations in pMSC supernatants (Figure 6A). Strikingly, whereas IDO activity was not significantly different among pADSC-PL, pBMMSC-PL, and pBMMSC-FCS, the 4 MSC batches inhibiting T cell proliferation by less than 50% (Figure 4A, left) also displayed a low IDO activity upon stimulation with inflammatory cytokines (ratio tryptophan/kynurenine <4). Moreover, we demonstrated a significant correlation between IDO activity detected in the 15 pMSC batches and the capacity of corresponding resting MSC to restrain T cell proliferation (Figure 6B). Conversely, neither *TSG6* and *PTGS2* mRNA nor the expression of CD200 or PD-L1 was positively correlated to the inhibition of T-cell proliferation thus confirming the central role of IDO in this process (data not shown).

Discussion

In this study, we have developed a panel of robust assays, validated in two independent immune monitoring laboratories, allowing the quantitative comparison of immunological properties of clinical-grade MSC. We identified the most critical variables to look for to ensure result consistency as: i) the references of antibodies; ii) the concentration of IFN- γ and TNF- α used to preactivate MSC with a careful control of the specific activity of IFN- γ that is highly variable depending on the distributor and batch; iii) the quality of responding cells, *i.e.* viability and proliferation rate in response to activation, especially for B cells (viability > 80%, proliferation >50%) and NK cells (viability > 50%, proliferation > 70%), in order to avoid interference with the antiapoptotic activity of MSC; iv) for the same reason, the use of the best stimulatory system for each purified cell subset (stimulatory cocktail composition including culture medium and batch to batch validation of non-clinical grade CD40 ligand and CpG, duration of the stimulation); v) the use of relative response index instead of cpm for measurement of proliferation by thymidine incorporation and the normalization of CFSE dilution with MSC to the CFSE dilution without MSC.

We applied these read-out systems to the evaluation of ADSC-PL, BMMSC-PL and BMMSC-FCS produced by three Cell Therapy Units according to GMP rules. We clearly demonstrated that MSC derived from various tissues and expanded through different culture procedures display some significant differences in their capacity to inhibit immune response, a property that is of utmost interest for their clinical application.

Several immune markers were proposed as differentially expressed on ADSC *versus* BMMSC and/or on MSC expanded in PL *versus* FCS. Among them, we found that CD274 and CD200, two immunosuppressive molecules that cooperate in blocking T cell activation, are poorly expressed by ADSC-PL unlike BMMSC-FCS. To date, a direct role of PD-1/PD-L1 pathway in T cell inhibition has been documented only for mouse BMMSC[23]. Furthermore, blocking of CD200/CD200R interaction does not prevent the decrease of T cell proliferation induced by MSC, even when using CD200^{hi} Wharton's jelly-derived MSC[44]. In agreement, our work confirms that CD274^{lo} CD200^{lo} ADSC-PL strongly inhibit proliferation of purified T cells, thus suggesting the implication of distinct dominant factors. CD54 and CD106 adhesion molecules were recently highlighted as crucial for MSC-driven immunoregulation, at least in mouse model[45]. Our data confirmed an opposite pattern of expression of CD54 *versus* CD106 on the various MSC subsets. Nevertheless, all resting MSC batches expressed at least one of these two molecules, whereas primed MSC displayed high levels of both. Interestingly, although CD54 has already been described as strongly expressed by ADSC[9], we further demonstrated that BMMSC-PL overexpress CD54 as compared to BMMSC-FCS. This could be related to the presence of very high levels of all three isoforms of platelet-derived growth factors (PDGF) in PL[32]. PDGF-BB upregulates CD54 expression on rat BMMSC through the activation of p38MAPK[46], indicating that PL could be involved in the induction of CD54 on BMMSC and in its increase on ADSC.

Composition of PL could influence many other MSC characteristics. Importantly, whole blood-derived PL used in Germany and apheresis-derived PL used in France have been previously compared and displayed very similar composition and capacity to sustain MSC growth[32]. Soluble VCAM-1 and soluble CD40L are two important components of PL[32] that could activate MSC, in particular ADSC. In fact, ADSC, unlike BMMSC, strongly expressed the VCAM-1-ligand VLA-4 that has been previously involved in murine MSC homing through the interaction with inflamed endothelium[47]. Moreover, we demonstrated that primed ADSC strongly express CD40, a marker that was also detected on native human BMMSC[48]. The role of CD40 signalling in MSC has never been explored, but CD40 is also inducible by IFN- γ on human fibroblasts where its ligation by CD40L enhances the secretion of inflammatory cytokines and chemokines and proangiogenic factors[49, 50]. Adipocytes also express functional CD40 molecules[51]. Given the major role of inflammatory chemokines in the immunosuppressive function of MSC[22], a detailed analysis of CD40 function on cultured MSC would be of great interest. Finally, we never detected HLA-DR expression by BMMSC-PL despite the use of unprocessed BM as starting material and culture with 8% PL containing about 500 pg/ml of FGF-2[32], suggesting that high amount of FGF-2 are required to drive HLA-DR expression.

It should be noted that this study was designed to compare validated clinical-grade processes currently used in several countries for therapeutic purposes making it not possible to directly conclude on the specific role of tissue origin in MSC properties. As an example, the total population doubling (PD) was similar for BMMSC obtained using both PL and FCS (see Supplementary data) but was higher for BMMSC than for ADSC, a discrepancy that could have some impact on MSC features. A parallel evaluation of autologous BMMSC and ADSC obtained using identical procedures would be mandatory to answer this important question.

Our functional assays first confirmed that IDO is the pivotal mechanism of T cell inhibition in all human MSC subtypes. Given the multiple levels of regulation of IDO, we propose to use the measurement of its enzymatic activity after stimulation by inflammatory cytokines to validate clinical-grade MSC. In fact, a good correlation exists between the expression by MSC of functional IDO after priming with IFN- γ and TNF- α and their capability of suppressing T cells. Conversely, we found no correlation between IDO mRNA level and T cell inhibition, whereas the quantification of IDO protein expression by Western Blot seems hardly realizable in terms of standardization for multicentric studies. In addition, IDO activity also contributes to the induction of IL-10-producing M2 macrophages by MSC[42], thus reinforcing the interest for its accurate evaluation. Blood or urinary IDO activity has been recently correlated to severity and outcome in several immune disorders[36, 52]. In addition to its interest as validation criteria of clinical-grade MSC, IDO activity should thus also be finely evaluated *in vivo* as a biomarker for the immunosuppressive activity of MSC in forthcoming MSC-based clinical trials. Of note, we previously demonstrated that IDO is also involved in the inhibition of B cell proliferation by MSC[37]. Accordingly, as activated human B cells do not produce IFN- γ , MSC priming by inflammatory stimuli was required to reveal their IDO-dependent B cell inhibitory potential[20]. We used an optimal cocktail to trigger IDO activity, so that all MSC that have been previously activated by IFN- γ + TNF- α inhibited both T cell and B cell proliferation in a secondary culture. In line with this, injected MSC seem to be highly effective in some mouse models of acute graft versus host disease (GVHD) only when the concentration of IFN- γ is sufficient to allow their *in vivo* licensing, or after *in vitro* priming by IFN- γ [53]. Surprisingly, whereas we could extend to ADSC-PL our previous data showing that resting BMMSC-FCS favor B cell growth, resting BMMSC-PL displayed no B cell supportive activity. Further studies targeting potential stroma-derived B cell growth factors would be helpful to understand this phenomenon.

There is accumulating evidence that BMMSC inhibit NK cell proliferation and cytotoxicity but could be killed by IL-2-activated NK[27, 41], a process that could contribute to their lack of long-term engraftment[54]. Whereas the general consensus describes high levels of CD112, CD155, and MICA/B on MSC, expression of ULBP remains still controversial[41, 55, 56]. As recently described for adult BMMSC-FCS[57], we found in our study a strong expression of ULBP-2, but not ULBP-1 and ULBP-3, on both ADSC and BMMSC. In addition, the use of PL instead of FCS did not alter the expression of NK activating ligands in contrast to previous reports[45]. Nevertheless, HLA-ABC was expressed on BMMSC-FCS at a lower level than on BMMSC-PL, in line with the capacity of FGF-2 to downregulate HLA class I expression[58]. This could contribute to a general decrease of NK cell degranulation and cytotoxicity induced by BMMSC-PL compared to BMMSC-FCS[45]. Priming of MSC rescues them from killing by NK cells, findings corroborated by others and associated with the higher expression of HLA class I[41]. Similar to Spaggiari *et al.*[41], we detected in primed BMMSC an increase in the expression of DNAM-1 ligands; *i.e.* CD112 and CD155, but not NKG2D ligands. However, CD155 was not induced on ADSC. Differential regulation of DNAM-1 ligand expression has been already reported in activated T cells or dendritic cells[59, 60]. Further investigations are required to definitively conclude on the origin and the functional consequences of this lower expression of CD155 by pADSC. Interestingly, we could not reverse the MSC-dependent inhibition of NK cell proliferation using IDO inhibitor (data not shown). In agreement with the reported implication of both IDO and Cox-2 in NK cell inhibition, resting BMMSC-FCS expressed higher levels of Cox-2 and strongly decreased NK cell proliferation[27].

Among the other proposed mechanisms of MSC-related immunosuppression, TSG-6 makes a major contribution to the decrease in neutrophil recruitment observed in response to MSC infusion in several mouse models of acute inflammation[29, 61], at least in part by reducing the release of inflammatory cytokines by macrophages[62]. We confirmed here that exposure to IFN- γ + TNF- α induces TSG-6 at the transcriptional level in MSC. Moreover, we identified ADSC-PL as strong producers of TSG-6, making them interesting candidates as anti-inflammatory MSC.

In conclusion, our study paves the way for the definition of quantitative and reproducible validation techniques to select the best GMP procedures and to establish release criteria for clinical-grade MSC production. Besides the well-known interindividual variability, differences among MSC produced from different tissues could be highlighted in different expansion media. In particular, ADSC-PL clearly emerge as a very interesting alternative to the classical BMMSC-FCS. In fact, we demonstrated that ADSC-PL produce high levels of IDO and TSG-6, thus targeting both innate and adaptative immunity, and inhibit reproducibly T cell proliferation. The next step will be to evaluate how these tools will be helpful to predict clinical response to MSC infusion in controlled clinical trials.

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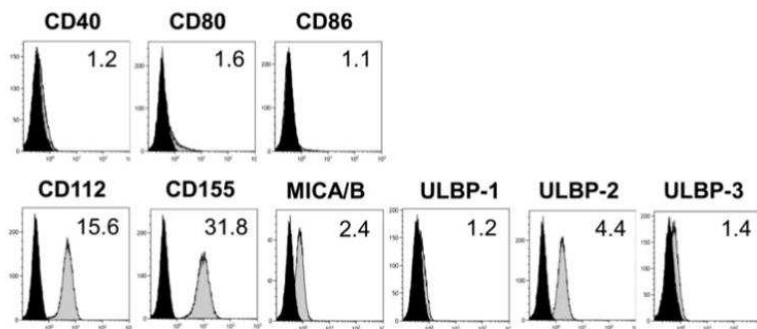
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Figure 1

Comparative phenotype of ADSC-PL, BMMSC-PL, and BMMSC-FCS

Thawed MSC were collected at the end of P2 and stained with appropriate antibodies (grey histogram) or isotype-matched controls (black histogram). Ratio of mean fluorescence intensity (rMFI) is indicated on the top right of each panel. One example representative of the 15 MSC batches is shown for commonly expressed markers (A). For differentially expressed markers, data for all MSC batches were shown. Bars: median (B)

A.



B.

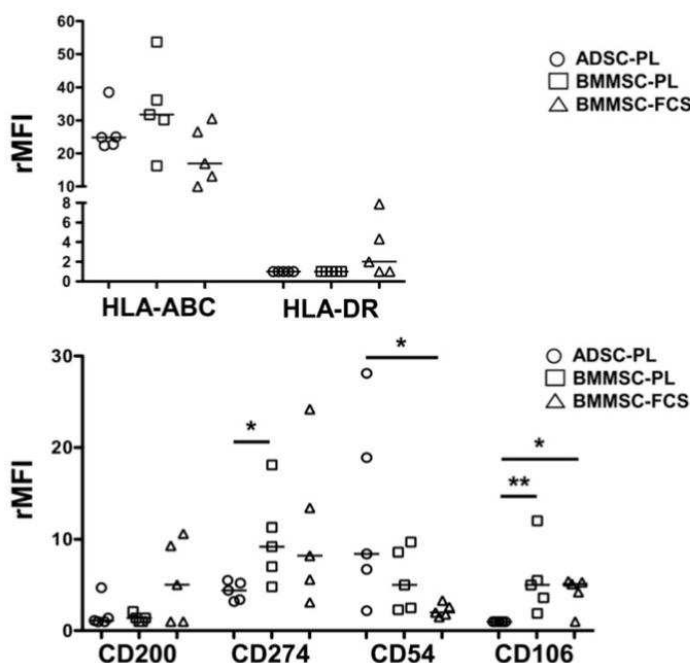


Figure 2

Phenotypic modifications induced on MSC by inflammatory stimuli

(A) Thawed MSC were stimulated by IFN- γ and TNF- α for 40 hours before staining with appropriate antibodies (grey histogram) or isotype-matched controls (black histogram). Ratio of mean fluorescence intensity is indicated on the top right of each panel. One example representative of the 15 MSC batches is shown. (B) The global expression of NK activating ligands on primed MSC (n=15) was obtained by combining the individual rMFI from MICA/B, ULBP-1, ULBP-2, ULBP-3, CD112, and CD155. This activating profile was then analyzed compared to the level of expression of HLA-ABC, the main NK inhibitory ligand. * $p < 0.05$; ** $p < 0.01$

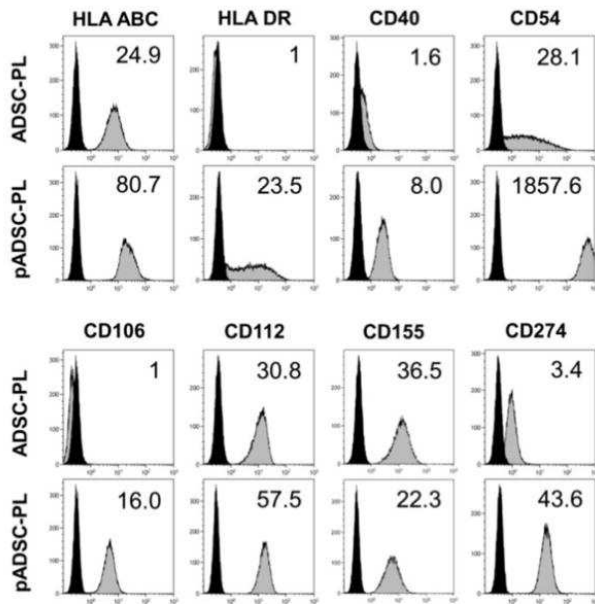
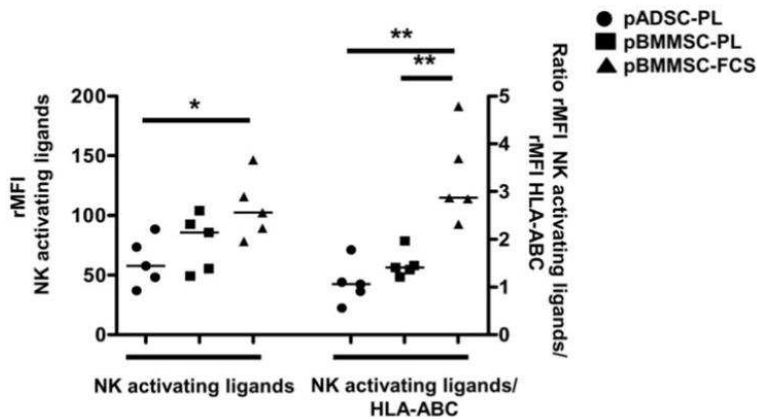
A.**B.**

Figure 3

Expression of immunosuppressive molecules on resting and primed MSC

Thawed MSC were stimulated or not by IFN- γ and TNF- α for 40 hours and *INDO*, *PTGS2*, *NOS2*, and *TNFAIP6* mRNA expression was measured by RQ-PCR in ADSC-PL (n=5), BMMSC-PL (n=5) and BMMSC-FCS (n=5) and their corresponding primed counterpart. The arbitrary value of 1 was assigned to a pool of PBMC. *INDO* and *NOS2* expression was undetectable in resting MSC samples. * $p < 0.05$; ** $p < 0.01$

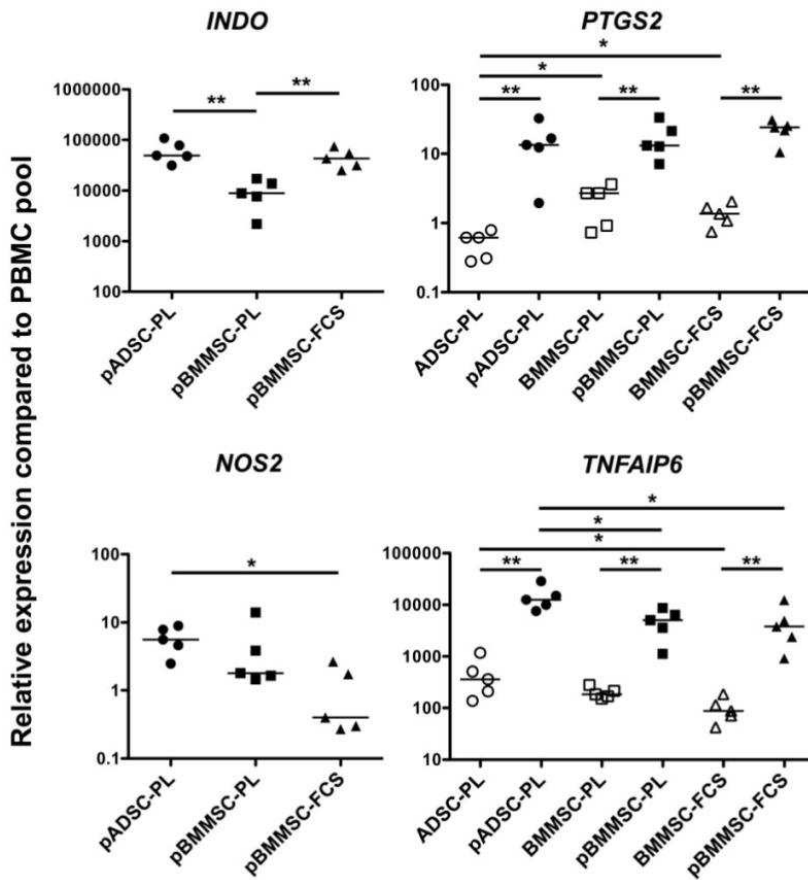


Figure 4

Immune properties of ADSC-PL, BMMSC-PL, and BMMSC-FCS

(A) Inhibition of T, B, and NK proliferation by primed and resting ADSC-PL (n=5), BMMSC-PL (n=5), and BMMSC-FCS (n=5) was assessed by the CFSE dilution method. Data are expressed as the percentage of inhibition of immune cell proliferation. (B) Responding T cells were stimulated with irradiated resting allogeneic MSC (5 ADSC-PL, 2 BMMSC-PL, and 3 BMMSC-FCS batches all used at 1/1 MSC/T ratio), with irradiated autologous PBMC (auto-MLR) as negative control, and with irradiated allogeneic PBMC (allo-MLR) and anti-CD3/anti-CD28 antibodies as positive controls. Each experiment was performed in sixplicate culture wells. Proliferation was assessed by the incorporation of tritiated thymidine ($^3\text{H-TdR}$). Results represent the mean \pm SD of the 10 experiments. (C) Lysis of resting *versus* primed MSC (3 ADSC-PL, 2 BMMSC-PL, and 2 BMMSC-FCS batches) by activated NK cells was assessed in a standard cytotoxicity assay. Results are represented as the mean for each MSC subtype. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

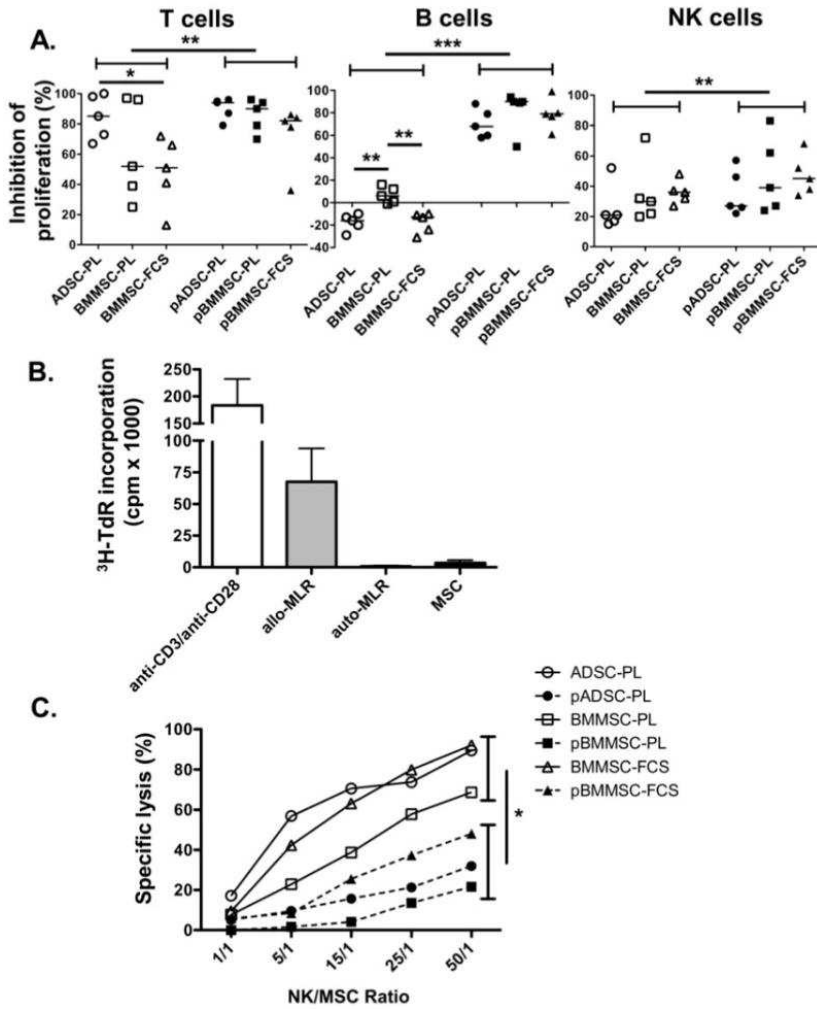


Figure 5

IDO is involved in the inhibition of T cell proliferation by MSC unlike NOS and PGE2

(A) Resting MSC (n=5) were cocultured at 10T/1MSC ratio with CFSE-labelled purified T cells stimulated with anti-CD3/anti-CD28 antibodies in the presence or not of L-N-monomethylarginin (L-NMMA), L-1-methyltryptophan (L-1MT), or NS398 to inhibit iNOS, IDO-1, or Cox-2 activity respectively or in the presence of IFN- γ blocking antibody. T cell proliferation was evaluated at day 6 and data are expressed relatively to T cells alone (assigned to 100%). Results are expressed as mean \pm SD of the 5 experiments. NS: not significant; ** p <0.01. (B) Representative example of IDO blockade in coculture of activated T cells with one ADSC-PL and one BMMSC-FCS. The IDO-2 inhibitor D-1MT is used as a negative control.

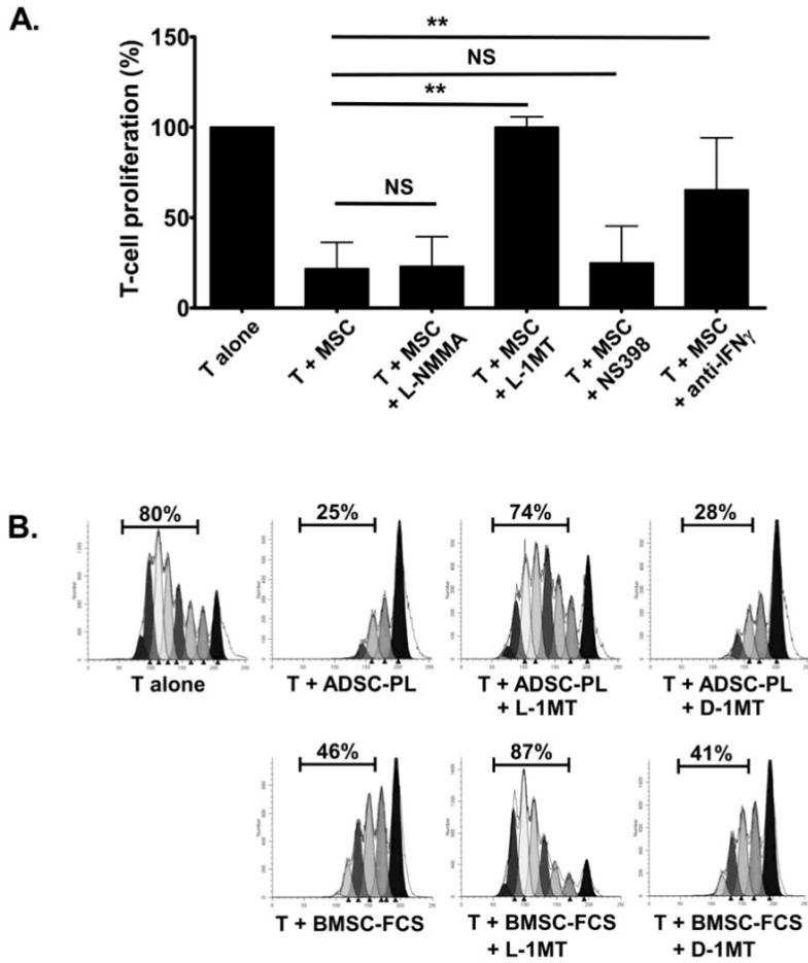


Figure 6

Correlation between IDO activity and T cell inhibition

MSC (n=15) were stimulated or not by IFN- γ + TNF- α for 40 hours. Culture supernatants were collected for IDO activity quantification, and MSC were cocultured with CFSE-labelled purified T cells stimulated with anti-CD3/anti-CD28 antibodies for 6 days. (A) IDO activity of primed MSC was evaluated as the ratio of kynurenine/tryptophan levels as determined by HPLC. Open symbols correspond to MSC batches that inhibit T cell proliferation by less than 50%. (B) The correlation between IDO activity produced by a given primed MSC and the capacity of corresponding resting MSC to inhibit T cell proliferation was determined by two-tailed Spearman's test. Each symbol corresponds to a different MSC batch with circles representing ADSC-PL (n=5); squares, BMMSC-PL (n=5); and triangles, BMMSC-FCS (n=5).

