The immunosuppressive activity of mesenchymal stem cells

Doctoral Thesis

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Budapest

2017

INTRODUCTION

Mesenchymal stem cells (MSCs) are tissue stem cells residing in virtually all tissues and organs supporting regenerative processes, hematopoiesis and immunomodulation. Since these cells lack MSC-specific cell surface markers, the International Society for Cellular Therapy established a set of minimal criteria to define human MSCs: 1.) plastic adherence and fibroblast-like morphology under standard culturing conditions; 2.) testing positive for CD105, CD73, CD90 cell surface markers, while negative for certain hematopoietic and endothelial markers, such as: CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA-DR; and 3.) the ability to differentiate to osteoblasts, adipocytes and chondroblasts *in vitro*.

The therapeutic use of MSCs is based on their ability to secrete a plethora of regeneration-promoting growth factors, cytokines and other soluble factors, and extracellular vesicles in response to tissue damage. Their other favorable characteristic is their ability to communicate with a wide range of immune cells favoring anti-inflammatory processes and immunosuppression. To be able to do so, they need to receive activating signals from the inflammatory microenvironment. They exert their immunosuppressive activity through several mediators, however, the exact mechanisms are still poorly understood with many contradictory results. Numerous MSC-derived soluble factors have been identified in MSC-mediated immunomodulation, these are prostaglandine-E2 (PGE-2), several cytokines (hepatocyte growth factor, transforming growth factor β), tumor necrosis factor-inducible gene 6 protein, nitric-oxide (NO), indolamine-2,3-dioxzgenase (IDO) enzyme, soluble human leukocyte antigen G (HLA-G), or cell contact-dependent molecules such as B7-H1 (PD-L1), B7-H4, Notch receptors or Jagged1.

MSCs are able to inhibit the activation and proliferation of T-cells. Activated T-cells (and the also present monocytes, macrophages and NK cells) secrete inflammatory cytokines (eg. IFNγ, TNFα and/or IL-1β) that induce MSCs to produce several factors (eg. PGE-2, NO) inhibiting T-cell proliferation. As a result, the cytokine production of T-cells shifts from the pro-inflammatory (IFNγ, TNFα, IL-6 és IL-17) to anti-inflammatory (IL-4 and IL-10) factors. However, the exact role of these mediators are yet to be elucidated. We also know that MSCs are able to inhibit the proliferation of both naïve and memory T-cells as well as CD4 and CD8 positive T-cells.

Our knowledge on the interaction of MSCs and macrophages (Mφs) are, however, even more limited. Based on a few publications, it is postulated that MSCs are able to induce the polarization of Mφs into an anti-inflammatory („alternatively activated") M2 phenotype – a group comprising regenerative M2a and anti-

inflammatory, regulatory M2b Mφs. Maggini et al. showed that the cytokine production of bacterial lipopolysaccharide (LPS) pre-treated, inflammatory M1 Mφs (TNFα, IL-6, IL-12p70, IFNγ) shifted towards a more anti-inflammatory profile (IL-10 and IL-12p40) in the presence of MSCs indicating the establishment of an M2 (regulatory, regenerative) Mφ phenotype. However, the few works in this topic did not distinguish if MSCs promote more of an M2a or M2b phenotype within the M2 anti-inflammatory Mφ group, yet, this could make the therapeutic use of MSCs more selective and precise, since distinct Mφ subpopulations can cause problems in different inflammatory and/or autoimmune disorders.

AIMS

In our work, we aimed to answer the following questions:

1. Can we establish stem cell cultures from the bone marrow of C57Bl/6 mice that show characteristics of mesenchymal stem cells?
2. Can we prove the immunosuppressive activity of these MSCs in mitogen- and alloantigen-induced T-cell proliferation experiments?
3. What major MSC-derived immunosuppressive mediators are responsible for the observed immunosuppressive effect?
4. How do certain pro-inflammatory cytokines – TNFα and IFNγ – affect the PGE-2-production of MSCs?
5. What is the mechanism behind the PGE-2 production of activated MSCs?
6. How does the phagocytic and antigen-presenting capacity, and the cytokine production – and thus the activation profile – of peritoneal Mφs change in the presence of MSCs?
7. Can we successfully establish bone marrow-differentiated Mφs from mouse bone marrow cells, and activate them into the main Mφ phenotypes (M1, M2a, M2b) with the help of the appropriate inductors?
8. How do MSCs affect the phagocytic capacity and cytokine production of bone marrow Mφs under M1, M2a and M2b induction?
9. Are bone marrow Mφs able to be re-polarized with the changing of the inductive environment and are MSCs able to affect these changes?
10. What main mediator is responsible for the effect of MSCs on Mφs?

METHODS

Isolation and culturing of mesenchymal stem cells

Bone marrow MSC cultures were established based on mainly their adherence. Briefly: bone marrow was flushed out of the femurs of 10-12 weeks old C57Bl/6 mice and the cells were placed in culture flasks. Constituents of the culturing medium (CM): DMEM/F-12 medium (1:1) supplemented with 10 v/v% FBS, 5 v/v% horse serum (HS), 2 mM L-glutamine, 50 U/ml penicillin and 50 µg/ml streptomycin. Cultures were kept at 37ºC in 5% CO_2 incubators. MSCs were used betwwen passages 7 and 15.

Purification of T-cells

Separation of T-cells were performed by the SpinSep Mouse $CD3^+$ T Cell Enrichment kit according to the manual. T-cells originated from the spleen of adult C57Bl/6 mice. Purity was tested with the help of CD3-specific monoclonal antibodies and flow cytometry.

Establishment of peritoneal- and bone marrow macrophage cultures

Peritoneal macrophages (Pe-Mφ) were gained by repeatedly flushing the peritoneal cavities of 10-12 weeks old C57Bl/6 mice with Hanks' balanced salt solution (HBSS) containing 10 NE/ml heparin. The cell suspension (PEC) was let to adhere to the culture plates and non-adherent cells were washed away after 1 hour of incubation. Cells were cultured in HS-free CM. Freshly isolated Pe-Mφs were used for each experiment.

Bone marrow macrophages (BM-Mφ) were differentiated from bone marrow cells that had been flushed from the femurs of 4-8 weeks old C57Bl/6 mice. Mφs were differentiated for 7 days in HS-free CM supplemented with 50 ng/ml M-CSF followed by a one day long rest in HS-free CM. Newly differentiated Mφs were used for each experiment.

Characterization of cells

The characterization of MSCs, Pe- and BM-Mφs was performed by flow cytometry. Cells were labeled with the appropriate antibodies against specific cell surface antigens as listed in the „Results" section.

For osteogenic induction of MSCs, confluent cells were cultured in DMEM containing 10% FBS, ß-glycerophosphate (10 mM), dexamethasone (10^{-8}

M) and ascorbic acid (0,3 mM) for 2 weeks. Deposited calcium was stained by Alizarin Rend dye. For adipogenic differentiation of MSCs, we used HS-free CM supplemented with dexamethasone (10^{-7} M) and 3-isobutyl-1-methylxanthin (0,5 mM) for 2 weeks. Intracellular lipid droplets were stained by Oil Red O dye.

Proliferation inhibition of activated T-cells

To investigate the immunosuppressive activity of MSCs, $2x10^5$ spleen cells or T-cells were activated by 5 μg/ml concanavalin A (ConA) in the presence or absence of $2x10^4$ MSCs.

Mixed lymphocyte reaction (MLR) was prepared using $2x10^5$ „responder" (C57Bl/6) and $2x10^5$ „stimulator" (30 Gy-irradiated Balb/c) spleen cells in the presence or absence of $2x10^4$ MSCs in HS-free CM. After 2 or 4 days of incubation (for ConA-stimulated or MLR cultures, respectively), proliferating cells were labeled with 1 μCi ^{3}H-thymidine for 6-12 hours, then cells were harvested and the count per minute values were measured by a liquid scintillator.

To test cell-to-cell contact dependency, $2x10^6$ T-cells were added to cultures of $2x10^5$ MSCs. T-cells were placed in MSC cultures directly or in inserts comprising a 1 μm pore-sized semi-permeable membrane („trans-well system").

Cytokine analysis

Supernatants collected from various experimental settings were tested for the presence of certain cytokines and mediators using the appropriate enzyme-linked immunosorbent assays.

Measurement of the prostaglandine-E2 production of MSCs

$2x10^5$ MSCs were seeded in culturing plates for 24 hours, then 10 or 50 ng/ml TNFα, 20 or 100 ng/ml IFNγ, 10μM indomethacin (Indo), 1mM N-methyl-L-arginine-acetate (L-NMA), 1 μM methyl-tryptophan (1-MT) or 80 μM nitrogen-monoxide donor molecule (NOC-18) were added to the cultures. After further 48-hour incubation, supernatants were collected.

Induction of macrophages into specific activation profiles

Activation of Pe-Mφs was achieved using 10 μg/ml lipopolysaccharide (LPS), or M1 induction medium (see later). 10^6 PEC were seeded into culturing

plates, then washed and the remaining cells were used for experiments (see above) to which $2x10^4$ MSCs were added.

For the induction of BM-Mφs, bone marrow cells were first seeded into culturing plates ($2x10^5$ cells/well), then according to the method described previously, cells were differentiated. Then, fot M1, M2a and M2b activation, the following cocktails were used: 1.) M1 induction medium: 100 ng/ml LPS + 10 ng/ml IFNγ in HS-free CM; 2.) M2a induction medium: 20 ng/ml IL-4 in HS-free CM; 3.) M2b induction medium: 100 ng/ml LPS + 100 μg/ml heat-aggregated mouse IgG (IgGa) in HS-free CM. Induction was performed in the presence or absence of $2x10^4$ MSCs.

Yeast- and apoptotic thymocyte phagocytosis of Mφs

To gain apoptotic thymocytes, the thymi of 6-8 weeks old C57Bl/6 mice were isolated and crushed. The resulting cell suspension was treated with 2 μM dexamethasone for 12 hours at 37°C.

MSCs, LPS, M1-, M2a- or M2b induction media were added to cultures of Pe-Mφs or BM-Mφs. After 48 hours of incubation at 37°C, $5x10^6$ heat-inactivated yeast cells *(Saccharomyces cerevisiae)* were added to the cultures for one hour. Cells were fixed then stained with Giemsa dye. Results were captured by digital photographs (random images), and the number of ingested yeast cells were counted (yeast cells/macrophage) in 100 macrophages in all treatment groups.

The apoptotic thymocyte uptake by Pe-Mφs was proven by specific 5(6)-CFDA/SE fluorescent labeling. The molecule converts into CFSE (carboxyfluorescein-succinimidyl-ester) inside the cells derived from the thymi of 6-8 weeks old C57Bl/6 mice. The labeled thymocytes were incubated in the presence of 2μM dexamethasone for 12 hours at 37°C, then were added to the cultures. MSCs were labeled by the lentiviral incorporation of the gene of mCherry (kindly provided by Dr. Katalin Német et al.). $5x10^5$ CFSE-labeled thymocytes and $2x10^4$ mCherry-expressing MSCs were added to Pe-Mφ cultures (founded using 10^6 PEC), cultures were incubated for 48 hours. Apoptotic cell uptake was proven by digital photographs and flow cytometry. In certain experiments, unlabeled apoptotic thymocytes or heat-inactivated yeast cells were used and cultures were incubated for 48 hours after one hour of phagocytosis. Supernatants were collected for cytokine analysis.

Antigen-presenting capacity of Pe-Mφs in cultures containing MSCs and T-cells

$4x10^3$ MSCs, $2x10^5$ PEC-derived Pe-Mφs, $2x10^5$ *in vivo* preactivated (by a 1:1 mixture of complete Freund adjuvant and ovalbumin) T-cells, and different concentrations of ovalbumin (OVA) (4; 20; 100 μg/ml) were cultured at 37°C for 5 days. Proliferating cells were labeled with 1 μCi ^{3}H-thymidine for 18 hours, then cells were harvested and the count per minute values were measured by a liquid scintillator.

Measurement of different T-cell subpopulations

$4x10^3$ MSCs, $2x10^5$ PEC-derived Pe-Mφs, $2x10^5$ *in vivo* ovalbumin-preactivated T-cells, and 20 μg/ml ovalbumin were cultured for 5 days at 37°C. Then, T-cells were collected from the cultures and the ratio of regulatory T-cells and helper 17 T-cells were determined by flow cytometry. Labeling for these cells was performed according to the manuals of the Mouse Regulatory T Cell Staining Kit #1 and the Mouse Th1/Th2/Th17 Phenotyping Kit.

Cell-to-cell contact dependency of BM-Mφ and MSCs in cocultures

$2x10^5$ BM-Mφs were seeded into culturing plates, then $2x10^4$ MSCs were either directly added to the cultures or they were placed in inserts comprising a 1 μm pore-sized semi-permeable membrane („trans-well system"). These cultures were then treated with M1-, M2a- or M2b induction media for 48 hours, then supernatants were collected.

Measuring the role of prostaglandine-E2 in BM-Mφ cultures

To inhibit the production of PGE-2, we added 10μM Resveratrol and/or 10μM Celecoxib as COX-1 or COX-2 inhibitors, respectively, to the cocultures of BM-Mφs and MSCs in M2b induction medium. After 48 hours of incubation, supernatants were collected. For the exogenously added PGE-2 experiments, 200, 400 or 800 pg/ml PGE-2 was added to BM-Mφ cultures and cultures were incubated for 48 hours in M2b induction medium, then supernatants were collected.

Statistics

Significance of results was determined using Student's t-test and non-parametric Kruskal-Wallis test. The significance level was set at $p<0.05$.

RESULTS

Characterization of the bone marrow mesenchymal stem cells

As the first step of our work, it was important to prove that our bone marrow-derived cultures fulfill the criteria for naming them MSCs. Our adherent cells showed an elongated, fibroblast-like morphology. The cell surface marker profile of these cells were in accordance with that of mouse MSCs: out of the tested markers, they expressed markers characteristic for MSCs and most mouse fibroblasts, namely Sca-1, CD44 and CD73, while they were negative for hematopoietic markers such as CD34, CD45R, CD11b, Gr1 and Ter117. Using specific inductors, our cells were able to differentiate into lipid droplet accumulating adipocytes and calcium-depositing osteoblasts *in vitro* after 14 days of incubation in adipogenic or osteogenic medium, respectively. This shows that our cells can be regarded as multi- or at least bipotent.

Measuring the immunosuppressive activity of bone marrow MSCs in systems containing activated T-cells

The immunosuppressive activity of MSCs was tested in alloantigen- and mitogen-activated lymphocyte systems. In mixed lymphocyte reaction, $2x10^5$ „responder" (capable of dividing, derived from C57Bl/6 mice) and the same amount of „stimulator" (irradiated, incapable of dividing, derived from Balb/c mice) spleen cells were cocultured with different number of MSCs: $1.25x10^3$, $2.5x10^3$, $5x10^3$, 10^4, or $2x10^4$. The proliferation of T-cells was followed by the incorporation of ^{3}H-thymidin. Our results showed that MSCs in the ratio of 1:40 significantly reduced the proliferation of alloantigen-activated T-cells, and the maximum of inhibition was achieved at 1:20 MSC:T-cell ratio. When we repeated the experiment in a mitogen-activated system using 5μg/ml ConA as a mitogen for T-cell activation, we observed similar trends, therefore, in the following experiments, we used MSCs and T-cells in the ratio of 1:20, respectively.

Mediators of the inhibitory effect of MSCs on T-cell proliferation

We wanted to elucidate what mediator(s) are responsible for the interaction of MSCs and activated T-cells. We used indomethacin (Indo) as inhibitors of cyclooxygenases (COX-1, COX-2) – enzymes that produce PGE-2 –, 1-methyl-tryptophane (1-MT) as an indolamine-2,3-dioxygenase (IDO) inhibitor, and N-methyl-L-arginine-acetate (L-NMA) as an inducible nitric-oxide (iNOS) and

thus nitric-oxide (NO) inhibitor to cultures of MSCs and ConA-activated T-cells. The inhibitory effect of MSCs on T-cell proliferation was again observed. This inhibitory effect was partially resolved by Indo and L-NMA, however, their effect was not additive. The effect of prostaglandins and NO was thus proven in the interaction of MSCs and activated T-cells. It has to be noted that their role is not exclusive, even their combined inhibition could only partially restore activated T-cell proliferation. Through 1-MT, the effect of the IDO enzyme was not detected in our experimental system.

The production of prostaglandine-E2 in the cocultures of MSCs and activated T-cells

After seeing that our results also verified the effect of PGE-2 in the MSC–T-cell interaction, we wanted to know how the PGE-2 production of MSCs is affected in the presence of activated T-cells. Our results showed that MSCs alone constitutively produce considerable amounts (1150 pg/ml) of PGE-2 (control). This production increased fourfold and tenfold in the presence of non-activated or ConA-activated T-cells, respectively. When physical contact between the two cell types was inhibited, neither non-activated, nor activated T-cells were able to enhance the control PGE-2 production of MSCs. Activated T-cell are thus able to augment the PGE-2 production of MSCs, but physical contact between the two cell types are essential to achieve this effect. The PGE-2-enhancing effect of activated T-cells was investigated in the presence of inhibitors Indo and L-NMA. As expected, Indo proved to be a strong inhibitor of PGE-2 synthesis, however, L-NMA had no inhibitory effect. Therefore, the PGE-2-enhancing effect of activated T-cells is mainly COX- and not iNOS- (and thus not NO-) dependent.

The mechanism of the PGE-2 production of mesenchymal stem cells in the presence of pro-inflammatory cytokines

To elucidate the mechanism behind the PGE-2 production of MSCs, we added two of the most important pro-inflammatory cytokines, interferon gamma (IFNγ) and tumor necrosis factor alpha (TNFα) to the MSC cultures. Higher doses of TNFα (50 ng/ml) was able to significantly enhance the PGE-2 production of MSCs while lower concentrations (10 ng/ml) had no effect. Additionally, neither higher, nor lower concentration of IFNγ (100 ng/ml and 20 ng/ml, respectively) were able to affect PGE-2 production, however, when IFNγ and TNFα were used concurrently, their effect became synergistic: even the lower concentration of IFNγ

was able to potentiate the effect of TNFα and enhanced PGE-2 production several folds. Therefore, IFNγ is able to augment the TNFα-induced PGE-2 production of MSCs.

Next, we used inhibitors of the enzymes (COX enzymes and iNOS) that could potentially have a role in PGE-2 signaling and added them to the previously described experimental setting. Baseline PGE-2 production of MSCs was almost completely abrogated in the presence of Indo (85% inhibition). The iNOS-inhibiting L-NMA was also a potent inhibitor in the system, however, its effect was weaker (around 60% inhibition) on PGE-2 synthesis than that of Indo. Then, taking the 50 ng/ml TNFα-induced PGE-2 production of MSCs as control, we observed that Indo was again a strong PGE-2 synthesis inhibitor (65% efficacy). However, in this activated state, L-NMA could not affect the PGE-2 synthesis of MSCs. Therefore, TNFα presumably acts through an iNOS-independent signaling pathway, and through the COX-enzymes, to induce the synthesis of PGE-2. When TNFα was used together with IFNγ, L-NMA also became a potent PGE-2 synthesis inhibitor. Its effect, however, was never as strong as that of Indo. This shows that IFNγ signaling also affects PGE-2 synthesis through the COX-enzymes and iNOS is an important partaker in the pathway upstream of the COX-enzymes. This is supported by our result that when MSCs got treated with a NO-donor molecule, NOC-18, their PGE-2 production was strongly enhanced.

The interaction between mesenchymal stem cells and peritoneal macrophages

Characterization of peritoneal macrophages

In the second part of our work, we focused on macrophages, since these cells are central partakers in inflammatory and/or autoimmune disorders. As described in the „Methods" section, we isolated macrophages from the peritoneal cavity of mice, and the resulting adherent cells were tested by flow cytometry. 49.6±7.3% of cells stained positive for the marker for mature macrophages (F4/80), of which 48.8±6.3% carried the CD11b monocyte-macrophage marker. We also found $CD19^+$ B-cells and $CD3^+$ T-cells in smaller quantities, while granulocytes could not be detected in our cultures. 69.8±5.4% of the examined cells were positive for MHC-II antigen, and 12.8±2.3% for Ly-6c. Both of these are markers of Mφ activation, however, some of the $MHC\text{-}II^+$ cells were probably B-cells (26.5±4.4%).

Yeast- and apoptotic thymocyte phagocytosis of peritoneal macrophages in the presence of MSCs

Pe-Mφs isolated by the method we used were successful at phagocytosing yeast cells, thus, we could establish cultures containing functional macrophages. The presence of MSCs significantly enhanced phagocytosis by Pe-Mφs. LPS, a Toll-like receptor 4 (TLR4)-ligand, is another potent Mφ-activator. Our results showed that treatment of Pe-Mφs with 10 μg/ml LPS largely decreased the yeast-uptake of Pe-Mφs, while the presence of MSCs significantly enhanced it even in the presence of LPS. After examining pathogen-uptake, we wanted to know how MSCs influence the internalization of apoptosing self-cells by Pe-Mφs. The uptake of apoptotic cells was followed by using dexamethasone-treated thymocytes that had been labeled with a fluorescent dye (CFSE). As proven by our fluorescent microscopy images and flow cytometry data, the Pe-Mφs managed to take up the apoptotic thymocytes: the mean fluorescence intensity (MFI) values decreased in the Pe-Mφ-containing cultures compared to the fluorescent values of free thymocytes (thymocyte-only cultures). In the presence of MSCs, Pe-Mφs internalized more apoptotic thymocytes, and a significant fraction of these Mφs gave higher fluorescent signal that showed the uptake of even higher numbers of thymocytes. Thus, Pe-Mφs become more potent at pathogen- and apoptotic self-cell uptake in the presence of MSCs.

We also wanted to know what changes occur in the cytokine production of Pe-Mφs when they internalize the two cell types (yeast cells or apoptotic thymocytes). To follow the changes, we measured the production of TNFα (a pro-inflammatory cytokine), IL-10 (an anti-inflammatory cytokine), and PGE-2 (an immunomodulatory lipid mediator) in the culture supernatants. The cytokine production of Pe-Mφs turned around in the presence of MSCs: they produced more IL-10 than TNFα, and this could also be observed in the presence of yeast cells or apoptotic thymocytes. Therefore, MSCs promote the establishment of an anti-inflammatory cytokine profile in Pe-Mφ cultures, and since the production of PGE-2 moved in parallel with the levels of IL-10, PGE-2 is a potential mediator in the process of MSC–Pe-Mφ interaction.

The effect of a pro-inflammatory environment on the interaction of peritoneal macrophages and MSCs

The polarization of classically activated, pro-inflammatory (M1) macrophages requires LPS and IFNγ as the most often used inductors, and we wanted to investigate how such M1 activated Mφs respond to MSCs. We treated Pe-

Mφ and MSC cultures with M1 induction medium. This induction resulted in a strongly TNFα-dominant cytokine production in Pe-Mφ-only cultures, while the presence of MSCs established an IL-10-dominant cytokine profile in Pe-Mφs treated with either CM or M1 induction medium, however, the TNFα-production of Pe-Mφs was not affected. Thus, MSCs are able to counteract the use of our pro-inflammatory mediators by enhancing the IL-10-production of Pe-Mφs.

The effect of MSCs on the antigen-presenting capacity of macrophages

The antigen-presenting capacity of Pe-Mφs was first examined during activation by LPS. Our results showed that Pe-Mφs became more potent antigen-presenting cells in the presence of LPS: the number of MHC-II$^+$ and CD86$^+$ cells was enhanced, and also, the number of these molecules increased on the surface of the cells. MSCs augmented the expression of MHC-II and CD86 molecules on the cell surface of both LPS-treated and untreated Pe-Mφs. Therefore, the presence of MSCs turns Pe-Mφs into more potent antigen-presenting cells even when LPS is present as an activator.

We further investigated the antigen-presenting capacity of Pe-Mφs by measuring ovalbumin- (OVA-) specific T-cell proliferation in the presence of different concentrations of OVA (4 μg/ml; 20 μg/ml; 100 μg/ml). According to our results, 20 mg/ml OVA was already a potent T-cell proliferation enhancer in cultures containing Pe-Mφs proving that our Pe-Mφs were potent antigen-presenting cells. The proliferation of OVA-specific T-cells was the most intense in cultures where MSCs and Pe-Mφs were both present. In these cultures, T-cells responded with increased proliferation to the higher concentrations of OVA in a dose-dependent manner. The presence of MSCs, therefore, was able to significantly enhance the antigen-presenting capacity of Pe-Mφs.

We also wanted to know, how the number of regulatory and helper 17 T-cells is affected in the above mentioned experimental setting. According to our results, the number of regulatory (FOXP3$^+$) T-cells increased among the CD4$^+$CD25$^+$ cells in the cultures containing both Pe-Mφs and MSCs compared to Pe-Mφ cultures without MSCs. At the same time, the number of Th17-cells decreased. MSCs are able to promote the differentiation of CD4$^+$ cells towards immunoregulatory T-cells, while they inhibit the evolvement of highly autoreactive, pro-inflammatory Th17-cells according to our results.

The interaction of mesenchymal stem cells and „naive" macrophages

Characterization of bone marrow macrophages

We wanted to examine the interaction between MSCs and Mφs in more detail, for which we needed pure, homogenous and non-activated Mφ cultures. We differentiated bone marrow Mφ (BM-Mφ) from the bone marrow cells of 4-8 weeks old C57Bl/6 mice. This method provided us with naive, non-activated (MHC-II$^-$ Ly-6c$^-$) F4/80 positive mature Mφs (F4/80$^+$CD11b$^+$) with a 98 ± 0,6% purity.

The yeast-phagocytosis of bone marrow macrophages activated into different macrophage phenotypes

BM-Ms were either cultured in CM as resting, naive (M0) Mφs, or they were activated into the three most well-known Mφ polarization profiles (M1 – pro-inflammatory, M2a – wound-healing and M2b – regulator) with the help of the appropriate induction cocktails, then the yeast-phagocytosis of these cells were measured. Resting M0 cells phagocytosed 12 yeast cells on average, this did not change considerably in the M2a cultures either. M1 and M2b Mφs, however, took up significantly less yeast particles. The presence of MSCs differently affected the yeast-phagocytosis of Mφs in the various activation profiles: yeast-uptake was decreased in M0 and M2a, while enhanced in M1 and M2b cultures.

The cytokine production of the different macrophage phenotypes in the presence of MSCs

We also wanted to know how MSCs affect the cytokine production of BM-Mφs in the different inductive microenvironments (M1, M2a and M2b). We measured the production of TNFα, IL-10 and chitinase-3-like protein 3 (Ym1) – factors produced only by Mφs in our experimental systems – to follow changes in Mφ activation. According to our results, naive M0 Mφs alone do not produce significant amounts of IL-10 and TNFα. During M1 and M2b activation, however, significant amounts of IL-10 and TNFα could be measured. Under M2b induction, higher levels of IL-10 and lower levels of TNFα was characteristic, this corresponds with the anti-inflammatory, regulatory Mφ profile. Under M1 induction, the ratio of the two cytokines was opposite and thus pro-inflammatory-dominant. This also corresponds with the appropriate pro-inflammatory M1 macrophage profile. M2a Mφs, similarly to naive M0, did not produce significant amounts of IL-10 and TNFα, however, characteristic to this phenotype, they produce considerable

amounts of Ym1. These results prove that we successfully established BM-Mφs with M1, M2a and M2b phenotypes. When MSCs were also present in these cultures, they influenced the cytokine profiles of the different Mφs through affecting the production of TNFα. In M1 and M2b inductive microenvironments, they significantly reduced, while in M2a cultures, they slightly enhanced the production of TNFα, while the levels of IL-10 and Ym1 was unaffected. Thus, the ratio of cytokines in M1 and M2b cultures was influenced in favor of IL-10, therefore the presence of MSCs promote an anti-inflammatory Mφ phenotype in such inductive environments. MSCs were not able to produce any of the measured soluble factors in these experimental settings.

The repolarization capacity of bone marrow macrophages

Next, we wanted to know whether Mφs are able shift several times between the distinct activation profiles and whether MSCs are able to influence these shifts. To investigate this, we first polarized naive BM-Mφs into M1, then M2a or M2b profiles, and MSCs were added at different steps of these inductions. The cytokine production of M2a and M2b Mφs induced from M1 Mφs were also compared to the cytokine production of M2a and M2b Mφs induced from naive BM-Mφs. BM-Mφs produced significant amounts of IL-10 and TNFα after M1 induction. BM-Mφs after an M1→M2a shift established the low IL-10 and TNFα, and high Ym1 production characteristic to M2a cells. These M2a Mφs did not differ from the M2a cells established from naive BM-Mφs – according to these three factors measured. M2b cells polarized from M1 Mφs also showed a characteristic M2b cytokine profile: TNFα-dominance in the TNFα/IL-10 ratio shifted towards IL-10-dominance. The established cytokine profile was similar that of M2b cells polarized from naive Mφs but IL-10 dominated more strongly in the supernatants of the repolarized cell. As it is characteristic to M1 and M2b cells, the production of Ym1 was not significant under either M0→M1→M2b, or M0→M2b inductions. Our results show that BM-Mφs are able to repolarize after pro-inflammatory M1 stimuli into M2a and M2b profiles. Interestingly, MSCs were not able to influence the cytokine production of M2a or M2b cells during repolarization from the M1 state.

The role of prostaglandin-E2 in the interaction of bone marrow macrophages and MSCs

We wanted to know how MSCs are able to influence the cytokine production of BM-Mφs. First, we wanted to investigate the role of cell-to-cell contact. To test this, BM-Mφs and MSCs were spatially separated from each other by a semi-permeable membrane under M1, M2a or M2b induction. This separation did not change the established IL-10 and TNFα ratios in the cocultures suggesting that mostly soluble mediators are responsible for the effect of MSCs on BM-Mφs.

According to literature data and our own results from Pe-Mφ experiments, PGE-2 can be a potential mediator playing a role in the MSC–Mφ interaction. To examine the role of PGE-2, we chose M2b Mφ-induction as an experimental system, since MSCs had the most profound effect on the cytokine production of BM-Mφs under this induction. During M2b induction, we used inhibitors (Resveratrol and Celecoxib) of two of the most important enzyme isoforms in PGE-2 synthesis (COX-1 and COX-2, respectively) in the BM-Mφ–MSC cocultures. Our results showed that the inhibition of the COX-2 isoform resulted in TNFα-dominance in the established TNFα/IL-10 ratios compared to control wells (without inhibitors), while the inhibition of COX-1 alone could not cause such a shift. These experiments were repeated under M1 induction where similar results were observed. Hence, PGE-2 is one of the key factors that are responsible for the effect of MSCs establishing an IL-10-dominant cytokine production in the BM-Mφ cocultures, and this relevant PGE-2 production occurs mainly with the help of the COX-2 enzyme. To further affirm this role of PGE-2, we used different concentrations of exogenously added PGE-2 (200 pg/ml; 400 pg/ml; 800 pg/ml) to simulate the effect of MSCs under M2b induction. PGE-2 was able to trigger the shift in the TNFα/IL-10 ratio in Mφ-only cultures towards IL-10 in a dose-dependent manner. Its effect was as strong as that of MSCs even in the lowest concentration (200 pg/ml) used. Our results prove that PGE-2 has an important role in the MSC–BM-Mφ interaction.

CONCLUSIONS

1. We successfully established cell cultures from the bone marrow of C57Bl/6 mice that showed fibroblast-like morphology, adipogenic and osteogenic differentiation potential, and expressed the appropriate cell surface markers so that they can be called mesenchymal stem cells.
2. We proved the immunosuppressive nature of our MSCs in mitogen- and alloantigen-induced T-cell proliferation systems, *in vitro*.
3. We revealed the role of direct cell-to-cell contact, and the role of PGE-2 and iNOS in the suppressive effect of MSCs on activated T-cells.
4. TNFα is alone able to enhance the PGE-2 production of MSCs, while IFNγ only capable to do so in the presence of TNFα. The effect of these two pro-inflammatory cytokines is synergistic.
5. TNFα and IFNγ acts through the induction of the COX-2 enzyme to increase the PGE-2 production of MSCs. iNOS and NO act – at least partially – through the IFNγ pathway, but they join the COX-2 induction pathway independent of TNFα signaling. The induction of PGE-2 production of MSCs is a redundant process.
6. The presence of MSCs enhances the phagocytic and antigen-presenting capacity of peritoneal Mφs and shifts their cytokine production from TNFα-dominance to IL-10-dominance, thus, Pe-Mφs acquire an anti-inflammatory, M2b-like phenotype when cocultured with MSCs. These effects are stable even in the presence of M1 or other pro-inflammatory activators.
7. We could successfully differentiate homogenous, non-activated, but functional Mφs from the bone marrow cells of mice and we successfully induced their activation into M1, M2a and M2b Mφ profiles according to their cytokine production.
8. MSCs enhance the phagocytic capacity of M1 and M2b Mφs, while they diminish it for M2a Mφs. MSCs affect the cytokine production of BM-Mφs only by influencing their TNFα-production: they decrease it for M1 and M2b Mφs, while slightly increasing it for M2a Mφs. The presence of MSCs promote the formation of an M2b-like regulator Mφ phenotype in both M1- and M2b-inducing environments.
9. Bone marrow Mφs are able to repolarize from M1 to M2a and M2b states, however, MSCs are not able to influence these shifts.

10. The effect of MSCs on bone marrow Mφs is exerted mainly through soluble mediators, and we could prove the role of PGE-2 and the COX-2 enzyme isoform in the process.

LIST OF PUBLICATIONS

Publications realted to the dissertation:

Kudlik G, Hegyi B, Czibula Á, Monostori É, Buday L, Uher F (2016) Mesenchymal stem cells promote macrophage polarization toward M2b-like cells. Experimental Cell Research 348:(1) 36-45.

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