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Different Culture Conditions Modulate the Immunological Properties of Adipose Stem Cells

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ABSTRACT

The potential of human adipose stem cells (ASCs) for regenerative medicine has received recognition owing to their ease of isolation and their multilineage differentiation capacity. Additionally, low immunogenicity and immunosuppressive properties make them a relevant cell source when considering immunomodulation therapies and allogeneic stem cell treatments. In the current study, immunogenicity and immunosuppression of ASCs were determined through mixed lymphocyte reactions. The immunogenic response was analyzed after cell isolation and expansion in fetal bovine serum (FBS), human serum (HS)-supplemented medium, and xeno-free and serum-free (XF/SF) conditions. Additionally, the immunophenotype and the secretion of CXC chemokine ligand 8 (CXCL8), CXCL9, CXCL10, C-C chemokine ligand 2 (CCL2), CCL5, interleukin 2 (IL-2), IL-4, IL-6, IL-10, IL-17A, tumor necrosis factor-α, interferon-γ, transforming growth factor-β1, indoleamine 2,3-deoxygenase, Galectin-1, and Galectin-3 were analyzed. The results showed that ASCs were weakly immunogenic when expanded in any of the three conditions. The significantly strongest suppression was observed with cells expanded in FBS conditions, whereas higher ASC numbers were required to display suppression in HS or XF/SF conditions. In addition, statistically significant differences in protein secretion were observed between direct versus indirect cocultures and between different culture conditions. The characteristic immunophenotype of ASCs was maintained in all conditions. However, in XF/SF conditions, a significantly lower expression of CD54 (intercellular adhesion molecule 1) and a higher expression of CD45 (lymphocyte common antigen) was observed at a low passage number. Although culture conditions have an effect on the immunogenicity, immunosuppression, and protein secretion profile of ASCs, our findings demonstrated that ASCs have low immunogenicity and promising immunosuppressive potential whether cultured in FBS, HS, or XF/SF conditions. STEM CELLS TRANSLATIONAL MEDICINE *2014;3:1220–1230*

INTRODUCTION

Human adipose tissue possesses an abundant source of multipotent mesenchymal stem cells (MSCs) known as adipose stem cells (ASCs). They are promising candidates for clinical applications because of their ability to differentiate toward several cell types of mesodermal origin [1, 2]. ASCs also have low immunogenicity [3], and they are capable of immunomodulation [4], which makes them an even more relevant cell source when considering clinical applications.

Several ongoing clinical trials are studying the suitability of ASCs in tissue repair and in treatment of autoimmune diseases or immunological disorders. For instance, the potential of ASCs is being evaluated in clinical trials for the treatment of craniofacial injuries of bone and soft tissue (NCT01633892), articular cartilage defects (NCT02090140), and urinary incontinence (NCT01799694; NCT01804153) (http://clinicaltrials.gov/). There is also a growing interest in clinical applications using the potential of ASCs to modulate immunity and inflammation, as reviewed by Casteilla et al. [5]. Specifically, the potential of ASCs is currently being evaluated in clinical trials for the treatment of Crohn's disease (NCT01157650; NCT01011244), osteoarthritis (NCT01739504; NCT01585857), graft versus host disease (GVHD) (NCT01222039), and multiple sclerosis (NCT01453764) (http://clinicaltrials.gov/).

The immunomodulatory capacity of ASCs has been studied by many research groups [4, 6–9], and interestingly, ASCs are considered immunologically privileged, lacking the expression of major histocompatibility complex (MHC) class II molecules, as well as T- and B-cell costimulatory molecules CD80, CD86, and CD40 [3, 9], required for complete T-cell activation. Nevertheless, immunological privilege of MSCs may not be absolute [10], and consequently the ability of MSCs to escape the immune system remains under debate. Still, ASCs have been shown to have

immunomodulatory properties, such as regulation of T-cell functions and modulation of cytokine secretion [3, 4, 7, 11, 12]. As shown by McIntosh et al. [3], the immunogenic properties of ASCs may also change as the passage increases. They showed that the stromal vascular fraction is more immunogenic when compared with higher passages that are also capable of immunosuppression. The immunomodulatory effect has also been demonstrated in vivo [8, 13, 14], where ASCs have been shown to suppress allogeneic lymphocytes and prolong allotransplant survival [8, 14]. In addition, the potential of MSCs to treat patients with GVHD has been reported in several clinical studies [15–18].

The mechanisms behind the immunosuppressive capacity are being investigated, so far mainly using bone marrow-derived mesenchymal stem cells (BMSCs). MSCs have been shown to actively inhibit the functions of several immune cells through cytokine secretion [19–21], and several soluble mediators have been suggested to be involved in the suppression process. These mediators include transforming growth factor β (TGF-β) [22] and prostaglandin E2 [23], both of which modulate interleukin 2 (IL-2) synthesis and T-cell activation, indoleamine 2,3-deoxygenase (IDO) [24, 25], and galectin-1 and -3 [26, 27]. In addition to soluble factors, direct cell-cell contact is an essential part of the interaction, but the mechanism has not been completely defined. Ren et al. [28] demonstrated that cell-cell adhesion mediated by intercellular adhesion molecule 1 (ICAM-1, CD54) and vascular cell adhesion molecule 1 (VCAM-1, CD106) is critical for MSC-mediated immunosuppression. In addition, the inflammatory environment has been shown to affect the suppression process, enhancing the immunosuppressive capacity of ASCs [4, 19, 24, 29]. Consequently, soluble factors, direct cell-cell contacts, and the inflammatory environment are all involved in the process, but more studies are required to fully determine the suppression mechanisms and to receive a comprehensive picture of the immunomodulatory potential of ASCs and other MSCs.

As the number of clinical therapies using ASCs increases, further requirements regarding the safety and efficiency of the cells arise. In fact, several reports have been published to fully characterize ASCs and to develop safe and efficient in vitro culture protocols for clinical cell therapies [30–34]. Completely defined xeno-free and serum-free (XF/SF) isolation and expansion protocols were recently developed for ASCs by our team [30] to replace traditional fetal bovine serum (FBS)-based culturing protocols, which should be avoided in clinical treatments because of safety concerns [35, 36]. Cells cultured in the presence of animal-derived components may transfer xenogeneic antibodies into the human body and trigger severe immune responses [37, 38]. By contrast, novel XF/SF culture conditions meet the current good manufacturing practice (GMP) requirements, and cells are cultured in fully defined media using only GMP grade reagents.

In this study, we evaluated the effect of the cell culture medium on the immunologic properties of ASCs. To our knowledge, this is a novel approach, and the aspect of the culture medium on cell immunologic properties has not been reported previously. However, in our previously published reports, we demonstrated that the culture conditions have an effect on the proliferation rate, as well as on the differentiation potential of the cells [30], and we hypothesized that the immunomodulatory properties also differ between cells isolated and expanded in different serum conditions and in XF/SF conditions. The isolation and expansion of ASCs was carried out in parallel in three different culture conditions: in an FBS-containing medium, in a human serum (HS)-containing medium, and in XF/SF conditions. After cell expansion in these conditions, ASCs were cocultured with peripheral blood mononuclear cells (PBMCs) in mixed lymphocyte reactions (MLRs) to evaluate the immunogenic properties of the cells in low (passage 2) and high (passage 5) passage numbers.

Materials and Methods

The collection of adipose tissue and peripheral blood was approved by the ethics committee of the Pirkanmaa Hospital District in Tampere, Finland (R03058). Adipose tissue samples were obtained from Tampere University Hospital, and the buffy coat samples were from the Finnish Red Cross Blood Service. All analyses were performed separately with four or five donor cell lines isolated in FBS, HS, and XF/SF conditions. The same five patient cell lines were used for MLR assays and for flow cytometric analyses (donors 1–5), whereas the protein secretion studies were performed with four different ASC lines (donors 6–9).

Isolation and Culture of ASCs

ASCs were isolated from adipose tissue samples (n = 9) collected from female donors (age 41 $\pm$ 10 years) undergoing elective surgical procedures in the Department of Plastic Surgery, Tampere University Hospital, Tampere, Finland. ASCs were isolated under three different culturing conditions: medium containing FBS, HS, or XF/SF culture conditions.

Isolation of ASCs from adipose tissue samples was carried out using a mechanical and enzymatic method as described previously [2, 31, 39]. Briefly, the adipose tissue was minced manually into small fragments and digested with collagenase NB 6 GMP Grade (SERVA Electrophoresis GmbH, Heidelberg, Germany, http://www.serva.de) in a water bath at 37°C under shaking conditions. The digested tissue was centrifuged and filtered in sequential steps through a 100-μm pore size filter to separate the ASCs from the surrounding tissue.

For FBS and HS conditions, Dulbecco's modified Eagle's medium (DMEM)/F-12 1:1 (Life Technologies, Rockville, MD, http://www.lifetech.com) was supplemented with 1% L-analyl-L-glutamine (GlutaMAX I; Life Technologies), 1% antibiotics (p/s; 100 U/ml penicillin, 0.1 mg/ml streptomycin; Lonza, Walkersville, MD, http://www.lonza.com) and either 10% FBS (Life Technologies) or 10% HS (human serum type AB; Lonza). ASCs isolated and expanded in FBS medium were detached using 1% trypsin (Lonza), and ASCs isolated in HS medium were detached using TrypLE Select (Life Technologies).

For XF/SF conditions, the cells were isolated under XF/SF conditions and seeded in carboxyl-coated flasks (PureCoat Carboxyl T75; BD Biosciences, Franklin Lakes, NJ, http://www.bdbiosciences.com) and expanded in STEMPRO MSC SFM (Life Technologies) supplemented with 1% GlutaMAX I, 0,3% antibiotics, and 10% StemPro MSC SFM Xeno-Free supplement as described previously [30]. From passage 1 onwards, XF/SF cells were expanded in STEMPRO MSC medium supplemented with CELLstart CTS coating (Life Technologies) according to the manufacturer's instructions. ASCs isolated and expanded in SF/XF medium were detached using TrypLE Select.

Isolation of PBMCs

Allogeneic human PBMCs were isolated from buffy coat samples (n = 7) by density gradient centrifugation using Ficoll-Paque PLUS

(density 1.077 g/ml; GE Healthcare, Little Chalfont, U.K., http://www.gehealthcare.com) according to manufacturer's instructions, aliquoted, and cryopreserved in the nitrogen gas phase until cocultures.

Immunogenicity and Immunosuppression Analyses

The one-way and two-way MLR assays were used to determine the immunogenic properties of ASCs after cell isolation and expansion in different culture conditions, in FBS, HS-containing medium, or XF/SF conditions. MLRs were performed separately with four to five ASC donor cell lines (donors 1–5) in passages 2 and 5. The MLRs were seeded on 96-well plates using DMEM/F-12 1:1 supplemented with 1% GlutaMAX I (Life Technologies), 1% antibiotics (p/s; 100 U/ml penicillin, 0.1 mg/ml streptomycin; Life Technologies), and 10% HS (PAA Laboratories, Pasching, Austria, http://www.paa.at). 10% HS (PAA Laboratories) medium was chosen to serve as a constant environment for MLR cultures because of low viability of PBMCs when cultured in XF/SF condition (data not shown). Therefore, prior to MLR assays, ASCs isolated and expanded in three different culture conditions, FBS, HS, (Lonza), and XF/SF conditions received the same treatment of medium change and were allowed to adjust in HS medium (PAA Laboratories) for 24 hours prior to coculture.

One-Way MLR Measuring the Immunogenicity of ASCs

PBMCs derived from three different donors were seeded at 2.5×10^5 cells per well and acted as responder cells. In addition to PBMC responders, various stimulator cells were added to the reactions: autologous PBMCs (baseline response) and allogeneic PBMCs (positive-control response), both plated at 1.0×10^4 cells per well, and the test ASCs, plated at 0.5×10^4, 1.0×10^4 and 2.0×10^4 cells per well. Stimulator PBMCs and ASCs were irradiated with γ-rays (40 Gy) prior to the coculture to inhibit the proliferation of the stimulator cells. ASCs in medium alone were plated as control cultures. In addition, control cultures of PBMCs alone were added as well as PBMCs supplemented with mitogen phytohemagglutinin (PHA, 1 μg/ml) to activate the responder PBMC lines to serve as a maximal positive control response in the one-way reaction. Quadruplicate reactions were performed from each treatment, and the cultures were incubated at 37°C in 5% CO_2 for 5 days in HS medium (PAA Laboratories). One-way MLRs were performed as described previously by McIntosh et al. [40].

Two-Way MLR Measuring the Immunosuppression of ASCs

Two different MLR combinations were formed from three different PBMC lines. For each MLR combination, cells from two donors were mixed at a time in equal amounts to activate the proliferative response of each PBMC line, and a total 2.5×10^5 cells were seeded per well. After seeding the MLRs, ASCs were added to the reactions at cell densities of 0.5×10^4, 1.0×10^4 or 2.0×10^4 cells per well. In addition, control wells containing only MLR combinations without ASCs, and ASCs alone were also seeded. Quadruplicate reactions were performed from each treatment group, and the cultures were incubated at 37°C in 5% CO_2 for 5 days in HS (PAA Laboratories) medium. Two-way MLRs were performed as described previously by McIntosh et al. [40].

Proliferation Assay

On day 4 of the MLRs, 10 μM bromodeoxyuridine (BrdU) was added to mono- and cocultures, and the cells were incubated for additional 16 hours at 37°C. On day 5, PBMC proliferation was assessed by BrdU enzyme-linked immunosorbent assay (ELISA) (Roche Applied Science, Penzberg, Germany, https://www.roche-applied-science.com) according to the manufacturer's instructions using a microplate absorbance reader (Victor 1429 Multilabel Counter; Wallac, Turku, Finland, http://www.perkinelmer.com).

Performed Calculations From Proliferation Assay's Absorbance Values

Prior to statistical analysis, the following calculations were performed for the raw absorbance data of BrdU ELISA. The average absorbance values of ASC control wells were calculated. Medium background was subtracted from the average ASC control values. These modified ASC control values were then subtracted from MLR absorbance values taking into account the used ASC numbers. Finally, these corrected MLR values were divided by the average autologous PBMCs value that was considered as baseline response in one-way MLR or by the average control value of wells containing only MLR cultures without ASCs, considered as baseline response in two-way MLR. According to the performed calculations, in one-way MLRs, the value 1 indicates the baseline response, and values above 1 indicate activation. Respectively, in two-way MLRs, a value of 1 represents a baseline response, and reaction values below 1 indicate suppression. Performed calculations are modified from the protocols described by McIntosh et al. [40].

Flow Cytometric Analyses

ASCs expanded in FBS, HS, and XF/SF media (n = 4–5, donors 1–5, passages 2 and 5) were analyzed by flow cytometry (FACSAria; BD Biosciences) to determine whether different culturing conditions affect the immunophenotype of the cells. Monoclonal antibodies against CD11a-allophycocyanin (APC), CD80-phycoerythrin (PE), CD86-PE, CD105-PE (R&D Systems Inc., Minneapolis, MN, http://www.rndsystems.com), CD-3 (PE), CD14-phycoerythrin-cyanine (PECy7), CD19-PECy7, CD45RO-APC, CD54-fluorescein isothiocyanate, CD73-PE, CD90-APC (BD Biosciences), and CD34-APC, HLADR-PE (Immunotools GmbH, Friesoythe, Germany, http://www.immunotools.de) were used. Analysis was performed on 10,000 cells per sample, and unstained cell samples were used to compensate for the background autofluorescence levels.

Quantitative Protein Measurements

Two-way MLR assay was used to evaluate the secreted protein expression in 24-well plate format. Four different donor PBMC lines were used as responder cells, in two different MLR combinations. PBMCs from two donors were mixed at a time in equal amounts to activate the proliferative response of each PBMC line, and a total cell number of 8.0×10^5 PBMCs was seeded per well. After seeding the MLRs, ASCs were added at densities of 3.0×10^4 cells per well (donors 6–9) either in direct coculture or using a semipermeable membrane inserts to prevent direct cell-cell contacts between ASCs and PBMCs. When using the inserts, PBMCs were pipetted into the inserts (pore size, 0.4 μm;

ThinCert, Greiner Bio-One, Frickenhausen, Germany, http://www.gbo.com/en), whereas ASCs remained on the bottom of the wells. In addition, control wells containing MLR cultures with no ASCs or ASCs alone were seeded. Similarly to immunogenicity assays, the reactions were performed with ASCs isolated and expanded in three different culture conditions (FBS, HS, and XF/SF conditions), and were subsequently let to adjust to the 5% HS (PAA Laboratories) medium for 24 hours prior to coculture. HS lot (PAA laboratories) was changed prior to cytokine studies, and 5% HS was chosen as a standard culturing condition to control excess cell growth during cytokine measurements. Quadruplicate reactions were performed from each treatment. Cell culture supernatants from mono- and cocultures were collected on day 5 and stored in −20°C until analysis. Cytokines and chemokines secreted by the cells were analyzed using Cytometric Bead Arrays (CBAs; BD Biosciences); human chemokine kit (CXC chemokine ligand 8 [CXCL8/IL-8], -9 [CXCL9/MIG], -10 [CXCL10/IP-10], C-C chemokine ligand 2 [CCL2/MCP-1], and -5 [CCL5/RANTES]), human Th1/Th2/Th17 cytokine kit (interleukin 2 [IL-2], -4 [IL-4], -6 [IL-6], -10 [IL-10], and -17A [IL-17A]; tumor necrosis factor α [TNF-α]; and interferon γ [IFN-γ]) and human TGF-β1 Single Plex Flex Set (TGF-β1). IDO and Galectin-1 and -3 were analyzed using colorimetric ELISA assays; ELISA kit for IDO (Cloud-Clone Corporation, Uscn Life Science Inc., Wuhan, People's Republic of China, http://www.uscnk.com), Human Galectin-1 Quantikine ELISA Kit (R&D Systems), and Human Galectin-3 Quantikine ELISA Kit (R&D Systems). Each colorimetric ELISA reaction was done in triplicate, and averages of the parallel reactions were then taken into account in statistical analysis. Substantially high concentrations of IL-6, CXCL8, CXCL9, CCL2, and CXCL10 were detected from the culture media, exceeding the detection limits of the human chemokine and human Th1/Th2/Th17 cytokine kits. As a consequence, the cell culture supernatants were diluted 1:150, and these cytokines were reanalyzed using multiplexed Flex Set array (BD Biosciences). CBA output data were analyzed using FCAP Array software version 3.0 (BD Biosciences) according to the manufacturer's instructions.

Statistical Analyses

Statistical analyses were performed with SPSS version 19 (IBM Corp., Armonk, NY, http://www.ibm.com). A nonparametric Kruskal-Wallis test with Mann-Whitney U post hoc test was used to compare different culture conditions and their effect on immunogenic proliferative response in one-way and two-way MLR, as well as for determining the effect of different culture conditions on cell surface protein expression of cytometric data. The cytokine secretion data were analyzed using a regression analysis with ranked values. The effect of culture condition on cytokine secretion was analyzed using a model in which the FBS or HS conditions were defined as reference. Secretion values of other two conditions were then compared with reference condition separately in direct and indirect cocultures. Differences in cytokine secretion between direct versus indirect cultures, as well as between cocultures and monocultures, were analyzed using a different model. In this model, monocultures (ASCs alone or MLR without ASCs) and indirect cocultures were compared with direct cocultures that were defined as reference. The results of MLRs, cytokine secretion studies, and flow cytometric analyses were reported as means and SD. When three different culturing conditions were compared, a Bonferroni correction was performed to avoid the problem of multiple comparisons. As a consequence, the p values were multiplied by 3 before the interpretation of statistical data. The results were considered statistically significant when the p value was under 0.05.

Results

ASCs Elicited a Weak Immunogenic Response on PBMCs in All Culture Conditions

One-way MLR assays were performed to assess the immunogenicity of ASCs (Fig. 1A, 1B). In Figure 1A and 1B, a value of 1 indicates the baseline response of PBMCs without ASCs, and values above 1 indicate activation.

ASCs expanded in the FBS-containing medium induced the lowest immunogenic response on PBMCs in both passages 2 and 5 (Fig. 1A, 1B). In passage 2, a significantly lower immunogenic response on PBMCs was observed in the FBS medium ($p < .05$) when compared with ASCs expanded in HS or XF/SF conditions, with 1.0×10^4 and 2.0×10^4 stimulator ASCs (Fig. 1A, a). In passage 5, a significantly lower PBMC immunogenic response was observed in the FBS medium ($p < .05$) compared with the HS medium, with 1.0×10^4 stimulator ASCs (Fig. 1B). Furthermore, the immunogenic response in passage 5 was significantly stronger in the HS medium ($p < .05$), with 2.0×10^4 stimulator ASCs, compared with both FBS and XF/SF conditions (Fig. 1B, b). However, although the different culture conditions of the ASCs had a significant effect to the immunogenicity of the cells, the ASCs expanded in all of the studied conditions elicited only a weak immunogenic response compared with the maximal positive control response of PBMCs activated with PHA mitogen (Fig. 1A, 1B).

ASCs Showed a Suppressive Effect on PBMC Proliferation

Differences between culture conditions were also evident regarding the suppression potential of ASCs (Fig. 1C, 1D). Coculture results in two-way MLRs were standardized using values from MLRs without ASCs. Therefore, in Figure 1C and 1D, a value of 1 represents the baseline, and values below 1 indicate suppression.

ASCs expanded in the FBS medium efficiently suppressed the proliferation of PBMCs stimulated in MLR in both passages 2 and 5 (Fig. 1C, 1D). ASCs expanded in the HS medium showed suppression of PBMCs in passage 2 (Fig. 1C) with 1.0×10^4 and 2.0×10^4 cells per well and in passage 5 (Fig. 1D) with 2.0×10^4 cells per well. By contrast, ASCs expanded in XF/SF conditions were not able to suppress the PBMC proliferation in passage 2 and suppressed the proliferation in passage 5 with only 1.0×10^4 or 2.0×10^4 cells per well (Fig. 1C, 1D). Some of these differences proved to be statistically significant. In passage 2, FBS showed significantly stronger suppression ($p < .05$), with 0.5×10^4 stimulator ASCs (Fig. 1C, a–c) compared with HS and XF/SF conditions that were not capable of inducing suppression. In addition, XF/SF-expanded ASCs were significantly less suppressive ($p < .05$) compared with HS and FBS conditions in passage 2 (Fig. 1C, c). In passage 5 (Fig. 1D), ASCs expanded in the FBS medium showed significantly stronger suppression ($p < .05$), with a cell number of 0.5×10^4 (Fig. 1D, a), compared with HS and XF/SF conditions. With a cell number of 1.0×10^4, a significantly stronger suppression was also observed in the FBS medium ($p < .05$) when compared with the HS medium. Therefore, the

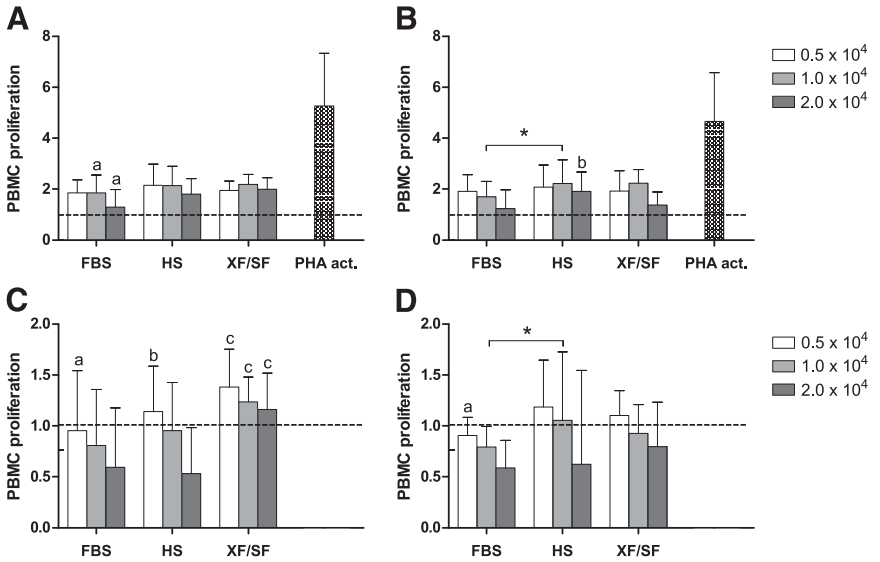

Figure 1. Immunogenicity and immunosuppression of adipose stem cells (ASCs). The immunogenicity **(A, B)** and suppression potential **(C, D)** of ASCs are presented in passages 2 **(A, C)** and 5 **(B, D)**. ASCs expanded in FBS, HS, and XF/SF conditions elicited only a weak immunogenic response compared with the maximal positive control response activated with PHA. Suppression potential was dependent on the ASC number, as well as the culture condition. The dashed line represents a baseline response; values above 1 indicate activation **(A, B)**, and values below 1 indicate suppression **(C, D)**. The results are presented as means ± SD. a, $p < .05$ when FBS is compared with HS and XF/SF conditions; b, $p < .05$ when HS is compared with FBS and XF/SF conditions; c, $p < .05$ when XF/SF is compared with FBS and HS conditions; ∗, $p < .05$ when FBS in compared with HS but not XF/SF conditions. Abbreviations: act., activated; FBS, fetal bovine serum; HS, human serum; PBMC, peripheral blood mononuclear cells; PHA, phytohemagglutinin; XF/SF, xeno-free/serum-free.

suppression potential of ASCs was dependent on the ASC number, as well as the culture condition.

Protein Secretion Profile Was Altered Depending on Direct Versus Indirect Cocultures and Expansion Conditions of ASCs

A panel of secreted proteins was analyzed from cell culture supernatants either in direct or indirect cocultures after the expansion of ASCs in different serum conditions: FBS, HS, and XF/SF conditions. Altered secretion profiles were observed between the different expansion conditions of the ASCs, but more evident was the effect of direct versus indirect contact between ASCs and PBMCs (Fig. 2).

Direct Cocultures

The secretion of IL-6 was increased in cocultures with ASCs expanded in FBS conditions compared with ASCs expanded in HS and XF/SF conditions, but no statistical significances were observed because of the large standard deviation. A significantly higher secretion of IFN-γ was detected in XF/SF conditions ($p < .05$) compared with cocultures with FBS- and HS-expanded ASCs. TNF secretion was higher in XF/SF conditions compared with the FBS and HS media, but no statistical significances were observed. However, statistically significant differences were seen between different culture conditions for the secretion of TGF-β1. Direct cocultures containing ASCs expanded in XF/SF conditions had the lowest TGF-β1 concentrations ($p < .05$) compared with either FBS or HS conditions. There was no significant difference between FBS and HS conditions.

The chemokine CCL2 secretion was significantly higher in cocultures with FBS-expanded ASC ($p < .05$) compared with HS-expanded ASCs. A significantly lower secretion of chemokine CCL5 was measured in HS conditions ($p < .05$) compared with XF/SF and FBS conditions. The secretion of chemokines CXCLX9 and -10 was strong in each culture condition, and the secretion of chemokine CXCL10 was significantly higher in cocultures with XF/SF-expanded ASCs ($p < .05$) compared with HS conditions. The chemokine CXCL8 was strongly secreted in cocultures with ASCs expanded in all of the studied conditions.

Statistically significant differences were also seen between different culture conditions for the secretion of IDO. Direct cocultures containing ASCs expanded in FBS conditions had the lowest IDO concentrations compared with either HS ($p < .05$) or XF/SF ($p < .001$) conditions. There was no significant difference between HS and XF/SF conditions. The concentration of galectin-1 was highest in the direct coculture containing ASCs expanded in HS conditions, but no statistically significant differences were seen between different culture conditions for galectin-1 or galectin-3.

Reactions containing only ASCs or PBMCs showed a significantly lower secretion of cytokines and chemokines IL-6, CCL2, CCL5, CXCL8, CXCL9, and CXCL10 ($p < .001$) compared with direct cocultures. IFN-γ and TNF-α secretion was not detected in reactions containing only ASCs. Therefore, a significantly lower secretion of IFN-γ and TNF-α ($p < .001$) was measured in reactions containing only ASCs, and a significantly lower secretion of IFN-γ ($p < .05$) was measured in reactions containing only PBMCs. A significantly lower secretion of TGF-β1 was detected in reactions containing PBMCs only compared with direct cocultures ($p < .05$). The secretion of TGF-β1 was also lower in reactions containing ASCs alone, but there was no significant difference compared with direct cocultures. Concentrations of IDO, galectin-1, and galectin-3 were significantly lower ($p < .001$) in reactions containing ASCs alone compared with direct cocultures. In addition, the concentration of galectin-1 ($p < .001$) was significantly

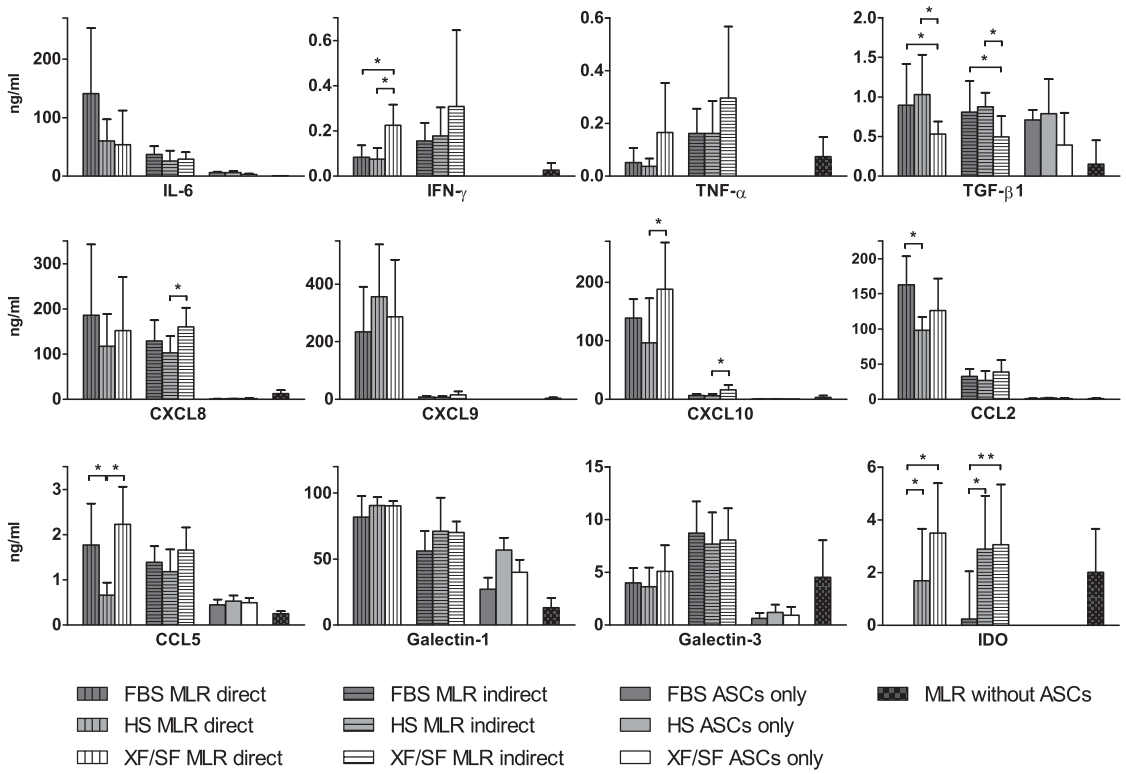

Figure 2. Protein secretion. Secreted proteins analyzed in direct cocultures and using separating membranes between ASCs and peripheral blood mononuclear cells. Prior to the cocultures, ASCs were expanded in different culture conditions: FBS, HS, and XF/SF. Altered secretion profiles were observed between the different expansion conditions of the ASCs. TGF-β1 and IDO amounts of medium alone were subtracted from quantitated concentrations of TGF-β1 and IDO in mono- and cocultures. The results are presented as means $\pm$ SD. $*$, $p < .05$; $**$, $p < .001$. Abbreviations: ASC, adipose stem cell; CCL2 and -5, C-C chemokine ligands 2 and 5; CXCL8, CXCL9, and CXCL10, CXC chemokine ligands 8, 9, and 10; FBS, fetal bovine serum; HS, human serum; IDO, indoleamine 2,3-deoxygenase; IFN-γ, interferon γ; IL-6, interleukin 6; MLR, mixed lymphocyte reaction; TGF-β1, transforming growth factor β1; TNF-α, tumor necrosis factor α; XF/SF, xeno-free/serum-free.

lower in reactions containing only PBMCs when compared with direct cocultures. However, there was no significant difference between direct cocultures and reactions containing PBMCs alone for the secretion of IDO and galectin-3. The secretion of cytokines IL-2, IL-4, IL-10, or IL-17A was not detected in cocultures or in reactions containing only ASCs or PBMCS in any of the studied culture conditions.

Indirect Cocultures

Compared with direct cultures, the secretion of IL-6 was significantly decreased ($p < .001$) in indirect cocultures when culture conditions were not assessed separately in the regression model. By contrast, the secretion of IFN-γ ($p < .05$) and TNF-α ($p < .001$) was significantly increased in the indirect coculture, when compared with the direct cultures. There was no significant difference in TGF-β1 secretion between direct and indirect cocultures. However, statistically significant differences in TGF-β1 secretion were also seen between different culture conditions in indirect cocultures. Similarly to direct cocultures, cultures containing ASCs expanded in XF/SF conditions had the lowest TGF-β1 concentrations ($p < .05$) compared with either FBS or HS conditions. There was no significant difference between FBS and HS conditions.

The chemokine CCL2 secretion was significantly lower ($p < .001$) in each culture condition when separating membranes were used. Chemokine CCL5 secretion was altered depending on the culture conditions. The secretion was decreased in indirect cultures in XF/SF and FBS conditions, whereas higher concentrations were measured in the HS medium in the indirect culture. Significantly lower secretion ($p < .001$) of chemokines CXCLX9 and -10 was measured in indirect cultures compared with the strong secretion that was measured in direct cocultures. Similarly to direct cultures, the secretion of chemokine CXCL10 was significantly higher in indirect cocultures with XF/SF-expanded ASCs ($p < .05$) compared with HS conditions. The chemokine CXCL8 was also strongly secreted in indirect cocultures with ASCs expanded in all of the studied conditions. Significantly higher secretion of CXCL8 was measured in XF/SF conditions ($p < .05$) compared with the HS medium using the indirect coculture.

Statistically significant differences in the secretion of IDO were also observed between different culture conditions in indirect cocultures. Cultures containing ASCs expanded in FBS conditions had the lowest IDO concentrations compared with either HS or XF/SF ($p < .05$) conditions. There was no significant difference between HS and XF/SF conditions.

Compared with direct cultures, the concentration of galectin-3 was significantly higher ($p < .001$) in indirect cocultures when culture conditions were not assessed separately in the regression model. By contrast, galectin-1 concentration was significantly lower ($p < .001$) in the indirect coculture compared with direct cultures. The concentration of galectin-1 was highest in

cocultures containing ASCs expanded in HS conditions, but no statistically significant differences were observed between different culture conditions for galectin-1 or for galectin-3.

Flow Cytometric Analyses

The cell surface marker expression of ASCs was analyzed by flow cytometry to compare the expression profile of cells expanded in different culture conditions, in FBS, HS, and XF/SF media. In general, the characteristic immunophenotype of ASCs was maintained in every condition, with some minor differences observed between XF/SF conditions and the serum-containing media, and in the expression of specific markers between passages 2 and 5. Flow cytometric results were very similar to our previously published results [30].

The largest variation between different culture conditions was seen in the expression of lymphocyte common antigen CD45 and the immune-related marker CD54 (Fig. 3). On average, ASCs lacked the expression (<2%) of CD45 in all conditions, except the average low expression (2% to ≤10%) in XF/SF conditions in both passages and in FBS conditions in passage 5. A significantly higher expression of CD45 was observed in XF/SF conditions ($p < .05$) compared with HS and FBS conditions in passage 2. Furthermore, the average moderate expression (10% to ≤25%) of CD54 was observed in FBS and HS conditions, whereas in XF/SF conditions, the cells lacked the expression in passage 2 and showed low expression in passage 5. A statistically significant difference between FBS and XF/SF conditions ($p < .05$) was observed in passage 2 for the expression of CD54.

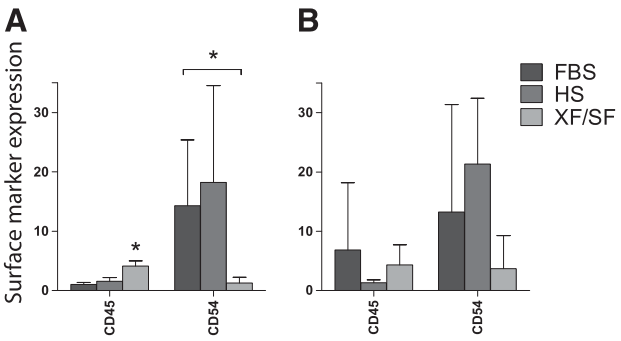

Figure 3. Surface-marker expressions of adipose stem cells. The expression of lymphocyte common antigen CD45 and immune-related marker CD54 on cells expanded in three different culture conditions (FBS, HS, and XF/SF) was investigated in passages 2 **(A)** and 5 **(B)**. A significantly higher expression of CD45 was observed in XF/SF conditions ($p < .05$) compared with HS and FBS conditions in passage 2. By contrast, significantly lower expression of CD54 was observed in XF/SF conditions ($p < .05$) compared with FBS conditions in passage 2. The data in the diagrams are presented as means ± SD; *, $p < .05$. Abbreviations: FBS, fetal bovine serum; HS, human serum; XF/SF, xeno-free/serum-free.

Discussion

Previous studies have demonstrated that MSCs are not strongly immunogenic and that they have an immunosuppressive capacity against T-cell functions and cytokine secretion [4, 7, 12]. The immunomodulatory properties of MSCs have been described both in vitro and in vivo; however, markedly more studies have been performed with BMSCs than with ASCs [17, 41–44]. In our previous study, we showed that culture conditions affect the proliferation rate and the differentiation potential of the cells [30]. In the current study, we demonstrate that different cell expansion conditions also affect the immunogenicity, immunosuppressive capacity, and immunogenicity-related protein secretion of ASCs. To our knowledge, this is a novel approach, and the effect of culture media on cell immunogenicity has not previously been reported.

Traditionally, the ASC culture medium has been supplemented with FBS, which is not a preferred option for clinical cell therapies because of safety concerns [35, 36]. Different alternatives to FBS have therefore been studied, such as human albumin [45], platelet-rich plasma [46], autologous HS [47], and a completely defined XF/SF culture medium [30, 31, 33, 48].

Our results show that ASCs elicit only a low immunogenic response to PBMCs in a one-way MLR, whether cultured in FBS, HS, or XF/SF conditions. This result is consistent with earlier studies performed with cells expanded in FBS medium [3, 9, 49]. However, statistically significant differences were observed between different culture conditions, and cells cultured in FBS medium proved the least immunogenic. It can be speculated that the reason for the low immunogenicity in the FBS medium is that FBS is harvested from the blood of bovine fetuses whose immune system is not fully mature at the point of harvest [50], and therefore immunomodulatory factors in the FBS medium may differ from the HS-supplemented medium. Nevertheless, a significantly lower activation of PBMCs was measured in MLR cultures with ASCs expanded in any of the studied conditions, when compared with a positive control response measured in PBMCs activated with PHA mitogen. This result suggests that ASCs elicit only a very low immunogenic response toward allogeneic PBMCs, and the different culture conditions do not affect the result substantially. Because of the low immunogenicity of ASCs, allogeneic stem cell treatments may be conceivable in the future, although in vivo studies are still required to systemically confirm the in vitro results shown in this study.

In addition to low immunogenicity, our present study confirms that ASCs have the potential for immunosuppression, as has been demonstrated previously [3, 4, 7, 8]. Our results revealed that the suppressive potential was affected by the culture condition of ASCs and that the suppression functioned in a dose-dependent manner. Two different MLR combinations were used in a two-way MLR assay, and these combinations were not identically reactive. This biological variation in the intensity of each MLR can be seen as the larger standard deviation after the coculture with ASCs. However, the strongest suppression was seen with ASCs cultured in FBS conditions, whereas ASCs cultured in HS or XF/SF conditions suppressed PBMC proliferation only with higher cell numbers. It was somewhat surprising that FBS-expanded ASCs elicited the strongest suppression on PBMC proliferation, but there are previous published studies that may explain this result. Certain adhesion molecules, such as ICAM-1 (CD54) and VCAM-1 (CD106), have been shown to play a key role in mesenchymal stem cell-mediated immunosuppression [4, 28, 51]. CD54 is reported to be present in endothelial cells, antigen-presenting cells, and some stromal cells. In our present study, a significantly higher expression of CD54 was observed on FBS-expanded ASCs, compared with cells cultured in XF/SF conditions, as detected by the flow cytometric analyses. In our previous published studies [24, 30] we have seen similar results when ASCs cultured in FBS- or HS-containing media have shown a significantly

higher expression of CD54 compared with XF/SF conditions. These findings are consistent with previous studies recognizing the importance of CD54 and CD106 in MSC-mediated immunosuppression. As demonstrated by Ren et al. [28, 51], CD54 and CD106 are required for lymphocyte-BMSC adhesion, and with the help of chemokines they induce immunosuppression mediated by BMSCs. Moreover, when the functions of these adhesion molecules were inhibited by gene knockout, BMSC-mediated suppression was significantly reversed, as shown by an in vivo study [52]. In contrast to BMSCs, ASCs have been shown to be negative for CD106 [31, 53], and thus it is likely that immunosuppression of ASC is CD54- but not CD106-mediated.

Previous studies have suggested that MSC-mediated immunosuppression would not be stable but rather induced by the inflammatory environment and cytokines, such as IFN-γ, TNF-α, and IL-6 [29]. Production of these cytokines is greatly increased under inflammatory conditions [24], and MSCs may respond to this increase by changing their immunomodulatory functions. IFN-γ has been shown to induce expression of MHC-I and to a lesser extent also MHC-II, increasing the antigen-presenting capacity and hence immunogenicity of MSCs [54]. Low levels of IFN-γ may induce MSCs to express MHC-II as antigen-presenting cells, whereas high levels can mediate a decreased expression of MHC-II [42]. These studies indicate the importance of local inflammatory conditions in the regulation of MSC plasticity. In our present study, we observed that the production of IFN-γ, TNF-α, and IL-6 was increased after coculture in a two-way MLR, as compared with monocultures of PBMCs and ASCs. IFN-γ and TNF-α levels were higher in the indirect culture compared with the direct coculture of ASCs and PBMCs. The result suggests that direct contact between ASCs and PBMCs inhibits the secretion of IFN-γ and TNF-α. Wu et al. [55] reported a similar phenomenon of reduced IFN-γ and TNF-α secretion of $CD4^+$T cells after coculture with MSCs. In their study, T cells were activated for higher IFN-γ and TNF-α secretion by PHA/IL-2 exposure, and reduced secretion of these factors was observed after coculture with MSCs. It can be speculated that similarly to PHA/IL-2, the indirect contact between PBMCs and ASCs changes the local inflammatory environment and activates PBMCs for higher secretion, as demonstrated by Crop et al. [24]. However, direct contact will reduce that activation.

In contrast to IFN-γ and TNF-α, direct contact between ASCs and PBMCs was required for higher production of IL-6, whereas lower levels of IL-6 were detected when the cells were separated by a membrane. Melief et al. [56] have shown that through production of IL-6, MSCs prevent monocyte differentiation toward antigen-presenting immunogenic cells and drive differentiation toward an anti-inflammatory IL-10-producing cell type. IL-6 can act to either support or suppress the inflammation depending on the context [57], and the anti-inflammatory effect is mediated through inhibition of TNF-α [58]. In our study, IL-6 secretion was significantly higher in FBS-expanded ASCs compared with XF/SF-expanded cells, which is in line with the stronger suppression potential observed in FBS conditions, as compared with XF/SF conditions. In addition, the secretion of TNF-α was low in FBS conditions compared with the higher secretion observed in XF/SF conditions. These results support the hypothesis proposed by Melief et al. [56], which suggests a connection between IL-6 and the anti-inflammatory environment.

Furthermore, signaling protein TGF-β1 has multiple functions in controlling cell growth, proliferation, differentiation, and apoptosis, and in addition, it is an important factor in maintaining immune tolerance [59]. Proliferation and activation of T cells can be diminished by the effect of TGF-β1 [60, 61]; similarly, it can downregulate many cytokines, including IFN-γ and TNFα [62]. The medium used in the MLRs contained a relatively large amount of TGF-β1, which may have obscured some of our results. Although there was a difference between reactions containing FBS- and HS-expanded ASCs, this was not significant. However, the significantly lower expression of TGF-β1 in cocultures containing XF/SF-expanded ASCs is in line with the proliferation assay results, and higher IFN-γ and TNFα and lower IL-6 concentrations that were measured in cocultures containing XF/SF-expanded ASCs.

Inflammatory mediators also induce the expression of CXCLX chemokines, and, for example, chemokine CXCL10 is secreted in response to higher IFN-γ levels. Accordingly, the significantly higher secretion of IFN-γ that we observed in XF/SF conditions induced significantly higher CXCL10 secretion in XF/SF-expanded ASCs compared with FBS and HS conditions. As we showed in this study, ASCs expanded in XF/SF conditions elicited a stronger immunogenic response in a one-way MLR compared with FBS conditions and proved to be less immunosuppressive in a two-way MLR compared with FBS and HS conditions. Thus, there may be a link between higher IFN-γ and CXCL10 levels in XF/SF conditions and the weaker suppression potential that was measured by the two-way MLR in XF/SF conditions, as compared with the HS and FBS media.

On the other hand, chemokines are needed to promote T-cell chemotaxis, and once T cells have made contact with MSCs, the function of T cells may be blocked by the combined action of soluble factors and direct cell-cell contact [20]. The expression of several chemokines has been shown to increase when ASCs are cultured in MLRs. Crop et al. [24] have demonstrated a strong increase in the expression of T-cell attractants; chemokine ligands CXCL9 and -10, as well as ligands with CC-motives CCL2 and -5, increased after MLR culture when compared with the control cultures of ASCs. In our studies, the secretion of chemokine ligands CXCL9 and -10, as well as CCL2, was increased after coculture in MLR, and significantly stronger secretion was observed in direct cocultures. This result suggests that direct contact between ASCs and PBMCs stimulates cells for stronger chemokine secretion to strengthen T-cell recruitment. Thus, the final determination of inflammatory responses may be elicited through a combined action of cell-cell contacts and soluble factors.

The secretion of CCL2 and CCL5 was significantly lower in HS conditions compared with FBS conditions (CCL2) and when compared with FBS and XF/SF conditions (CCL5). In addition, significantly lower secretion of CXCL8 was observed in the HS medium when compared with XF/SF conditions. Chemokines CCL2 and CCL5 have been shown to be involved in MSC chemotaxis and cell migration to the sites of injury or inflammation [41]. The increase in the production of chemokines such as CXCL8, CCL2, and CCL5 may also induce extracellular matrix degradation. These chemotactic factors are produced by immune cells, and the phenomenon is facilitated by the increased migration of human MSCs through the extracellular matrix. Based on our results, it is still difficult to conclude how the lower levels of CCL2 and CCL5 that we observed in HS conditions may affect the immunogenicity of ASCs. The effect of CCL2 and CCL5 on cell homing to inflammation sites could probably be better demonstrated in vivo.

Similarly to TGF-β1, the reaction medium contained a relatively large amount of IDO. Our results still show a significantly higher secretion of IDO in cocultures compared with reactions containing ASCs only. In reactions in which ASCs were cultured alone,

the measured IDO was lower than in the medium controls, which suggests that ASCs do not readily express IDO without activation [63]. When considering the potent immunosuppressive effects of IDO, our results are somewhat opposed to the two-way MLR assay results. The lowest levels of IDO were in the cocultures containing ASCs expanded in the FBS medium, although the stronger suppression potential was also observed in FBS conditions. This might be due to the high initial concentration of IDO or due to the fact that complete secretion of IDO would have required a longer coculture, and thus other factors have a stronger effect than IDO in the reactions performed here [64].

Galectin-1 and -3 were included in the secreted protein panel because of their reported importance in regulating immune reactions and reported function as mediators of immune suppression caused by MSCs [26, 65]. Galectin-1 is clearly linked to immunosuppression, and galectin-3 also has T-cell proliferative functions in addition to suppressive functions [66]. Thus, it is not surprising that our results on galectin-1 and -3 are partially opposing. Galectin-1 was low in the reactions containing only PBMCs, and although ASCs alone expressed some galectin-1, the expression was higher in direct and indirect cocultures. Galectin-1 induces apoptosis in T cells and suppresses immune responses, which is in line with the suppression of proliferation and elevated galectin-1 concentrations in the cocultures. Similar to galectin-1, galectin-3 was upregulated in cocultures and compared with reactions with ASCs alone; there were no significant differences between the cocultures containing ASCs expanded in different conditions. Unlike with galectin-1, the galectin-3 concentration was relatively high in the reactions with PBMCs only, and it was also higher in indirect cocultures than in direct cocultures. The higher concentrations of galectin-3 that were measured in cocultures are the opposite of the proliferation assay results, but might well be due to the relatively high expression seen already in reactions with PBMCs only and the very low expression in reactions containing only ASCs. These results might also be partially explained by the differences in overall expression patterns and functions between the two galectins [67], but according to our results, galectin-3 expression is not as good an indicator of immune suppression as galectin-1: galectin-1 concentrations correlated well with the proliferation assay results. Figure 4 summarizes the key signaling proteins investigated in the present study. The increase or decrease in protein secretion is presented in the direct coculture compared with the indirect culture and vice versa.

Furthermore, flow cytometric analyses revealed that the cell surface marker expression profile of ASCs was largely similar in cells grown under different culture conditions. However, statistically significant differences were still found for the expression of CD45, which was more expressed in XF/SF conditions in passage 2. CD45 is a receptor-linked protein-tyrosine phosphatase that is expressed on all leukocytes playing a crucial role in the function of these cells, and the expression has been shown to be essential for differentiation and antigen receptor-mediated signal transduction in leukocytes [68, 69]. XF/SF-expanded ASCs showed a higher expression of CD45, and in line with higher expression, they also proved to be less immunosuppressive in XF/SF conditions in passage 2. However, because of large patient variation, no significant differences were seen in passage 5, and more studies are needed to draw a conclusion and show a link between immunosuppression capacity and the expression of CD45 in ASCs.

The suppression capacity of MSCs holds promise for the future treatments of severe immunological disorders, such as GVHD. Several recognized clinical studies have thus been performed to investigate the potential of BMSCs for the treatment of GVHD [15, 70–73]. In addition, both preclinical and clinical studies have been performed to evaluate the use of ASCs to prevent acute GVHD, as reviewed by Leto Barone et al. [74]. Nonetheless, if ASCs are used for patients for immunomodulatory purposes, it is important to carefully assess the risk for the possibility of tumor formation connected with ASC-mediated suppression. There is evidence that tumors are potential sites for inflammatory cytokine and chemokine production, which may enable MSCs to home in on tumor sites and as a result immunomodulate the tumor environment [75, 76]. Furthermore, if MSCs may play a role in tumor growth and metastasis as has been stated [77, 78], there is a potential risk in the use of MSCs in cell-based therapies. Compte et al. have shown that tumor angiogenesis in particular may be enhanced by MSCs, which is critical considering tumor development [79]. Taking these previous results into account, the possible supportive effect on the pathogenesis and progression of tumors should be clarified carefully before further clinical applications of immunomodulation.

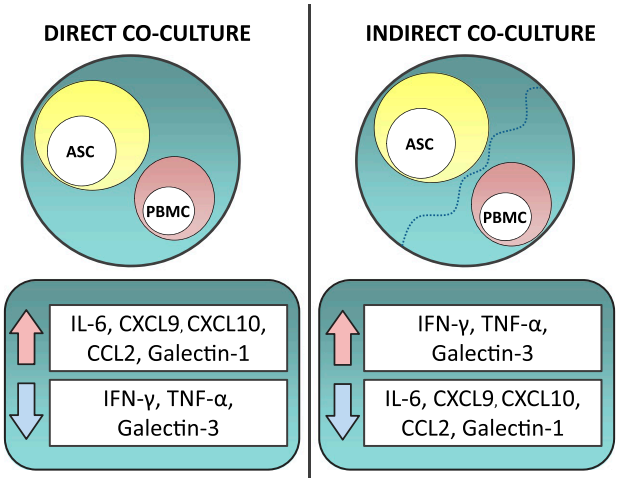

Figure 4. Schematic overview highlighting the key signaling proteins of our study. The increase or decrease in protein secretion is presented in direct coculture compared with indirect culture. All changes in secretion between direct versus indirect cocultures were statistically significant ($p < .05$). Abbreviations: ASC, adipose stem cell; CCL2, C-C chemokine ligand 2; CXCL9 and CXCL10, CXC chemokine ligands 9 and 10; IFN-γ, interferon γ; IL-6, interleukin 6; PBMC, peripheral blood mononuclear cell; TNF-α, tumor necrosis factor α.

CONCLUSION

Our findings demonstrated that the isolation and expansion conditions of ASCs have an effect on immunogenicity, suppressive potential, and protein secretion profile of the cells. Nevertheless, ASCs elicit only a low immunogenic response whether cultured in FBS, HS, or in XF/SF conditions, and suppressive potential is detectable in every condition with high ASC numbers.

The strongest immunosuppression and lowest immunogenicity was observed with ASCs expanded in the FBS-supplemented medium. High IL-6 and low IFN-γ secretion was observed in FBS conditions, which may have a link to stronger suppressive potential. By contrast, ASCs expanded in XF/SF conditions induced the strongest proliferative response in PBMCs and showed less immunosuppressive potential, that was in line with low IL-6

and TGF-β1 and high IFN-γ and TNF-α secretion in XF/SF conditions. However, the differences between culture conditions were minor, especially for immunogenicity. In addition to low immunogenicity, the safety and efficacy of allogeneic ASC treatments should be evaluated based on the proliferation and differentiation potential of the cells in these different conditions. Therefore, considering allogeneic stem cell treatments, FBS may not be the optimal culture condition for clinical use. For immunosuppressive purposes, FBS-expanded cells may have potential, but there are several factors affecting the immunosuppression process. The mechanism behind the suppressive capacity involves direct cell-cell contact, secretion of soluble factors, and the modulation inflammatory environment. Moreover, by increasing the ASC number, suppression can also be achieved with HS- or XF/SF-expanded cells. In conclusion, ASCs have potential for immunomodulation therapies and allogeneic stem cell treatments in the future. Still, the effect of the culture medium on cell characteristics should not be disregarded when clinical applications are considered.

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Author Contributions

M.P.: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript; J.S.: conception and design, collection and/or assembly of data, data analysis and interpretation, final approval of manuscript; H.H. and M.H.: data analysis and interpretation, final approval of manuscript; F.S.: collection and/or assembly of data, final approval of manuscript; B.M. and S.M.: conception and design, administrative support, data analysis and interpretation, final approval of manuscript.

Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

References

1 Zuk PA, Zhu M, Ashjian P et al. Human adipose tissue is a source of multipotent stem cells. Mol Biol Cell 2002;13:4279–4295.
2 Gimble J, Guilak F. Adipose-derived adult stem cells: Isolation, characterization, and differentiation potential. Cytotherapy 2003;5:362–369.
3 McIntosh K, Zvonic S, Garrett S et al. The immunogenicity of human adipose-derived cells: Temporal changes in vitro. Stem Cells 2006;24:1246–1253.
4 Kronsteiner B, Wolbank S, Peterbauer A et al. Human mesenchymal stem cells from adipose tissue and amnion influence T-cells depending on stimulation method and presence of other immune cells. Stem Cells Dev 2011;20:2115–2126.
5 Casteilla L, Planat-Benard V, Laharrague P et al. Adipose-derived stromal cells: Their identity and uses in clinical trials, an update. World J Stem Cells 2011;3:25–33.
6 Crop MJ, Baan CC, Korevaar SS et al. Human adipose tissue-derived mesenchymal stem cells induce explosive T-cell proliferation. Stem Cells Dev 2010;19:1843–1853.
7 Lee JM, Jung J, Lee HJ et al. Comparison of immunomodulatory effects of placenta mesenchymal stem cells with bone marrow and adipose mesenchymal stem cells. Int Immunopharmacol 2012;13:219–224.
8 Kuo YR, Chen CC, Goto S et al. Modulation of immune response and T-cell regulation by donor adipose-derived stem cells in a rodent hind-limb allotransplant model. Plast Reconstr Surg 2011;128:661e–672e.
9 Niemeyer P, Kornacker M, Mehlhorn A et al. Comparison of immunological properties of bone marrow stromal cells and adipose tissue-derived stem cells before and after osteogenic differentiation in vitro. Tissue Eng 2007;13:111–121.
10 Eliopoulos N, Stagg J, Lejeune L et al. Allogeneic marrow stromal cells are immune rejected by MHC class I- and class II-mismatched recipient mice. Blood 2005;106:4057–4065.
11 Puissant B, Barreau C, Bourin P et al. Immunomodulatory effect of human adipose tissue-derived adult stem cells: Comparison with bone marrow mesenchymal stem cells. Br J Haematol 2005;129:118–129.
12 Yoo KH, Jang IK, Lee MW et al. Comparison of immunomodulatory properties of mesenchymal stem cells derived from adult human tissues. Cell Immunol 2009;259:150–156.
13 McIntosh KR, Lopez MJ, Borneman JN et al. Immunogenicity of allogeneic adipose-derived stem cells in a rat spinal fusion model. Tissue Eng Part A 2009;15:2677–2686.
14 Niemeyer P, Vohrer J, Schmal H et al. Survival of human mesenchymal stromal cells from bone marrow and adipose tissue after xenogenic transplantation in immunocompetent mice. Cytotherapy 2008;10:784–795.
15 Le Blanc K, Frassoni F, Ball L et al. Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: A phase II study. Lancet 2008;371:1579–1586.
16 Ringdén O, Uzunel M, Rasmusson I et al. Mesenchymal stem cells for treatment of therapy-resistant graft-versus-host disease. Transplantation 2006;81:1390–1397.
17 Resnick IB, Barkats C, Shapira MY et al. Treatment of severe steroid resistant acute GVHD with mesenchymal stromal cells (MSC). Am J Blood Res 2013;3:225–238.
18 Kim N, Im KI, Lim JY et al. Mesenchymal stem cells for the treatment and prevention of graft-versus-host disease: Experiments and practice. Ann Hematol 2013;92:1295–1308.
19 Waterman RS, Tomchuck SL, Henkle SL et al. A new mesenchymal stem cell (MSC) paradigm: Polarization into a pro-inflammatory MSC1 or an Immunosuppressive MSC2 phenotype. PLoS One 2010;5:e10088.
20 Ren G, Zhang L, Zhao X et al. Mesenchymal stem cell-mediated immunosuppression occurs via concerted action of chemokines and nitric oxide. Cell Stem Cell 2008;2:141–150.
21 Krampera M, Cosmi L, Angeli R et al. Role for interferon-gamma in the immunomodulatory activity of human bone marrow mesenchymal stem cells. Stem Cells 2006;24:386–398.
22 Melief SM, Zwaginga JJ, Fibbe WE et al. Adipose tissue-derived multipotent stromal cells have a higher immunomodulatory capacity than their bone marrow-derived counterparts. Stem Cells Translational Medicine 2013;2:455–463.
23 Cui L, Yin S, Liu W et al. Expanded adipose-derived stem cells suppress mixed lymphocyte reaction by secretion of prostaglandin E2. Tissue Eng 2007;13:1185–1195.
24 Crop MJ, Baan CC, Korevaar SS et al. Inflammatory conditions affect gene expression and function of human adipose tissue-derived mesenchymal stem cells. Clin Exp Immunol 2010;162:474–486.
25 Burr SP, Dazzi F, Garden OA. Mesenchymal stromal cells and regulatory T cells: The Yin and Yang of peripheral tolerance? Immunol Cell Biol 2013;91:12–18.
26 Sioud M. New insights into mesenchymal stromal cell-mediated T-cell suppression through galectins. Scand J Immunol 2011;73:79–84.
27 Sioud M, Mobergslien A, Boudabous A et al. Mesenchymal stem cell-mediated T cell suppression occurs through secreted galectins. Int J Oncol 2011;38:385–390.
28 Ren G, Zhao X, Zhang L et al. Inflammatory cytokine-induced intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 in mesenchymal stem cells are critical for immunosuppression. J Immunol 2010;184:2321–2328.
29 Shi Y, Hu G, Su J et al. Mesenchymal stem cells: A new strategy for immunosuppression and tissue repair. Cell Res 2010;20:510–518.
30 Patrikoski M, Juntunen M, Boucher S et al. Development of fully defined xeno-free culture system for the preparation and propagation of cell therapy-compliant human adipose stem cells. Stem Cell Res Ther 2013;4:27.

31 Lindroos B, Boucher S, Chase L et al. Serum-free, xeno-free culture media maintain the proliferation rate and multipotentiality of adipose stem cells in vitro. Cytotherapy 2009; 11:958–972.

32 Chase LG, Yang S, Zachar V et al. Development and characterization of a clinically compliant xeno-free culture medium in good manufacturing practice for human multipotent mesenchymal stem cells. STEM CELLS TRANSLATIONAL MEDICINE 2012;1:750–758.

33 Santos F, Andrade PZ, Abecasis MM et al. Toward a clinical-grade expansion of mesenchymal stem cells from human sources: A microcarrier-based culture system under xeno-free conditions. Tissue Eng Part C Methods 2011;17:1201–1210.

34 Rajala K, Lindroos B, Hussein SM et al. A defined and xeno-free culture method enabling the establishment of clinical-grade human embryonic, induced pluripotent and adipose stem cells. PLoS One 2010;5:e10246.

35 Mackensen A, Dräger R, Schlesier M et al. Presence of IgE antibodies to bovine serum albumin in a patient developing anaphylaxis after vaccination with human peptide-pulsed dendritic cells. Cancer Immunol Immunother 2000; 49:152–156.

36 Selvaggi TA, Walker RE, Fleisher TA. Development of antibodies to fetal calf serum with arthus-like reactions in human immunodeficiency virus-infected patients given syngeneic lymphocyte infusions. Blood 1997;89:776–779.

37 Spees JL, Gregory CA, Singh H et al. Internalized antigens must be removed to prepare hypoimmunogenic mesenchymal stem cells for cell and gene therapy. Mol Ther 2004;9:747–756.

38 Heiskanen A, Satomaa T, Tiitinen S et al. *N*-Glycolylneuraminic acid xenoantigen contamination of human embryonic and mesenchymal stem cells is substantially reversible. STEM CELLS 2007;25:197–202.

39 Haimi S, Moimas L, Pirhonen E et al. Calcium phosphate surface treatment of bioactive glass causes a delay in early osteogenic differentiation of adipose stem cells. J Biomed Mater Res A 2009;91:540–547.

40 McIntosh KR. Evaluation of cellular and humoral immune responses to allogeneic adipose-derived stem/stromal cells. Methods Mol Biol 2011;702:133–150.

41 Anton K, Banerjee D, Glod J. Macrophage-associated mesenchymal stem cells assume an activated, migratory, pro-inflammatory phenotype with increased IL-6 and CXCL10 secretion. PLoS One 2012;7:e35036.

42 Chan JL, Tang KC, Patel AP et al. Antigen-presenting property of mesenchymal stem cells occurs during a narrow window at low levels of interferon-gamma. Blood 2006;107:4817–4824.

43 Chen PM, Yen ML, Liu KJ et al. Immunomodulatory properties of human adult and fetal multipotent mesenchymal stem cells. J Biomed Sci 2011;18:49.

44 Jacobs SA, Roobrouck VD, Verfaillie CM et al. Immunological characteristics of human mesenchymal stem cells and multipotent adult progenitor cells. Immunol Cell Biol 2013;91:32–39.

45 Trivedi HL, Vanikar AV, Thakker U et al. Human adipose tissue-derived mesenchymal stem cells combined with hematopoietic stem cell transplantation synthesize insulin. Transplant Proc 2008;40:1135–1139.

46 Tzouvelekis A, Koliakos G, Ntolios P et al. Stem cell therapy for idiopathic pulmonary fibrosis: A protocol proposal. J Transl Med 2011;9:182.

47 Thesleff T, Lehtimäki K, Niskakangas T et al. Cranioplasty with adipose-derived stem cells and biomaterial: A novel method for cranial reconstruction. Neurosurgery 2011;68:1535–1540.

48 Dromard C, Bourin P, André M et al. Human adipose derived stroma/stem cells grow in serum-free medium as floating spheres. Exp Cell Res 2011;317:770–780.

49 Ren ML, Peng W, Yang ZL et al. Allogeneic adipose-derived stem cells with low immunogenicity constructing tissue-engineered bone for repairing bone defects in pigs. Cell Transplant 2012;21:2711–2721.

50 Sharma AA, Jen R, Butler A et al. The developing human preterm neonatal immune system: A case for more research in this area. Clin Immunol 2012;145:61–68.

51 Ren G, Roberts AI, Shi Y. Adhesion molecules: Key players in mesenchymal stem cell-mediated immunosuppression. Cell Adhes Migr 2011;5:20–22.

52 Shimada Y, Hasegawa M, Kaburagi Y et al. L-selectin or ICAM-1 deficiency reduces an immediate-type hypersensitivity response by preventing mast cell recruitment in repeated elicitation of contact hypersensitivity. J Immunol 2003;170:4325–4334.

53 De Ugarte DA, Alfonso Z, Zuk PA et al. Differential expression of stem cell mobilization-associated molecules on multi-lineage cells from adipose tissue and bone marrow. Immunol Lett 2003;89:267–270.

54 Chan WK, Lau AS, Li JC et al. MHC expression kinetics and immunogenicity of mesenchymal stromal cells after short-term IFN-gamma challenge. Exp Hematol 2008;36:1545–1555.

55 Wu M, Han ZB, Liu JF et al. Serum-free media and the immunoregulatory properties of mesenchymal stem cells in vivo and in vitro. Cell Physiol Biochem 2014;33:569–580.

56 Melief SM, Geutskens SB, Fibbe WE et al. Multipotent stromal cells skew monocytes towards an anti-inflammatory interleukin-10-producing phenotype by production of interleukin-6. Haematologica 2013;98:888–895.

57 Scheller J, Chalaris A, Schmidt-Arras D et al. The pro- and anti-inflammatory properties of the cytokine interleukin-6. Biochim Biophys Acta 2011;1813:878–888.

58 Ulich TR, Yin S, Guo K et al. Intratracheal injection of endotoxin and cytokines: II. Interleukin-6 and transforming growth factor beta inhibit acute inflammation. Am J Pathol 1991;138:1097–1101.

59 Wahl SM, Wen J, Moutsopoulos N. TGF-beta: A mobile purveyor of immune privilege. Immunol Rev 2006;213:213–227.

60 Tiemessen MM, Kunzmann S, Schmidt-Weber CB et al. Transforming growth factor-beta inhibits human antigen-specific CD4+ T cell proliferation without modulating the cytokine response. Int Immunol 2003;15:1495–1504.

61 Gilbert KM, Thoman M, Bauche K et al. Transforming growth factor-beta 1 induces antigen-specific unresponsiveness in naive T cells. Immunol Invest 1997;26:459–472.

62 Letterio JJ, Roberts AB. Regulation of immune responses by TGF-beta. Annu Rev Immunol 1998;16:137–161.

63 Meisel R, Zibert A, Laryea M et al. Human bone marrow stromal cells inhibit allogeneic T-cell responses by indoleamine 2,3-dioxygenase-mediated tryptophan degradation. Blood 2004; 103:4619–4621.

64 Aggarwal S, Pittenger MF. Human mesenchymal stem cells modulate allogeneic immune cell responses. Blood 2005;105:1815–1822.

65 Gieseke F, Böhringer J, Bussolari R et al. Human multipotent mesenchymal stromal cells use galectin-1 to inhibit immune effector cells. Blood 2010;116:3770–3779.

66 Hernandez JD, Baum LG. Ah, sweet mystery of death! Galectins and control of cell fate. Glycobiology 2002;12:127R–136R.

67 Perillo NL, Marcus ME, Baum LG. Galectins: Versatile modulators of cell adhesion, cell proliferation, and cell death. J Mol Med (Berl) 1998 76:402–412 [BERL].

68 Altin JG, Sloan EK. The role of CD45 and CD45-associated molecules in T cell activation. Immunol Cell Biol 1997;75:430–445.

69 Matsuda A, Motoya S, Kimura S et al. Disruption of lymphocyte function and signaling in CD45-associated protein-null mice. J Exp Med 1998;187:1863–1870.

70 Lazarus HM, Koc ON, Devine SM et al. Cotransplantation of HLA-identical sibling culture-expanded mesenchymal stem cells and hematopoietic stem cells in hematologic malignancy patients. Biol Blood Marrow Transplant 2005;11:389–398.

71 Le Blanc K, Rasmusson I, Sundberg B et al. Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells. Lancet 2004;363:1439–1441.

72 Papewalis C, Topolar D, Gotz B et al. Mesenchymal stem cells as cellular immunotherapeutics in allogeneic hematopoietic stem cell transplantation. Adv Biochem Eng Biotechnol 2013;130:131–162.

73 Vaes B, Van't Hof W, Deans R et al. Application of MultiStem(®) allogeneic cells for immunomodulatory therapy: Clinical progress and pre-clinical challenges in prophylaxis for graft versus host disease. Front Immunol 2012;3:345.

74 Leto Barone AA, Khalifian S, Lee WP et al. Immunomodulatory effects of adipose-derived stem cells: Fact or fiction? Biomed Res Int 2013; 2013:383685.

75 Zimmerlin L, Park TS, Zambidis ET et al. Mesenchymal stem cell secretome and regenerative therapy after cancer. Biochimie 2013; 95:2235–2245.

76 Hall B, Dembinski J, Sasser AK et al. Mesenchymal stem cells in cancer: Tumor-associated fibroblasts and cell-based delivery vehicles. Int J Hematol 2007;86:8–16.

77 Karnoub AE, Dash AB, Vo AP et al. Mesenchymal stem cells within tumour stroma promote breast cancer metastasis. Nature 2007; 449:557–563.

78 Djouad F, Plence P, Bony C et al. Immunosuppressive effect of mesenchymal stem cells favors tumor growth in allogeneic animals. Blood 2003;102:3837–3844.

79 Compte M, Cuesta AM, Sánchez-Martín D et al. Tumor immunotherapy using gene-modified human mesenchymal stem cells loaded into synthetic extracellular matrix scaffolds. STEM CELLS 2009;27:753–760.

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