Mesenchymal stem cells promote macrophage polarization toward M2b-like cells

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Abstract

Mesenchymal stem or stromal cells (MSCs) act on different components of the immune response including macrophages (MΦs). Therefore this study has been committed to explore how MSCs may modify the effect of MΦ polarization upon an inductive environment using mouse bone marrow (BM)-derived "naïve", unpolarized MΦs. Phagocytosis of various MΦ subtypes was different since M1 and M2b showed poorer, while M2a higher rate of phagocytosis. MSCs significantly promoted yeast ingestion by M1 and M2b and diminished it by M2a cells. Under polarizing conditions, MSCs profoundly affected the TNFα production of MΦ subtypes since M1 and M2b MΦs produced less and M2a produced higher amount of TNFα while the amount of IL-10 was not affected. The most striking effect of MSCs was registered on M2b cells since the inflammatory TNFα dominance remarkably shifted to the immunosuppressive IL-10. Prepolarized M1 cells readily converted to M2a and M2b states when polarizing conditions changed from M1 to M2a or M2b induction, respectively. Repolarizing from M1 to M2a resulted in the decline of IL-10 and TNFα and defined elevation of Ym1 similar to levels characteristic to M2a primarily polarized from naïve BM-MΦs. Similarly, polarization of M1 to M2b MΦs was successful showing increase in IL-10 and reduction in TNFα levels characteristic to M2b cells. However, when co-culturing with MSCs, M1–M2a or M1–M2b transition was not affected. Crosstalk between MΦs and MSCs depended on PGE-2 since COX-1 and COX-2 inhibition reduced the effect of MSCs to establish an IL-10-dominant cytokine production by MΦs.

Introduction

Mesenchymal stem or stromal cells (MSCs) are multipotent fibroblast-like cells that were first isolated from the bone marrow [1] and later identified in various tissues of the body such as adipose tissue, heart, liver, spleen or thymus [2, 3]. Their *in vivo* functions are assumed to be providing support for hematopoiesis, contribution to tissue maintenance and regeneration, and modulation of immune responses [4]. Through various soluble and cell surface molecules such as TGFβ, HGF, IL-6, PGE-2, B7-H1 or Jagged-1, they are able to communicate with a wide range of immune cells and usually the net outcome of these interactions is the inhibition of immune effector cells and the promotion of regulatory cell functions [5]. These characteristics make MSCs potent candidates in the treatment of inflammatory and/or autoimmune diseases such as acute graft versus host disease, multiple sclerosis or Crohn's disease [6, 7].

Macrophages (MΦs) are major partakers in inflammatory and regenerative processes and thus in inflammation-related degenerative disorders. As opposed to our former knowledge of MΦs that they generally acquire an inflammatory profile, recently, several other, even suppressive functional phenotypes of these cells have been described after different activation stimuli [8, 9]. "Naïve" M0 MΦs emerge from committed myeloid progenitors in the presence of M-CSF [10] characterized by the expression of CD11b and F4/80, a monocyte/macrophage and a mature MΦ marker, respectively, and readily phagocytose cellular debris or pathogens. They lack the expression of antigen presenting (MHC-II) and co-stimulatory (B7) molecules [10]. Classically activated or "M1" MΦs bear an immune phenotype of MHC-II^{hi}B7^{hi} and have high inducible nitric oxide synthase (iNOS) activity and low chitinase (Ym1) production [11]. iNOS is responsible for the production of nitric oxide, a free radical with a central role in MΦ-mediated cytotoxicity [12]. Thus, M1 MΦs are acting as pro-inflammatory cells by producing large amounts of TNFα, IL-1β and IL-12, performing antigen presentation towards

adaptive immune cells, and orchestrating Th1 function [9]. Other types of MΦs, termed M2, are mostly antagonistic to M1 MΦs in their effect although their characteristics sometimes partially overlap with the pro-inflammatory phenotype [13]. Recently, subdivisions in the M2 activation profile have arisen from different immune phenotypes and functions. Hence, alternatively activated, wound healing or M2a MΦs are described as MHC-II^{lo}B7^{lo} cells with low iNOS and high Ym1 activity promoting wound healing and tissue remodeling [13]. Ym1 is a secreted chitinase-like protein with proposed pathogen-binding function and role in the deposition of extracellular matrix during wound-healing [14]. It serves as a good marker for the distinction of alternatively activated murine M2a MΦs from M1 MΦs as Ym1 is upregulated in the presence of Th2 cytokines serving as inductors of the phenotype [9, 11]. Regulatory or M2b MΦs bear an MHC-II^{hi}B7^{hi} phenotype with increased iNOS activity just like their M1 counterparts, however, they secrete relatively low levels of pro-inflammatory cytokines, such as TNFα and IL-12, compared to their elevated IL-10 production [13]. These characteristics empower M2b MΦs to be able to act as immune regulator cells favoring Th2 activation and function and dampening pro-inflammatory responses [13, 15]. The ratio of IL-10 to TNFα or IL-12 is often used to distinguish M1 from M2 MΦs [16, 17]. Although more activation profiles have been identified for MΦs *in vivo* [18, 19], M1, M2a and M2b are the best described functional states. According to Edwards et al., these activation states can be generated *in vitro* by applying the appropriate inductors: TLR-ligands, such as LPS, together with IFNγ for M1, IL-4 or IL-13 for M2a and LPS together with immune complexes or apoptotic cells for M2b MΦ phenotypes [9, 13]. The helper T cell-derived cytokines as well as LPS and immune complexes are proposed to account for the *in vivo* physiological activation of MΦs into the above mentioned functionally distinct states [19].

Although the interaction between MSCs and T lymphocytes has been extensively studied in the past decades [5], the possible communication between MSCs and MΦs has only been

gaining more attention in the last years [20, 21]. Former studies mostly focused on the interplay between monocytes or tissue-resident, mainly peritoneal MΦs (Pe-MΦs) and MSCs while the true nature of the effect of MSCs has not been profoundly elucidated on "naïve" (M0) MΦs.

Therefore, in this study we have compared the purity, phenotype and response of bone marrow derived-MΦs (BM-MΦs) and Pe-MΦs to the presence of MSCs. BM-MΦs comprised of a mature, naïve, homogenous cell population while, using the standard method for MΦ isolation from the mouse peritoneum based on plastic adherence [22], Pe-MΦs were contaminated with other cells and were largely in active state. Based on the results from these experiments we chose BM-MΦs as *in vitro* model system for the further studies. The appropriate inducing cocktails stimulated the polarization of the M0 MΦs into M1, M2a and M2b states. Polarization of MΦs into different subpopulations was differently affected by MSCs. The presence of MSCs resulted in the reduction of TNFα both in M1 and M2b MΦs and increase in M2a cells while no significant change in IL-10 was observed. However, the TNFα/IL-10 ratio was remarkably shifted to the immunosuppressive IL-10 secretion only in the case of M0/M2b polarization. PGE-2 was identified as an important regulatory factor in this process. Moreover, transition of M1 to M2a or M2b direction under changing stimulatory environment was successful, nevertheless, MSCs did not modify trans-polarization.

Materials and Methods

Animals

In this study, adult (8-12 weeks old) C57Bl/6 (H-2b) mice (National Institute of Oncology, Budapest, Hungary) were used according to the guidelines of the Animal Care and Use Committee of the National Blood Service (Budapest, Hungary).

Establishment and characterization of MSC cultures

The establishment and characterization of MSC cultures was performed according to the methods described by Hegyi et al. [23]. Briefly, 10-12 weeks old C57Bl/6 (H-2b) mice were sacrificed by cervical dislocation then femurs were collected. Bone marrow (BM) cells were obtained by flushing out femurs with complete medium (CM) containing DMEM/Ham's F-12 medium (Invitrogen, Carlsbad, CA, USA), 10% fetal bovine serum, 5% horse serum (Invitrogen), 50 U ml^{-1} penicillin, 50 μg ml^{-1} streptomycin (Sigma-Aldrich) and 2 mM L-glutamine (Invitrogen) supplemented with heparin at a final concentration of 5 U ml^{-1}. The resulted suspension was then filtered using a 60 micron nylon mesh and washed twice with Hank's balanced salt solution (HBSS) (Invitrogen). Cells were seeded in a 25 cm^2 culture flask (BD Falcon, Bedford, MA) in CM in a density of 1-2x10^5 cells cm^{-2} and incubated in a humidified 5% CO_2 incubator at 37˚C. After 72 hours, non-adherent cells were removed by replacing CM. Reaching cell confluence, primary cultures were washed with cold HBSS then cells were retrieved by trypsinization using 0.25% Trypsin-EDTA solution (Invitrogen) and seeded into 75 cm^2 culture flasks (BD Falcon). Further passages were carried out by subculturing cells in a 1:5 ratio. Cells were used for experiments between passage 6 and 12 to avoid hematopoietic contamination.

MSC characterization occurred by the analysis of their cell surface markers and differentiation capacity as described earlier [24]. Briefly, cells were incubated with monoclonal antibodies against mouse Sca-1, CD44, CD73, CD90, CD45, CD11b and Gr-1 (all from BD Pharmingen, San Diego, CA, USA) for 20 min at 4°C in a buffer containing PBS with 0.5% v/w BSA. After incubation and two rounds of wash in the same buffer, labeling was analyzed by a FACScan flow cytometer using the CellQuest software (Becton Dickinson, San Diego, CA, USA). Osteogenic differentiation was induced by culturing confluent MSCs for 2 weeks in medium supplemented with dexamethasone (10^{-8} M), β-glycerophosphate (10 mM), and ascorbic acid (0.3 mM) (all from Sigma-Aldrich). To observe calcium deposition, cultures were stained with alizarin red S stain (Sigma-Aldrich). To induce adipogenic differentiation, confluent MSCs were cultured for 2 weeks in complete medium supplemented with dexamethasone (10^{-7} M) and 3-isobutyl-1-methylxanthine (0.5 mM) (Sigma-Aldrich). The cells were then fixed with 10% formalin and stained with oil red O (Sigma-Aldrich) and analyzed with microscopy.

Generation of bone marrow- and peritoneal macrophages

Bone marrow macrophages (BM-MΦs) were generated by flushing the femurs of 4-8 weeks old mice. Then the bone marrow cells were incubated for 7 days in BM-MΦ differentiation medium (BMDM): CM without horse serum containing 50 ng/ml M-CSF (Miltenyi Biotec, Germany). Then the cells were left in BMDM without M-CSF for a further 24 hours.

Peritoneal MΦs (Pe-MΦs) were isolated from the peritoneal cavity of 10-12 weeks old mice by washing the peritoneal cavity with HBSS supplemented with 10 U/ml heparin (Richter Gedeon, Budapest, Hungary). Peritoneal exudate cells (PEC) were washed twice with HBSS then seeded into 24-well flat-bottom plates (BD Falcon) and incubated for 1 hour in CM without horse serum in a 5% CO_2 incubator at 37°C. After 1 hour, non-adherent cells were

removed by two rounds of HBSS wash and the remaining adherent cells were used for experiment.

Characterization of MΦs

For characterization of Pe-MΦs and BM-MΦs by flow cytometry using FACScan instrument and CellQuest software (Becton Dickinson) the cells were retrieved by trypsinization (0.25% Trypsin-EDTA) then tested for the presence of F4/80, CD11b, MHC-II, and Ly-6c using fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated monoclonal antibodies against mouse cell surface markers (anti-F4/80 from AbD Serotec Ltd., Oxford, UK and all others from BD Pharmingen). Labelling occurred in dark for 20 minutes at 4°C.

Induction of MΦs into different activation profiles

Bone marrow cells ($2x10^5$ cells/well) were seeded into 24-well flat-bottom plates. For polarization into various MΦ subpopulations, 100 ng/ml LPS + 10 ng/ml IFNγ (Miltenyi Biotec) were used as M1 inducers, 20 ng/ml IL-4 (Miltenyi Biotec) was used as M2a activator, and 100 ng/ml LPS + 100 μg/ml heat-aggregated mouse IgG (IgG_a) was used for M2b induction. Heat-aggregated mouse IgG was prepared as follows: mouse IgG1, IgG2a, IgG2b, and IgG3 (all from Sigma) were mixed in equal amounts, filtered through a 0.22 micron nylon mesh and heated in a 63°C water-bath for 20 minutes. Induction was carried out in the presence or absence of $2x10^4$ MSCs. For experimental setups using Pe-MΦs, 10^6 PEC/well and $2x10^4$ MSCs were used.

Measurement of yeast-phagocytosis

Heat inactivated yeast particles were added to cultures of $5x10^6$ BM-MΦ/well or 10^6 Pe-MΦs/well, and incubated for 1 hour at 37°C. After removing free yeast particles with HBSS

wash, cells were fixed by 8% PBS-buffered formalin for 10 minutes and stained with Giemsa. Finally, digital photos were taken of each well by a Nikon Coolpix 4500 digital camera (Nikon GmbH, Düsseldorf, Germany) connected to an Olympus CK2 inverted microscope (Olympus, Tokio, Japan). Phagocytosis of yeast cells/macrophage was determined by counting ingested yeast particles in 100 macrophages then means were calculated and the average ingested yeast particles per one macrophage for each treatment are shown.

Transwell experiments

For transwell experiments, $2x10^5$ BM-MΦs were seeded into 24-well flat bottom plates (BD Falcon) and $2x10^4$ MSCs were placed into 0.1 micron inserts (BD Falcon). Cultures were induced by M1, M2a or M2b activators and then incubated for 48 hours at 37°C. Finally culture supernatants were collected and amounts of TNFα and IL-10 were analyzed as described below.

Cytokine analysis

Cell culture supernatants were tested for amounts of TNFα, IL-10, chitinase 3-like 3 (Ym1) and PGE-2 using Quantikine™ mouse TNF-α ELISA Kit, mouse IL-10 ELISA Kit, mouse CHI3L3/ECF-L ELISA Kit and Parameter™ PGE2 ELISA Kit (all from R&D Systems, Inc., Minneapolis, MN) according to the manufacturer's instructions.

For inhibition of PGE-2 synthesis 10μM Resveratrol (Sigma) and/or 10μM Celecoxib (Sigma) as COX-1 and COX-2 inhibitors, respectively were added to the cultures for 48 hours at 37°C then culture supernatants were collected and used for analysis of TNFα and IL-10. For exogenous PGE-2 experiments, 200, 400 or 800 pg/ml PGE-2 (Sigma) was added to the cultures for 48 hours at 37°C then supernatants were collected and again levels of TNFα and IL-10 were measured.

Statistical analysis

Each graph was composed of data of three independent experiments. Significance of data was evaluated by two-tailed Student's t-test with a significance level set at $p<0.05$. Data are expressed as mean ± SD. For yeast-phagocytosis, variables did not show normal distribution, hence the non-parametric Kruskal-Wallis test was used setting the significance level at $p<0.05$.

Results

Characterization and comparison of MΦs isolated from bone marrow and peritoneum

Our purpose was to find the best source of MΦs used for further experiments. The requirements included purity, and mature but inactive state. MSCs used in these experiments met the criteria for murine MSCs according to morphology, flow cytometry analysis of cells surface markers and differentiation potential (as we described earlier [24]). Treatment of bone marrow nucleated cells with M-CSF for one week resulted in a homogenous population of mature ($CD11b^+F4/80^+$) and naïve ($Ly\text{-}6c^-MHC\text{-}II^-$) MΦs (BM-MΦs) (Fig. 1). In contrast, Pe-MΦs were partially activated expressing Ly-6c (11.5%) and MHC-II (71%) (Fig. 1). The MHC-II positive cells did not only represent activated MΦs. Rather, one part of $MHC\text{-}II^+$ cells belonged to $CD19^+$ B cells (26.5±4.4%, data not shown). Additionally, Pe-MΦ cultures also contained 4.8±3.1% of $CD3^+$ T cells (data not shown). Presence of B and T cells and the partial appearance of cells with MΦ markers, CD11b and F4/80, confirmed the impurity of the Pe-MΦ cultures. The cells of both sources were functionally active since heat-inactivated yeast particles were readily phagocytosed (Fig.2A). Phagocytosis by BM-MΦs and Pe-MΦs was significantly inhibited and promoted by MSCs, respectively (Fig. 2B). Cytokine production of BM-MΦs and Pe-MΦs was also differently regulated by MSCs since they did not modify IL-10 and TNFα production by BM-MΦs while Pe-MΦs secreted ~10-fold more of the immunosuppressive IL-10 in the presence of MSCs (Fig. 2C and D). Non-MΦ cells and MΦs in an already activated state could also be regulated by MSCs [25] that may hinder or mask the direct MΦ response to the regulatory effect of MSCs in Pe-MΦ cultures. All of these results showed that BM-derived MΦs were highly superior as source of MΦs as these

cells were homogenous regarding purity and naïve, non-activated state. Therefore we used BM-MΦs in further studies.

MSCs differentially affect yeast-phagocytosis by distinct MΦ subpopulations

M1 and M2b MΦs induced with LPS+IFNγ and LPS+IgG_a, respectively, exerted similar phagocytic activity on heat-inactivated yeast while M2a, polarized with IL-4, showed significantly higher phagocytosis than M1 and M2b cells. The presence of MSCs differently affected the phagocytic activity of the various MΦ subpopulations. Hence, activity of M1 and M2b MΦs significantly increased while uptake by M2a declined (Fig. 3.).

The presence of MSCs shifts the cytokine production of M2b cells from TNFα to IL-10

Polarization of BM-MΦs was successful as M1 and M2b cells secreted high amounts of TNFα and IL-10 (Fig. 4A and 4B) in accordance with literature data [13]. Characteristically [9, 11], M2a cells lacked pronounced production of these two cytokines while producing substantial amounts of Ym1 (Fig. 4D). Under polarizing conditions, MSCs profoundly affected the TNFα production of MΦ subtypes since M1 and M2b MΦs produced less while M2a higher amount of TNFα, although TNFα production by M2a remained at low level (Fig. 4B), while the amount of IL-10 was not affected (Fig. 4A). The most striking effect of MSCs was registered on M2b but not on M1 or M2a cells since the inflammatory TNFα was remarkably shifted to the immunosuppressive IL-10 (Fig.4C). In contrast, Ym1 production by either MΦ subpopulation was not affected by MSCs (Fig. 4D).

Transition between MΦ subpopulations is not affected by MSCs

As Fig. 4. showed, induction of MΦ polarization occurred upon specific culture conditions and MSCs enhanced the immunosuppressive properties of the M2b subpopulation, shifting

the cytokine production to IL-10 prevalence. The question remained whether polarization represented a terminal differentiation of the MΦ subpopulations or under altered microenvironment, the polarization might shift toward another polarization state and MSCs could modify this process. To resolve this question, prepolarized M1 MΦs were created and placed into a distinct polarizing stimulation with or without being co-cultured with MSCs. During M1–M2a transition, IL-10 and TNFα production was highly reduced while Ym1 level was remarkably increased (Fig. 5A). Contrarily, during M2b polarization from M1 cells, IL-10 highly increased and TNFα- strongly diminished while Ym1 remained at a very low level (Fig. 5B). Both transition directions from M1 cells indicated that under altered polarizing environment, M1 cells cordially transformed to M2a or M2b MΦs. Polarization of M1 to M2b also occurred generating an IL-10 predominance, however, MSCs did not affect the levels of IL-10 or TNFα (Fig. 5B) as it was observed for M0–M2b transition (Fig. 4). The reason for the absence of any effect by MSCs could be accounted for the very low TNFα production in the absence of MSCs that could not be further reduced.

Requirement of PGE-2 in regulation of MΦ functions by MSCs

Cross-talk between MSCs and MΦs may occur by direct cell-cell interaction and/or by soluble factors. To analyze this question a transwell system was applied. The physical separation of MSCs and MΦs did not affect IL-10 (Fig. 6A) or TNFα production (Fig. 6B) indicating the role of soluble factors in the process.

As shown on Fig. 4, M2b cells responded most profoundly to MSCs by shifting their cytokine production from TNFα to IL-10. To determine whether an important MSC-derived immunoregulatory factor PGE-2 [26] plays a role in the modulation of M2b polarization, PGE-2 production has been inhibited in M2b–MSC cocultures (Fig. 7A). Resveratrol, a COX-

1 inhibitor did not influence, while the COX-2 inhibitor Celecoxib or the combination of COX-1 and COX-2 inhibitors shifted the cytokine production of M2b MΦs to TNFα.

To affirm the role of PGE-2 in the MSC–M2b MΦ interaction, MSCs were replaced with different concentrations of PGE-2 (Fig. 7B). The addition of PGE-2 shifted the TNFα/IL-10 ratio of M2b MΦs towards IL-10 in a concentration-dependent manner confirming its major role in the effect of MSCs on M2b MΦs. Hence, PGE-2 was found to be an important soluble factor in the MΦ–MSC interaction.

Discussion

Orchestrated macrophage activation and function during inflammation initiation, resolution and subsequent reparative processes are key events in responding to challenge and returning to homeostasis. Numerous pathological conditions show impaired MΦ response [27-29], hence targeting MΦs with MSC therapy can be of great importance. However, the effect of MSCs on MΦ functions has not clearly been explored yet. So far, the majority of studies have used adherent cells from peritoneal exudates or spleen isolates [30, 31]. Using the standard method for MΦ isolation from the mouse peritoneum based on plastic adherence [22], these cultures contain at least 5-10% of non-MΦ cells [25, 32] and these contaminating cells may also be affected by MSCs masking the MΦ response or modifying the results of co-culture experiments. Also the isolated MΦs might have acquired an unknown fate *in vivo* in the past, hence, these cultures likely contain different unrecognized subpopulations of MΦs in different activation states that may affect the conclusions drawn from the results. Moreover, certain tissue resident MΦ populations, such as Pe-MΦs, have recently been recognized as mostly consisting of a self-renewing, embryonic-yolk-sac-derived cell population, and therefore can differ from the continuously forming, monocyte-derived MΦs [33, 34]. Indeed, comparing BM- and Pe-MΦs, we found that while MΦs from bone marrow were a homogenous

population of naïve cells with mature MΦ phenotype, Pe-MΦs were contaminated with non-MΦ cells and showed a remarkable activated status. Moreover, Pe-MΦs responded with highly elevated IL-10 production to the presence of MSCs supporting that PEC contained MSC-responsive non-MΦ cells even after 1 hour of plating while BM-MΦs did not secrete increased amount of this immunosuppressive cytokine. To avoid the above disadvantages, we have applied BM-MΦs. Appropriate inducing cocktails triggered the generation of MΦ subpopulations, M1, M2a and M2b [13, 17, 35]. This model has been used to answer two major questions: 1) how MSCs may modify the effect of an inductive environment; 2) whether the shift from one to another MΦ polarization state induced by the instantaneous microenvironment represents an irreversible or a reversible process.

As shown, the various MΦ subtypes were distinguishable in their phagocytic capacity since M1 and M2b showed poorer, while M2a higher phagocytosis. This function was modified by MSCs since yeast ingestion by M1 and M2b was elevated while it was decreased for M2a MΦs. To date, most of our knowledge regarding phagocytic capacity as well as phagosome maturation concerns M1 and M2a MΦs [36]. Recent works showed slower phagosome maturation for M1 than M2a MΦs [36] serving as an explanation for the delayed particle uptake by M1 compared to M2a macrophages. Increased zymosan and apoptotic thymocyte phagocytosis by mouse MΦs in the presence of MSCs was described by Maggini et al. [30]. The mechanism of this effect, however, is unclear and need further investigation. The functional changes during the induction of MΦ subpopulations were reflected in the alterations in the production of different factors: M1 and M2b polarization resulted in remarkable rise of IL-10 and TNFα, cytokines that were not secreted by naive MΦs, while the level of these factors did not change in M2a cultures. Moreover, chitinase (Ym1) expression found only in M2a cells [9] increased to a high level under M2a polarizing conditions. These results were in accordance with previous data [37]. The presence of MSCs did not cause a

change in the level of IL-10 or Ym1, however significantly downregulated TNFα in M1 and M2b cells and upregulated in M2a MΦs although to a very low level. Tumor growth factor beta 1 (TGF-β1), another potent immunosuppressive molecule produced by both MΦs [38] and MSCs [39] was also analyzed in the MSC–MΦ interaction, however, none of the MΦ subsets responded with changes in TGF-β1 when cocultured with MSCs (data not shown). The most important consequence of the presence of MSCs was their effect on the ratio of TNFα/IL-10 in M2b cells since cytokine production shifted toward the immunosuppressive IL-10. An emerging dogma in regenerative biology suggests that M1 MΦs are the predominant population being present during the first few days after injury, corresponding to the early inflammatory and proliferative phases, whereas alternatively activated MΦs, M2a and M2b, are the main effectors of the later stages of tissue repair and remodeling [27]. Accordingly, M1 cells readily transformed into M2a or M2b cells when changing the microenvironment. Interestingly, MSCs did not affect the cytokine production by M2b cells transformed from M1. A plausible explanation for this finding is that during M1 to M2b polarization, TNFα level is dramatically reduced and MSCs that primarily act on TNFα production are not able to further reduce this cytokine. This decline of TNFα may stem from endotoxin tolerance [40] since both M1 and M2b polarization is induced partially with LPS. The second LPS stimulation can inhibit TNFα expression [41]. It is also apparent from our results that the presence of MSCs is permissive for TNFα production although in diminished quantities. Certain amounts of TNFα, and a controlled, dynamic change in TNFα/IL-10 levels seem to play important roles in regenerative physiological processes. Anti-inflammatory and proinflammatory cytokine expression, however, may be induced simultaneously at early time points during tissue repair. For example, at 48h after acute liver injury in mice, MΦs simultaneously express IL-10 and TNFα [42]. During skeletal muscle repair, satellite cells are major targets of TNFα signaling that promotes the early proliferative stage of myogenesis

[43]. In the case of heart, TNFα has a cardioprotective function in mice [44]. On the other hand, mononuclear phagocytes engage in a bidirectional interaction with MSCs [20]. MΦ-derived TNFα is able to induce tumor necrosis factor alpha-stimulated gene 6 (TSG6) production by MSCs. The secreted TSG6 is one of the most important, MSC-derived soluble mediator with anti-inflammatory and immunosuppressive activity *in vitro*, as well as *in vivo* [45]. It is also important to note that changes in the cytokine levels we measured reflect alterations in the activation of MΦs only as several laboratories proved that MSCs are not capable of producing IL-10 [46] or TNFα even after treatment with TLR agonists [47]. In accordance with these findings, we could not detect TNFα and IL-10 production in MSC control wells even when LPS or IFNγ was present. In another work of our laboratory, we also proved that MSCs do not produce either of these cytokines when cocultured with microglial cells, another type of macrophage, using transwell coculturing systems [48]. Thus, activation of the TNFα or IL-10 promoter in MSCs in the presence of macrophages is highly unlikely.

In agreement with several earlier results [21, 49, 50], the crosstalk between MΦs and MSCs occur primarily by soluble factors, since cytokine production during physical separation of the two cell types was similar to those measured in direct co-cultures. One of the soluble factors produced by both MΦs and MSCs is PGE-2. The immunologically relevant synthesis of PGE-2 depends mostly on COX-2 but not COX-1 enzyme activity with COX-2 being the inducible and COX-1 the constitutively active isoforms [51]. Our results support these literature data, since the inhibition of COX-2 but not COX-1 resulted in a shift of cytokine production towards TNFα in our M2b MΦ–MSC cocultures. The importance of PGE-2 in the cross-talk between MΦs and MSCs was further affirmed when exogenous PGE-2 was added to M2b cells instead of MSCs and PGE-2 acted similarly to MSCs on M2b cells. These results indicate that PGE-2 plays a pivotal role in MSC-modified MΦ polarization, as already shown by several other laboratories [30, 52, 53]. Although extensive *in vivo* studies are needed to

further verify in vitro results, in the light of these findings, MSCs seem to be promising candidates for the manipulation of MΦ polarization towards a more M2b-like, IL-10-dominant immunosuppressive phenotype during tissue regeneration and in the treatment of various inflammatory and/or autoimmune diseases.

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Figure legends

Fig. 1. Characterization of bone marrow- and peritoneal cavity-derived MΦs. The expression of monocyte-macrophage marker CD11b, mature macrophage marker F4/80, and MΦ activation markers MHC-II and Ly-6c were measured on BM- and Pe-MΦs using flow cytometry. Light grey shaded histograms and continuous lines represent the results given by isotype matched controls and the indicated specific antibodies, respectively.

Fig. 2. Comparison of functions of BM- and Pe-MΦs. (A) MΦs phagocytosing yeast particles for one hour were fixed then stained with Giemsa and analyzed with light microscopy.(B) The number of ingested yeast particles per MΦ were counted using light microscopy images of Giemsa stained cultures and was evaluated in at least 100 MΦs per treatment. Results are representative of three independent experiments and are shown as mean ± SE. (C) The amount of IL-10 and (D) TNFα secreted by BM- or Pe-MΦs in the presence or absence of MSCs was measured using ELISA assays. Results are shown as mean ± SD, n=3.

Figure 3. Yeast ingestion by M1, M2a or M2b polarized BM-MΦs. MΦs were induced with M1, M2a or M2b medium in the presence or absence of MSCs and yeast ingestion was analyzed as described under Fig 2B. Results are shown as mean ± SD, n=3.

Figure 4. Cytokine analysis of MΦs subpopulations. Supernatants harvested from polarized M1, M2a and M2b MΦ cultures after 48 hours of induction of polarization were used the detect the concentrations of IL-10 (A), TNFα, (B) and Ym1 (D) using ELISA. Results are expressed as mean ± SD, NS: not significant, n=3. The relative TNFα/IL-10 ratio (C) was calculated compared with TNFα/IL-10 ratio in MΦ-only cultures that is represented as one.

Figure 5. Trans-polarization of M1 cells to M2a or M2b. Naïve M0 MΦs were treated with M1, M2a or M2b inducing agents (A and B) in the presence or absence of MSCs for 48 hours (A and B). For M1-M2a (A) and M1-M2b (B) transitions, prepolarized M1 cells were washed twice with HBSS and media were replaced with CM containing either M2a (A) or M2b (B) inducing cocktails with or without MSCs. After 48 hours of incubation, supernatants were collected and concentrations of IL-10, TNFα and Ym1 were analyzed with ELISA. Results are expressed as mean ± SD, n=3; NS, not significant.

Figure 6. Physical separation of MSCs and MΦs in transwell system. One micron pore-sized transwell chambers were used to inhibit direct MΦ-MSC interaction. MΦs were seeded into 24-well flat bottom plates and polarized with the appropriate inductors while MSCs were placed in the transwell chambers. Cell culture supernatants were collected after 48 hours of incubation. IL-10 (A) and TNFα (B) concentrations were measured with ELISA. Results are expressed as mean ± SD, n=3; NS, not significant.

Figure 7. Role of PGE-2 in the establishment of TNFα/IL-10 ratio by M2b MΦs. (A) MΦs and MSCs were cocultured in M2b inducing medium in the absence or presence of cyclooxigenase inhibitors, 10μM Resveratrol (COX-1) and/or 10μM Celecoxib (COX-2) for 48 hours. (B) MΦs in M2b medium were either cocultured with MSCs or treated with 200, 400 or 800 pg/ml PGE-2 for 48 hours. TNFα and IL-10 concentration in the culture supernatants were measured using ELISA assays. The relative TNFα/IL-10 ratio was calculated in proportion to TNFα/IL-10 ratio in MΦ–MSC cocultures with no inhibitors that is represented as one (A), or in proportion to TNFα/IL-10 ratio in MΦ-only cultures that is represented as one (B), respectively. Results are expressed as mean ± SD, n=3; NS, not significant.