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Immunogenicity and Immunomodulatory Properties of Umbilical Cord Lining Mesenchymal Stem Cells

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We here present an immunologic head-to-head comparison between human umbilical cord lining mesenchymal stem cells (clMSCs) and adult bone marrow MSCs (bmMSCs) from patients >65 years of age. clMSCs had significantly lower HLA class I expression, higher production of tolerogenic TGF-β and IL-10, and showed significantly faster proliferation. In vitro activation of allogeneic lymphocytes and xenogeneic in vivo immune activation was significantly stronger with bmMSCs, whereas immune recognition of clMSCs was significantly weaker. Thus, bmMSCs were more quickly rejected in immunocompetent mice. IFN-γ at 25 ng/ml increased both immunogenicity by upregulation of HLA class I/ HLA-DR expression and tolerogenicity by increasing intracellular HLA-G and surface HLA-E expression, augmenting TGF-β and IL-10 release, and inducing indoleamine 2,3-dioxygenase (IDO) expression. Higher concentrations of IFN-γ (>50 ng/ml) further enhanced the immunosuppressive phenotype of clMSCs, more strongly downregulating HLA-DR expression and further increasing IDO production (at 500 ng/ml). The net functional immunosuppressive efficacy of MSCs was tested in mixed lymphocyte cultures. Although both clMSCs and bmMSCs significantly reduced in vitro immune activation, clMSCs were significantly more effective than bmMSCs. The veto function of both MSC lines was enhanced in escalating IFN-γ environments. In conclusion, clMSCs show a more beneficial immunogeneic profile and stronger overall immunosuppressive potential than aged bmMSCs.

Key words: Mesenchymal stem cells (MSCs); Immunogenicity; Immunomodulation; Stem cell transplantation

INTRODUCTION

Mesenchymal stem cells (MSCs) have been identified a promising cell population not only for tissue regeneration (15,30,40), but also for immunomodulatory therapy (31,42). An emerging body of data indicates that MSCs may play specific roles as immunomodulators in maintenance of peripheral tolerance, transplantation tolerance, autoimmunity, and tumor immunobiology (25). MSCs were shown to interact with various immune cell types including T cells (9,18,41), B cells (1,7,11), dendritic cells (DCs) (14,48), and natural killer (NK) cells (17,37). Multiple mechanisms, requiring dynamic cross-talk between MSCs and immune cells and involving soluble immunomodulatory factors, cause a shift of the immune response towards tolerance or anergy (11,28,46). Release of transforming growth factor-β (TGF-β), hepatocyte growth factor (HGF), and induction of the tryptophan catabolizing enzyme indoleamine 2,3-dioxygenase (IDO) has been suggested to mediate the immunosuppressive effects of MSCs (9,22).

The immunosuppressive properties of MSCs have been examined in a variety of animal models of autoimmunity, solid organ, or stem cell transplantation. MSCs

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have been reported to ameliorate experimental autoimmune encephalomyelitis (46,47) in mice. Infusion of host-derived MSCs has been shown to decrease the rejection of allogeneic bone marrow (BM) stem cells in a murine transplant model (26). Even more impressive, MSC administration sufficiently reversed ongoing graft versus host disease (GVHD) (45) in mice, an effect that could already be verified in human disease (19,33). Modest success in prolonging the survival of allogeneic skin transplants by MSC infusion has already been demonstrated in baboons (3). However, controversies exist on the efficiency of MSC infusion to prolong solid organ graft survival (13,50). The involved mechanisms of MSC-based immunomodulation may favor the suppression of a more generalized state of immune activation in BM transplant, GVHD, or autoimmune disease compared to the more localized immune response in organ transplantation (2). Still, recommendations have been formulated on how to move MSC-based tolerance strategies forward toward clinical application in solid organ transplantation (8).

Because MSCs can be isolated from various tissues including BM, adipose tissue (51), umbilical cord (34), placenta, liver, and spleen (4), and these cell populations may vary in their functional capacities, the optimal source of MSCs for cell-based immunomodulatory therapies remains to be determined. Patients in need of stem cell-based therapies for cardiovascular diseases are usually aged and approaches using autologous adult stem cells have to deal with aged cells. Allogeneic stem cell strategies can utilize fetal stem cells, but may encounter problems with immune activation and cell rejection. We here present an immunologic head-to-head comparison between human umbilical cord lining MSCs (clMSCs) and aged adult BM MSCs (bmMSCs) to help clarify which strategy offers better immunomodulatory properties.

MATERIALS AND METHODS

Animals

Six- to 8-week-old male Balb/c and Scid-beige (CB17.Cg-Prkdc^{scid} Lyst^{bg}/Crl) mice were purchased from Charles River Laboratories (Sulzfeld, Germany) and received humane care in compliance with the Guide for the Principles of Laboratory Animals.

MSC Culture and Cell Transplantation

Female neonatal clMSCs (kindly provided by Cell Research Corporation Pte Ltd, Singapore) were isolated from the outer amniotic lining of the umbilical cord and were cultured in PTT4 media (CellResearch). bmMSCs were isolated from the bone marrow of three different healthy female donors more than 65 years of age (Promocell, Heidelberg). Therefore, the bone marrow was flushed from femoral heads and isolated using plastic adherence. Cells were grown in their specific growth media [CMRL-1066 + antibiotics + L-glutamine (Cell ResearchCorp) for clMSCs and mesenchymal stem cell growth media (Promocell) for bmMSCs]. Cells were split at a confluency of 80–85% by mechanically lifting the cells from the tissue culture plastic surface using a cell lifter (Costar, Corning). Before transplantation, MSCs were dissociated by collagenase type I solution at a concentration of 0.025% for 5 min at 37°C, and resuspended in sterile PBS at 1×10^6 cells per 50 µl. MSC viability was approximately 95% as determined by trypan blue staining. MSC transplantation was performed by direct injection of 1×10^6 MSCs into the right gastrocnemius muscle of recipient mice using a 27-gauge syringe. In vitro experiments with MSCs were also performed at a confluency of 80–85%.

Multipotent Capacity

Adipogenic Differentiation. Cells were plated onto 24-well plates at 6×10^4 cells per well (3.15×10^4 cells/cm^2) and differentiation was induced at 80–90% confluency by using mesenchymal stem cell adipogenic differentiation medium (Promocell) for 14 days at 37°C and 5% CO_2. Medium was changed every third day. After 2 weeks, cells were washed with PBS (Gibco) and fixed using 10% formalin for 30 min at room temperature. The Oil Red O stock solution was freshly prepared by dissolving 300 mg Oil Red O in 100 ml 99% isopropanol. Fixed cells were washed with distilled water (Gibco), incubated with isopropanol for 5 min followed by incubation with filtered Oil Red O working solution for 5 min. Hematoxylin stain was added for 30 s, followed by 1% acetic acid. Lipids appear red and nuclei appear blue on a phase contrast microscope.

Chondrogenic Differentiation. Cells (5×10^5) were plated into a 96-well U-bottom suspension culture plate using ready-to-use mesenchymal stem cell chondrogenic differentiation medium (Promocell) in an incubator with an atmosphere of 5% CO_2 at 37°C. Spheroids spontaneously formed within 24–48 h. Cells were incubated for 21 days; medium was changed every third day without aspirating the spheroids.

Spheroids were placed into 10% formalin for 1 h, dehydrated, and paraffin embedded. Paraffin sections (5 µm) were prepared, and slides were deparaffinized, hydrated, and stained with toluidine blue (1 g toluidin blue in 100 ml 70% ethanol) for 3 min. Slides were dehydrated through 95% and 100% ethanol, and cleared in xylene. Chondrogeneic spheroids appear blue on a phase contrast microscope.

Osteogenic Differentiation. Cells (6×10^4) were plated onto a 24-well tissue culture plate (3.15×10^4 cells/cm^2) and differentiation was induced after cells

reached >100% confluency (after 24–72 h) in an incubator at 37°C and 5% CO_2. Cells were incubated with mesenchymal stem cell osteogenic differentiation medium (Promocell) for 21 days; medium was changed every third day. Cells were fixed by removing the media, washing with PBS, and incubating with 10% formalin for 10 min. Alizarin Red S solution (2 g Alizin red S in 100 ml dest. water) was incubated for 2 min. The osteogenic lineage was identified by dark red staining on a phase contrast microscope.

MSC Proliferation Assay

MSCs were seeded in 24-well plates (3,000 cells/well) and grown in their specific growth media. Daily cell counts were determined using the CellTiter 96 Cell Aqueous One Solution Proliferation Assay (Promega, Madison, WI). Triplicates were performed and the absorbance at 490 nm was measured with the Magellan ELISA Reader and Software (Tecan Systems Inc, San Jose, CA).

TGF-β and IL-10 Elispot Assays

The cytokine release for TGF-β and IL-10 was analyzed using human Elispot assays (R&D Systems). clMSCs or bmMSCs (5×10^5) were plated in their cell-specific growth media onto TGF-β and IL-10-coated Elispot plates for 48 h in an incubator at 37°C and 5% CO_2.

Spot developing was performed according to the manufacturer's protocol (BD Biosciences). Spots were automatically enumerated using an Elispot plate reader (CTL, OH) for scanning and analyzing.

IDO Western Blots

Cell extract was prepared in RIPA buffer (Sigma). Amount of protein was determined and electrophoresis was performed by loading an 8 μg protein sample into each well. Proteins were then blotted onto PVDF membrane in transfer buffer (3.75 g glycin, 7.25 g Tris base, 2.5 ml methanol was dissolved in total 2.5 L). The membranes were incubated in 5% BSA/TPBS solution prepared in PBST buffer (1× PBS, pH 7.0, 1 ml Tween-20/L) at room temperature for 2 h and then primary antibodies were added overnight at 4°C in 1:5000 dilution for IDO. Once the primary antibody labeling was performed, the blots were washed with PBST and the secondary antibody labeling (goat anti-mouse conjugated to horseradish peroxidase) was performed at room temperature for 1 h at 1:5000 dilution in 5% BSA in PBST. After 1 h of washing at room temperature with PBST, the ECL Western Blot System (Amersham, Piscataway, NJ) was used to visualize proteins using bioluminescence imaging (Caliper, IVIS 200), and photons per second were measured. Membrane-bound antibodies were stripped using restore Western blot stripping buffer (Pierce) for 15 min at room temperature. Membranes were blocked for 2 h using in 5% BSA/TPBS buffer, and primary antibody for GAPDH was added overnight at 4°C.

Bioluminescence Imaging (BLI)

BLI is a noninvasive, molecular imaging modality that allows for quantitative and repetitive imaging of targeted cells, transfected with plasmid vectors encoding for firefly luciferase (Fluc), in living organisms (44). In the presence of ATP and O_2, Fluc converts exogenously administered D-luciferin to the optically active metabolite oxyluciferin. Light emission can be monitored with an ultrasensitive CCD camera. In the setting of cell transplantation, BLI signals correlate with the number of living $Fluc^+$ cells. For this study, MSCs were transduced with Adv-Fluc (CMV) at a multiplicity of infection of 10 to stably express Fluc. Briefly, after MSCs were seeded, viral aliquots were added to 0.75 ml of their cell-specific growth media containing 8 μg/ml polybrene at a multiplicity of infection of 10, and the plate was then placed into a cell incubator at 37°C and 5% CO_2 for 24 h. Medium was changed after 24 h. Transduction efficacy was confirmed using Adv-GFP.

BLI was performed using the Xenogen in vivo imaging system (Caliper Life Sciences, Hopkinton, MA) as reported previously (5). Mice were anesthetized with 2% isoflurane and D-luciferin (Caliper Life Sciences) was administered IP at a dose of 375 mg/kg of body weight. At the time of imaging, animals were placed in a light-tight chamber, and photons emitted from FLuc-expressing MSCs were counted. The recipient mice were scanned repetitively on days 0, 1, and then every other day until the signal dropped into the background. BLI signals were quantified in units of photons per second (total flux) and are presented on a logarithmic scale.

Cell Surface Molecule Expression

MSCs grown in their growth media with or without additional recombinant human IFN-γ at 25 ng/ml (low-dose; PeproTech) were harvested to assess their expression of various surface markers. Cells were dissociated by collagenase type I solution at a concentration of 0.025% for 5 min at 37°C, and 5×10^5 cells were stained for 45 min at 4°C in 100 μl of 0.1% BSA in PBS containing an appropriate dilution of a desired PE-conjugated antibody. Primary PE-conjugated antibodies (all BD Bioscience unless stated otherwise) used were anti-human-CD31 (clone WM59), CD44 (clone G44-26), CD34 (clone 563), CD45 (clone H130), CD90 (clone 5E10), CD105 (clone 166707), CD117 (clone 104D2), SSEA-1 (clone MC-480), SSEA-4 (clone MC813-70), TRA-1-60 (clone TRA-1-60), HLA class I (clone

DX17), β_2-microglobulin (clone TÜ99), HLA-DR (clone WR18; abcam), HLA-G (clone MEM-G/9; Santa Cruz), HLA-E (clone MEM-E/06; Santa Cruz), CD40 (clone 5C3), CD80 (clone L307.4), and CD86 (clone 2331). IgG3 (clone 133316) isotype control was purchased from R&D Systems (Minneapolis, MN), IgG2a isotype control from Abcam (clone MOPC-173; Cambridge, MA), IgG1 (clone MOPC-21), and IgM (clone G155-228) from BD Biosciences. For the detection of intracellular HLA-G, cells were made permeable using the fix and perm cell permeabilization kit (BD Bioscience).

Flow cytometry was performed on a FACSCalibur system with Cell Quest Pro software (Becton Dickinson, Heidelberg, Germany); 10,000 events were collected. Analysis of data was performed using Flowjo (Tree Star, Ashland, OR). In the histograms, the isotype controls are represented by the gray solid areas and the antigens of interest by solid lines. Mean fluorescence intensities (MFI) were calculated. Molecule expression is defined as the ratio (MFI of antigen)/(MFI of negative isotype control), with 1 equaling no expression.

Elispot Assays

The cellular in vivo immune responses in Balb/c mice were studied after cell transplantation of either clMSCs or bmMSCs. The spleen of recipient animals was harvested 5 days after MSC transplantation to isolate recipient splenocytes. Elispot assays using 5×10^5 mitomycin-inhibited MSCs (Sigma Aldrich, St. Louis, MO) as stimulator cells and 5×10^6 recipient splenocytes as responder cells were performed according to the manufacturer's protocol (BD Biosciences) using IFN-γ and IL-4-coated plates.

The immunogenicity of MSCs to elicit an in vitro immune response was investigated by incubating 1×10^5 clMSCs or bmMSCs with 1×10^6 human peripheral blood mononuclear cells (PBMCs) for 7 days on a coated Elispot plate. Human PBMCs from healthy donors were previously isolated by Ficoll gradient centrifugation.

The immunosuppressive properties of MSCs in vitro were assessed by their potential to suppress a one-directional allogeneic mixed lymphocyte response in an allogeneic setting. Mitomycin-inhibited stimulator lymphocytes (1×10^5, freshly isolated from healthy donors by Ficoll) and 1×10^6 allogeneic effector lymohocytes were incubated with 1×10^5 clMSCs or bmMSCs in an IFN-γ, IL-4, or IL-2-coated Elispot plate for 7 days. Plates were developed according to the manufacturer's protocol (BD Bioscience) and spots were automatically enumerated using an Elispot plate reader (CTL, OH) for scanning and analyzing.

For IL-2 Elispots, clMSCs and bmMSCs were used either untreated or pretreated with 25 or 500 ng/ml IFN-γ for 48 h.

The High-IFN-γ Veto Function of MSCs

MSCs grown in their growth media with or without additional recombinant human IFN-γ at 25 ng/ml (low dose) or 500 ng/ml (high dose). Cells were harvested to assess their surface expression of HLA class I, HLA-DR, HLA-G, and HLA-E by flow cytometry as described above. Also, their IDO production was quantified in Western blot assays.

Statistical Analysis

Data are presented as mean ± SD. Comparisons between two groups were done by independent sample *t*-tests. Comparisons between groups were done by analysis of variance between groups (ANOVA) with LSD Post Hoc tests. Differences were considered significant for $p < 0.05$. Statistical analysis was performed using SPSS statistical software for Windows (SPSS, Chicago, IL).

RESULTS

Phenotypic and Functional Characteristics of MSCs

Both clMSCs and bmMSCs were positive for the mesenchymal stem cell markers CD90 and CD105 and negative for hematopoietic stem cell markers such as CD34, CD45, and CD117 (Fig. 1A). They were positive for the cell–cell interaction protein CD44 and negative for CD31. Both MSC lines were mildly positive for SSEA-4, an embryonic stem cell marker, and remained negative for SSEA-1, a marker for maturity, and the keratan sulfate antigen TRA-1-60, a human embryonic carcinoma marker.

In culture, both clMSCs and bmMSCs had fibroblast-like spindle-shaped morphology (Fig. 1B), although clMSCs were mildly bigger. After trypsination, the rounded clMSCs had diameters of 15.8 ± 3.1 μm and the bmMSCs of 11.8 ± 4.1 μm ($p < 0.001$). Both MSC lines were easily differentiable towards the chondrogenic, adipogenic, and osteogenic lineages. No differences between both MSC lines were noted regarding the time required for cell differentiation.

When grown in their cell specific media, cell proliferation of clMSCs was much faster than of bmMSCs (Fig. 1C). Differences between groups were statistically significant ($p < 0.05$) for day 3 and later time points. Thus, cell cultures with clMSCs required splitting approximately every 3 days, whereas bmMSCs were split on average every 10 days.

Antigeneic, Costimulatory, and Tolerogenic Properties of MSCs

MSCs were screened for their surface expression of HLA antigens, costimulatory factors, and immune toler-

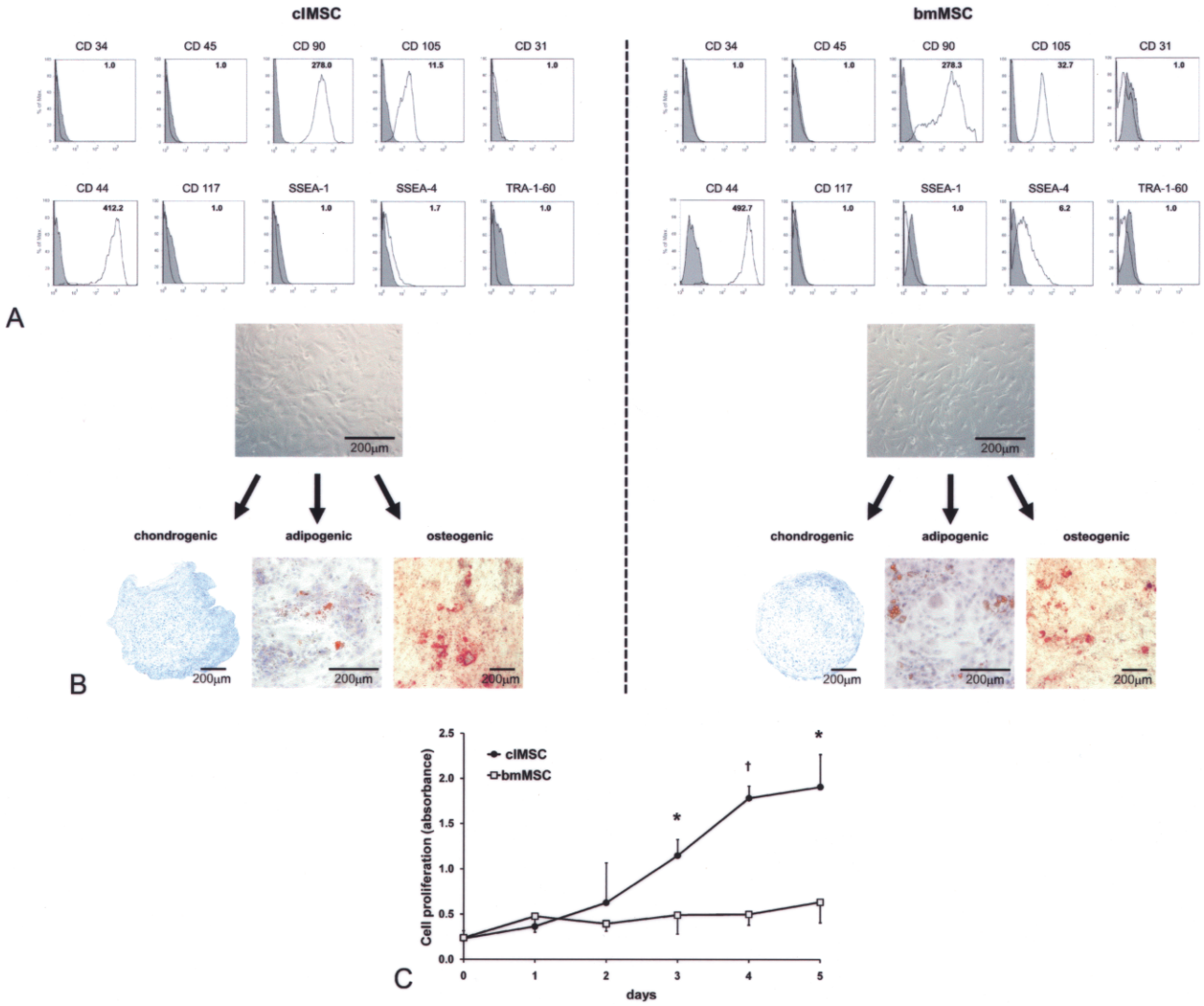

Figure 1. Phenotypic and functional characteristics of MSCs. clMSCs and bmMSCs were positive for CD90, CD105, and CD44, mildly positive for SSEA-4, and negative for CD31, CD34, CD45, CD117, SSEA-1, and TRA-1-60 (A; isotype control: gray solid area, antigens of interest: solid line; molecule expression is provided in the right upper corners). Both MSC lines grew in fibroblast-like spindle shape during cell culture conditions (original magnification 200×) and were differentiable into chondrocytes (original magnification 100×), adipocytes (original magnification 200×), and osteocytes (original magnification 100×, Leica microscope with QWin acquisition software, B). In vitro, clMSCs proliferation over a 5-day period was much faster than proliferation of bmMSCs (C; $*p < 0.05$, $\dagger p < 0.01$).

ance molecules at rest and in an inflammatory milieu with IFN-γ at 25 ng/ml. clMSCs had far lower expressions of HLA class I molecules ($p < 0.001$) and β_2-microglobulin ($p = 0.001$) than bmMSCs. Both MSCs completely lacked HLA-DR expression and were negative for the costimulatory factors CD40, CD80, and CD86 (Fig. 2A). After stimulation with IFN-γ, the levels of HLA class I ($p = 0.014$ and $p = 0.015$) and HLA-DR ($p < 0.001$ each) significantly increased for clMSCs and bmMSCs, respectively. All costimulatory factors remained negative after IFN-γ challenge. Interestingly, surface expression of HLA-E ($p = 0.012$ for clMSCs and $p = 0.033$ for bmMSCs), but not HLA-G (not shown) was significantly upregulated with IFN-γ in both MSC lines. Intracellular HLA-G, however, was constitutively expressed in both MSCs, although at higher levels in clMSCs ($p = 0.034$). HLA-G was upregulated by IFN-γ ($p = 0.005$ for clMSCs and $p = 0.043$ for bmMSCs).

The release of the tolerogenic cytokines TGF-β and IL-10 was quantified in Elispot assays. At rest, both MSC lines produced TGF-β, but only clMSCs released detectable amounts of IL-10 (Fig. 2B). After IFN-γ stim-

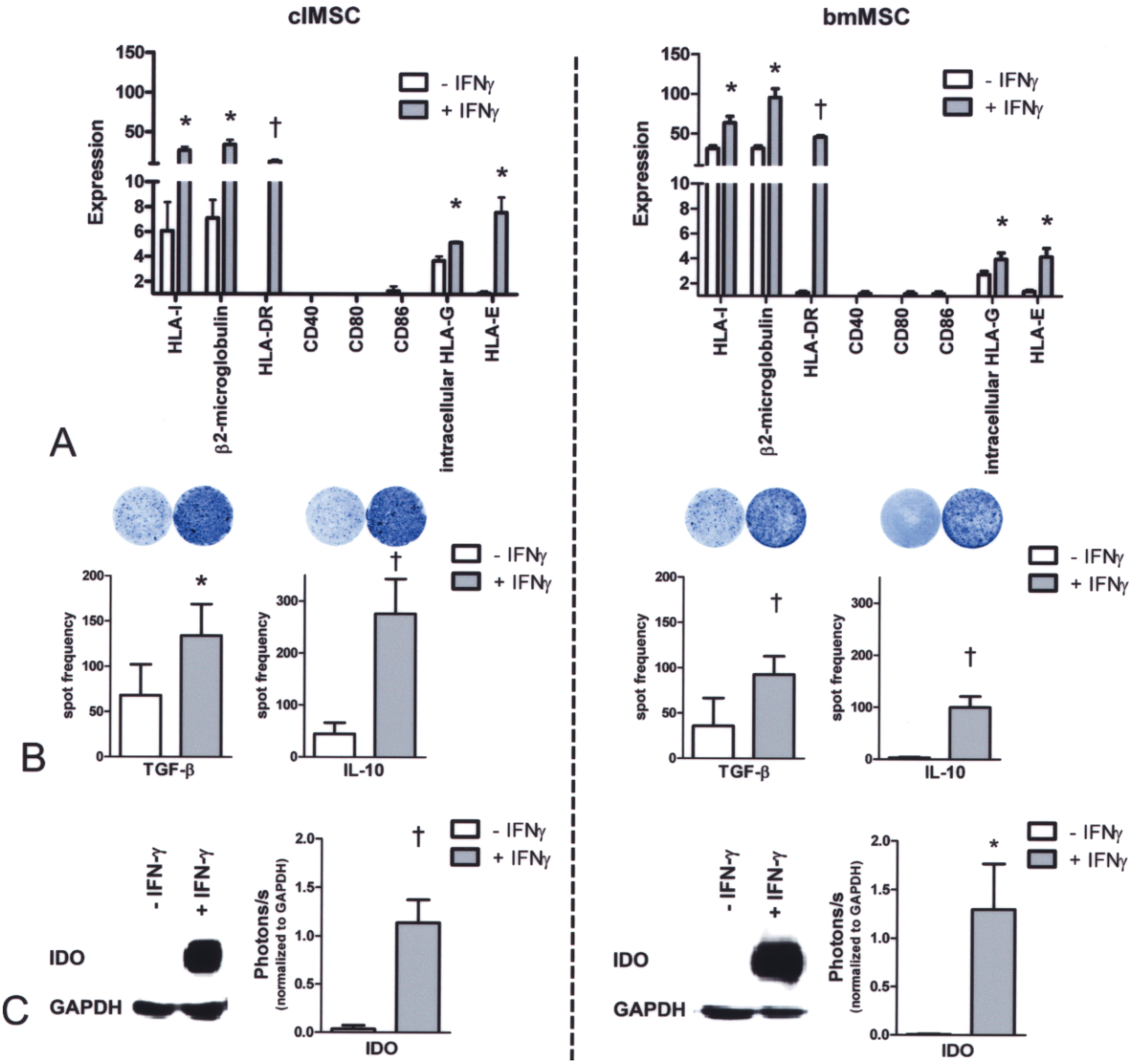

Figure 2. Antigeneic, costimulatory, and tolerogenic properties of MSCs. Surface molecule expression of clMSCs and bmMSCs was assessed by flow cytometry in the presence and absence of IFN-γ at 25 ng/ml (A; $*p < 0.05$, $\dagger p < 0.01$). TGF-β and IL-10 production by MSCs was quantified in Elispot assays (B; $*p < 0.05$, $\dagger p < 0.01$). With and without IFN-γ, the cytokine release was significantly higher in clMSC cultures. The enzyme IDO was hardly detectable in resting MSCs using Western blots, but IDO expression was strongly upregulated by IFN-γ in both clMSCs and bmMSCs (C; $*p < 0.05$, †xml;0.01).

ulation, the release of both TGF-β ($p = 0.018$ and $p = 0.003$) and IL-10 ($p < 0.001$ and $p < 0.001$) was significantly increased by clMSCs and bmMSCs, respectively. Absolute values for TGF-β and IL-10 release were significantly higher in clMSC cultures ($p = 0.03$ and $p < 0.001$, respectively) than with bmMSCs. IDO expression was hardly detectable in resting MSCs, but was strongly upregulated by IFN-γ in clMSCs ($p < 0.001$) and bmMSCs ($p = 0.041$) (Fig. 2C).

Immunogenicity of MSCs

Because clMSCs and bmMSCs were shown to have differences in their expression of immunogenic molecules as well as in their release of tolerogenic factors, it was tested if they were immunogenic enough to elicit an immune response in vivo and in vitro. In preparation for BLI, clMSCs and bmMSCs tolerated the FLuc transduction well and both showed a high transduction efficacy

of >95% as confirmed by Adv-GFP/DAPI double staining (not shown).

Elispot assays, performed 5 days after xenogeneic MSC transplantation, showed that both MSC lines triggered some cellular immune activation. However, bmMSCs were much more immunogenic and the provoked Th1 and Th2 responses were much stronger. Spot frequencies for IFN-γ and IL-4 were 10.8-fold (95% CI: 6.6–17.8) and 3.4-fold (95% CI: 2.2–5.2) higher with bmMSCs, respectively ($p < 0.001$ each vs. clMSCs) (Fig. 3A). Concordantly, bmMSCs were rejected more quickly than clMSCs in immunocompetent Balb/c mice. BLI signals dropped into the background after 7.2 ± 0.9 days for bmMSCs and after 10.9 ± 1.2 days for clMSCs ($p < 0.001$) (Fig. 3B). In immunodeficient SCID-beige mice, cell survival was prolonged and similar (14.7 ± 2.5 and 17.0 ± 2.8 days, respectively, $p = 0.205$).

Allogeneic immune activation was assessed in Elispot assays after incubation of MSCs with human PBMCs. Both MSC lines showed five out of six possible HLA-A, HLA-B, and HLA-DR mismatches with the used effector lymphocytes, but all cells shared the HLA-A24 epitope (Table 1). We found only very weak in vitro T-cell activation by clMSCs (PBMC + clMSC) with non-significant increases in IFN-γ spots (ANOVA $p = 0.002$, LSD post hoc $p = 0.419$) and IL-2 spots (ANOVA $p = 0.042$, LSD post hoc $p = 0.060$) versus PBMC control. Allogeneic in vitro lymphocyte activation, however, was stronger for bmMSCs (PBMC + bmMSC) with significantly increased IFN-γ (LSD post hoc $p = 0.001$) and IL-2 spots (LSD post hoc $p = 0.014$) (Fig. 3C) versus PBMC.

These data demonstrate that although both clMSCs and bmMSCs are recognized by allogeneic and xenogeneic lymphocytes, clMSCs are less immunogenic, cause less immune activation, and get rejected more slowly.

Immunosuppressive Properties of MSCs

HLA molecule expression of MSCs was further assessed in escalating IFN-γ environments. Although both clMSCs and bmMSCs upregulated HLA-DR expression upon low-dose IFN-γ stimulation at 25–50 ng/ml, HLA-DR was downregulated again with higher doses (100–500 ng/ml) (Fig. 4A). Throughout the IFN-γ range of 25–500 ng/ml, HLA-DR expression and HLA-I expression were significantly lower in clMSCs compared to bmMSCs ($p \leq 0.020$). Although HLA-I expressions also showed mild reductions with increasing IFN-γ concentrations, the downregulation remained statistically insignificant for both clMSCs and bmMSCs. Intracellular HLA-G showed a mild increase with rising IFN-γ that reached significance for the 500 ng/ml concentration compared to unstimulated conditions in both cell lines. HLA-E surface expression was already significantly upregulated by 25 ng/ml IFN-γ and remained at similar levels in higher IFN-γ milieus.

With increasing IFN-γ concentrations from 25 to 500 ng/ml, IDO expression further significantly increased from 1.2 ± 0.1 to 2.3 ± 0.1 ($p < 0.001$) in clMSCs (Fig. 4B). No significant differences were observed between 25 and 100 ng/ml IFN-γ in clMSCs and throughout the IFN-γ range in bmMSCs.

To test their net immunosuppressive effects, MSCs were incubated with allogeneic mixed PBMC cultures at different IFN-γ concentrations. Stimulator and effector lymphocytes had three out of four possible HLA-A/B mismatches and one out of two possible HLA-DR mismatches (Table 1). Effector lymphocyte activation was assessed by IL-2 Elispot assays (Fig. 4C). Both clMSCs ($p < 0.001$) and bmMSCs ($p < 0.001$) significantly reduced the IL-2 spot frequencies of mixed PBMC cultures in the absence of IFN-γ, although clMSCs were significantly more potent that bmMSCs ($p < 0.001$). Efficacy of bmMSCs was significantly improved in a low-dose IFN-γ environment (25 ng/ml; $p < 0.001$) and IL-2 spots were significantly lower with 500 versus 25 ng/ml IFN-γ in both MSCs ($p < 0.001$ each).

DISCUSSION

There are fundamental biological and practical differences between clMSCs and bmMSCs for cell-based immunomodulation. Whereas bmMSCs can be individually tailored for autologous use in chronic diseases, the retrieval of sufficient amounts of multipotent cells from BM of aging patients becomes increasingly problematic. Individualized cell production and storage would be very costly. On the other side, clMSCs are abundantly available, and can easily be isolated and expanded for a commercial off-the-shelf product for allogeneic use. The effect of MSCs seems not to be donor restricted, because the survival of third-party grafts could also be prolonged and third-party MSCs have also been shown to alleviate the rejection of skin grafts in baboons (3). MSCs were shown to modulate mixed lymphocyte reactions independent of MHC matching status (20) and third-party MSCs were shown to facilitate engraftment after umbilical cord blood transplantation (16). Third-party MSC infusion in hematopoietic stem cell transplant patients did not induce immunological memory to the infused MSCs (39). These characteristics support the possibility of exploiting universal "one cell fits all" MSC lines for therapeutic applications.

Both clMSCs and bmMSCs used in our study met phenotypical and functional criteria required for the definition of MSCs (12,25). They express CD105, CD90, and CD44, and are devoid of hematopoietic and endo-

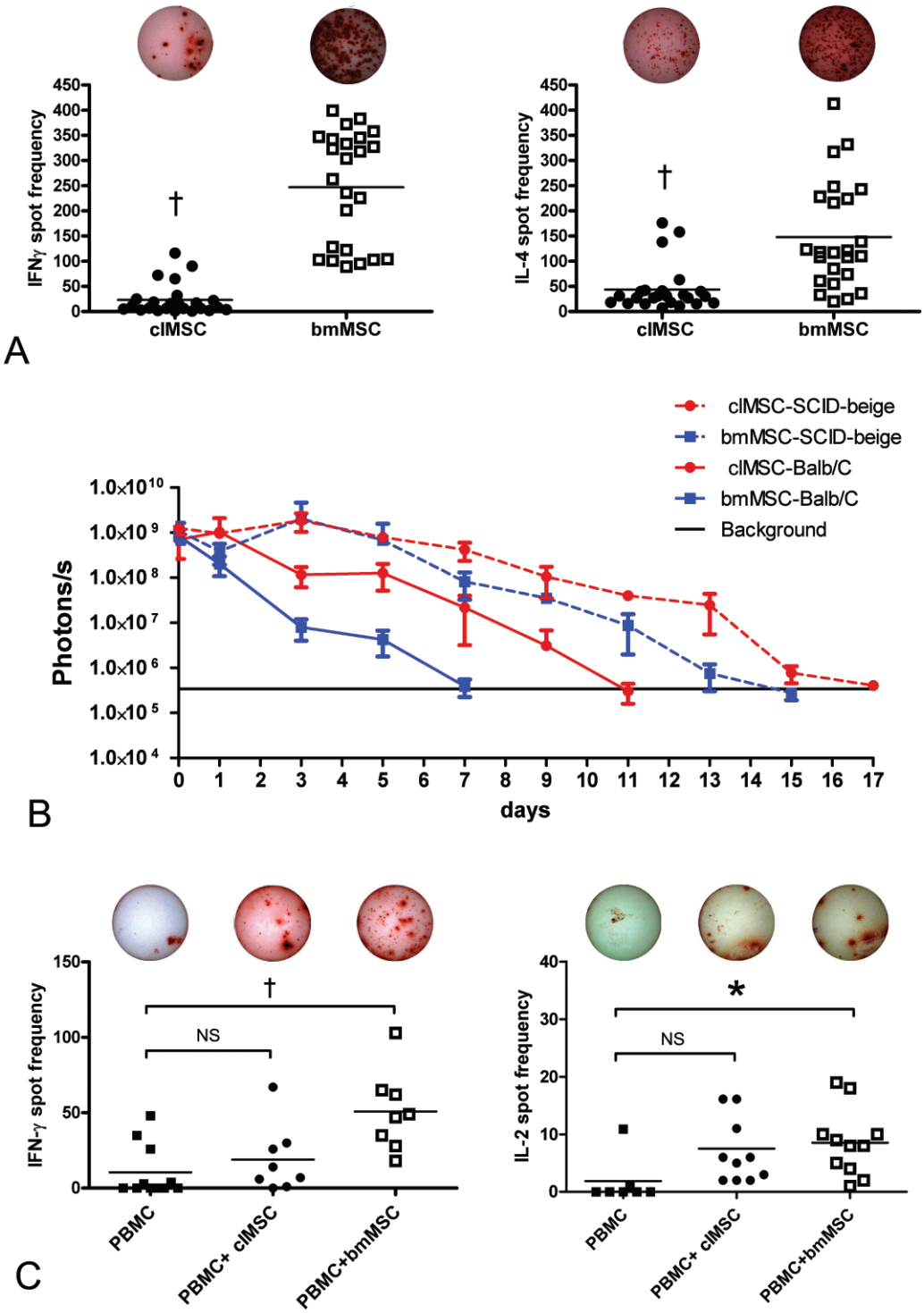

Figure 3. Immunogenicity of MSCs. Five days after MSC transplantation into Balb/c mice, Elispot assays for IFN-γ and IL-4 demonstrated that bmMSCs were much more immunogenic than clMSCs (A; †$p < 0.01$). Survival of FLuc$^+$ MSCs in Balb/c mice and in SCID-beige mice was longitudinally followed by BLI (B). bmMSCs were rejected more quickly than clMSCs in immunocompetent Balb/c mice, whereas MSC survival was similar and significantly longer in immunodeficient SCID-beige mice. Allogeneic immune activation in vitro after incubation of MSCs with human PBMCs showed very weak T-cell activation by clMSCs but a significant lymphocyte activation by bmMSCs (C; *$p < 0.05$, †$p < 0.01$).

thelial markers, such as CD31, CD34, CD45, and CD117. Because of the lack of specific MSC markers, additional antigens like the glycolipids SSEA-1 and SSEA-4 as well as the keratin sulfate TRA-1-60 are usually screened for. In line with previous reports (10,32), both of our MSC lines were positive for the embryonic stem cell-associated marker SSEA-4, although contrary findings were reported (36).

Additionally, clMSCs and bmMSCs allowed adipogeneic, osteogeneic, and chondrogeneic differentiation in vitro. Neither clMSCs nor bmMSCs were positive for Oct4, Sox-2, or TRA-1-60 (data not shown), essential factors for maintenance of pluripotency and reflecting the primitiveness of embryonic stem cells (21,49). Even after serum deprivation, we and others (49) could not induce Oct4 expression, as has been reported earlier (27). In accordance with previous reports on osteogenic capacity of stem cells (49), we could show the far superior proliferation capacity of clMSCs over adult bmMSCs.

Our data show that MSCs may be considered hypoimmunogenic due to their relatively low MHC I expression, but they are not intrinsically immunoprivileged and undergo immune rejection in an immunocompetent host. The reduction in MSC survival times between immunodeficient SCID-beige mice and Balb/c mice must be attributed to host immune responses. We show that clMSCs express significantly lower levels of HLA-I at rest and after stimulation with IFN-γ. Recent evidence indicates that MSCs can function as antigen presenting cells (APC) themselves, with phagocytic function and the ability to induce T-cell responses (6,38). We demonstrate that, under resting cell culture conditions, both MSCs were negative for HLA-DR. In the presence of IFN-γ, however, HLA-DR was upregulated, especially in bmMSCs, in which significantly higher expression values were achieved compared to clMSCs. Because cell culture conditions can impact HLA-DR expression (35), all experiments were performed at similar cell densities. Both MSC lines were negative for costimulatory molecules. The lower HLA class I and II expression profile on clMSCs impedes immune recognition, promotes immune ignorance, and delays immune rejection in vivo. We found significantly longer survival times of clMSCs in Balb/c mice compared to bmMSCs. Also, xenogeneic in vivo immune activation of Th1 and Th2 cells upon MSC transplantation was weaker for clMSCs. In accordance with our xenotransplant results, allogeneic in vitro lymphocyte activation was mild for clMSCs and thus much weaker than for bmMSCs, which triggered considerable IFN-γ and IL-4 production. Although it has been reported that clMSCs from Wharton's jelly completely fail to induce allogeneic in vitro PBMC activation (43), we have used more sensitive Elispot assays and have found at least mild immune activation with all MSC lines tested in our lab so far. However, MSCs gain tolerogenic potential through IDO expression (22), HLA-G production (24), and IL-10 and TGF-β secretion (23). These properties firstly alleviate immune recognition and diminish MSCs rejection and secondly may be used to suppress ongoing immune processes in the close vicinity of the administered or homed MSCs. We show that clMSCs produce larger amounts of tolerogenic IL-10 and TGF-β and have higher amounts of intracellular HLA-G.

IFN-γ dose-dependently exerts opposing effects on MSCs, enhancing their immunogenicity at low doses and increasing the production of tolerogenic cytokines and the expression of IDO at higher doses (6). It was previously shown that low-dose IFN-γ is produced by untreated MSCs, which may serve as endogenous autocrine stimulation (6) to enable their APC function. During inflammation, GVHD, and immune rejection, high levels of inflammatory mediators like IFN-γ are hypothesized to create a microenvironment in which the veto properties of MSCs dominate and MSCs suppress the ongoing immune reaction (6,29). We show that repression of HLA-DR is much more effective than of HLA-I in both clMSCs and bmMSCs. Head-to-head comparison between MSC lines shows that upregulated HLA-DR expression by 25 ng/ml IFN-γ remains much lower in clMSCs, and that HLA-DR downregulation by IFN-γ at concentrations beyond 50 ng/ml is also more effective in clMSCs. IDO production increases with rising IFN-γ, and shows no decrease at higher concentrations.

The net immunosuppressive effect of MSCs was tested in mixed PBMC cultures. The main findings are that clMSCs are more immunosuppressive than bmMSCs in the absence of IFN-γ and that the immunosuppressive effect of MSCs can be enhanced by IFN-γ. In an inflammatory milieu, MSCs seem to get further activated,

Table 1. Both MSC Lines Show Five Out of Six Possible HLA-A, HLA-B, and HLA-DR Mismatches With the Used Effector Lymphocytes, But All Cells Share the HLA-A24 Epitope

	HLA-A	HLA-B	HLA-DR
Effector lymphocytes	**03**01	**07**02	**15**01
	2402	—	—
Stimulator lymphocytes	**01**01	**15**01	**15**01
	2402	**44**05	**16**01
clMSCs	**11**01	**35**05	**11**01
	2407	**38**02	**12**02
bmMSCs	**02**01	**39**01	**11**01
	2402	**51**01	**13**01

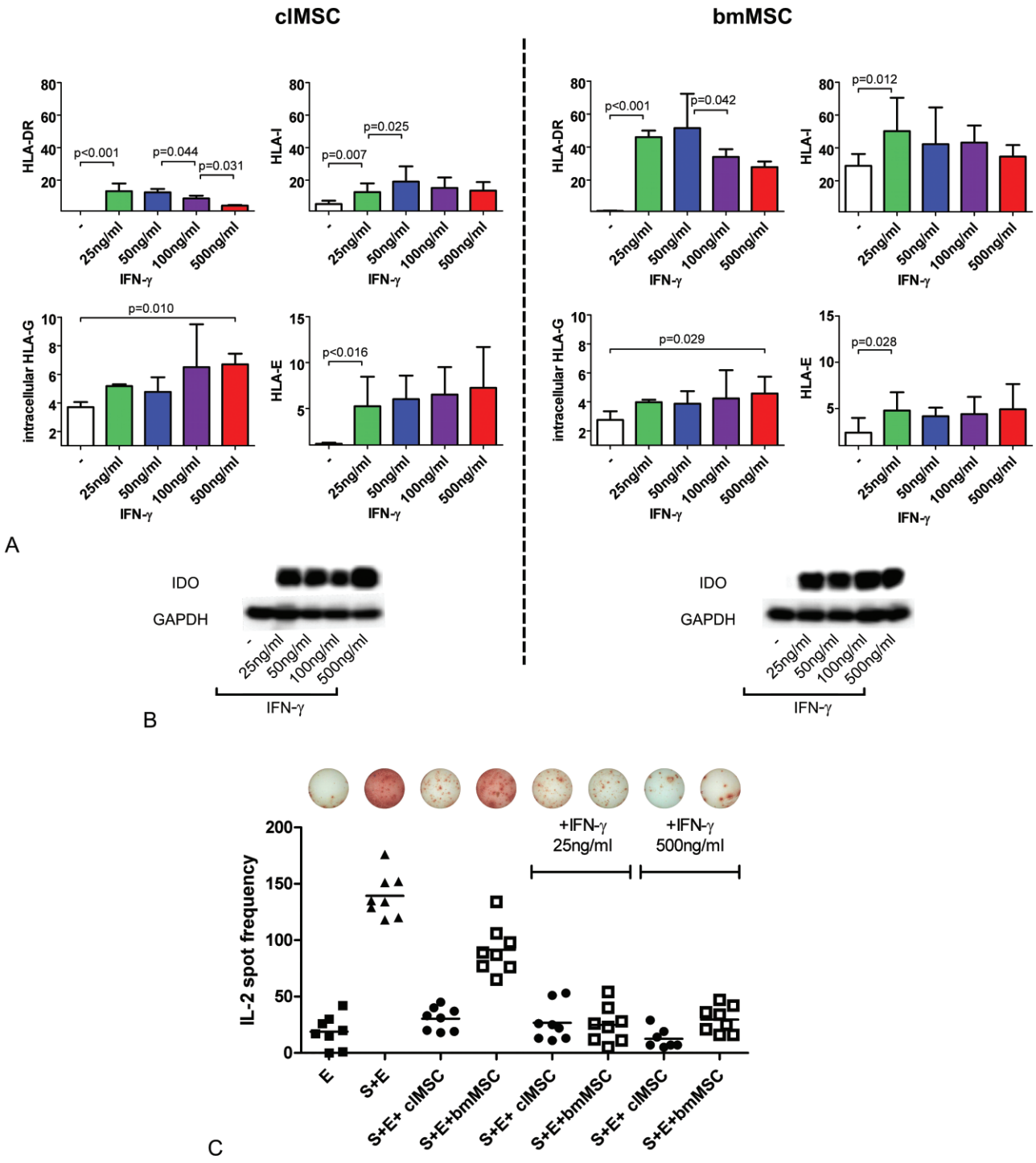

Figure 4. Immunosuppressive properties of MSCs. An increase of the IFN-γ concentration beyond 50 ng/ml significantly downregulated clMSC and bmMSC HLA-DR expression, whereas absolute HLA-DR values remained significantly lower in clMSCs at all IFN-γ concentrations (A). IDO expression was significantly increased from 25 to 500 ng/ml IFN-γ in clMSCs and moderately, nonsignificantly in bmMSCs (B). The net immunosuppressive effects of MSCs at different IFN-γ concentrations was assessed by in vitro IL-2 Elispot assays in allogeneic mixed PBMC cultures (C; S: stimulator PBMC, E: effector PBMC). clMSCs were significantly more potent in reducing IL-2 spot frequencies. The efficacy of bmMSCs significantly improved with rising IFN-γ concentrations.

which enhances their immunosuppressive potential and makes them promising tools for future clinical use.

SUMMARY

We show that MSCs are not inherently immune evasive and immunoprivileged and that clMSCs and aged bmMSCs show relevant differences in their immunogeneic profile and their capacity to respond to inflammatory stimuli and to suppress allogeneic immune activation. Altogether, clMSCs are superior cells for immunomodulation, because of their higher proliferative capacity, their lower immunogenicity, and their stronger production of soluble tolerogenic factors, contributing to their stronger overall immunosuppressive potential.

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