Mesencyhmal stromal-like cells set the balance of stimulatory and inhibitory signals in monocyte-derived dendritic cells

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ABSTRACT

The major reservoir of human multipotent mesenchymal stem/stromal cells (MSC) is the bone marrow (BM) with the capability to control hematopoietic stem cell (HSC) development. The regenerative potential of MSC is associated with enhanced endogenous repair and healing mechanisms that modulate inflammatory responses. Our previous results revealed that MSC-like (MSCl) cells derived from pluripotent human embryonic stem cells resemble BM-derived MSC in morphology, phenotype and differentiating potential. Here we investigated the effects of MSCl cells on the phenotype and functions of dendritic cells (DC). To assess how anti-viral immune responses could be regulated by intracellular pattern recognition receptors (PRR) of DC in the presence of MSCl cells we activated DC with the specific ligands of retinoic acid-inducible gene I (RIG-I) helicases and found that activated DC co-cultured with MSCl cells exhibited reduced expression of CD1a and CD83 cell surface molecules serving as phenotypic indicators of DC differentiation and activation, respectively. However, RIG-I-mediated stimulation of DC via specific ligands in the presence of MSCl cells resulted in significantly higher expression of the co-stimulatory molecules CD80 and CD86 than in the presence of BM-MSC. In line with these results the concentration of IL-6, IL-10 and CXCL8 was increased in the supernatant of the DC-MSCl co-cultures, while the secretion of TNF-α, CXCL10, IL-12 and IFNγ was reduced. Furthermore, the concerted action of mechanisms involved in the regulation of DC migration resulted in the blockade of cell migration indicating altered DC functionality mediated by MSCl cell-derived signals and mechanisms resulting in a suppressive microenvironment.

INTRODUCTION

Mesenchymal stromal cells (MSC) developing from multipotent hematopoietic stem cells (HSC) are preferentially localized to the bone marrow [1], but can also be obtained from umbilical cord, adipose tissue, placenta, muscle, vein wall, peripheral blood [2] and corneal stroma[3]. The main functions of MSC involve the maintenance of the bone marrow niche via inhibiting the differentiation of HSC through direct cell-to-cell contacts [4] and the release of galectin-1, angiopoetin-1, osteopontin, thrombospondin-1 and 2 together with other factors to support tissue repair through regulating cell differentiation and immunomodulation [5]. Based on their wide spread physiological functions MSC were also considered in tissue engineering and the first successful therapy based on the intravenous infusion of haplo-identical MSC for treating graft vs host disease was published in 2004 [6-8]. The inhibitory effects of MSC on DC functional activities and antigen-specific T- and B-lymphocyte expansion through blocking G0/G1 transition to the S phase were also reported [9-12]. Recently, different approaches have been developed for using MSC in clinical trials [13] (www.clinicaltrials.gov). However, the establishment and the production of reproducible, stable and well characterized human MSC has remained a challenge hampered by the heterogeneity of tissue-derived MSC [14]. Other studies focused to the phenotypic and functional characterization of MSC-like cell lines derived from different human tissues [15] and to confirm pluripotency of human ES cell-derived MSC by generating un-limited numbers of early passage MSC of consistent quality and immune suppressive properties [16]. In the present study we used a previously characterized human MSCl cell line generated from pluripotent HUES9 embryonic stem cells. The phenotype, morphology and functional attributes of these cells have previously been described [17,18] and its stability up to 15 passages has been verified. They expressed the typical cell surface markers of MSC including

CD44, CD73, CD90, CD105, but were negative for the hematopoietic markers CD34, CD14, CD133, CD45 and the embryonic markers Oct4, Nanog, ABCG2, PODXL and SSEA4. They were able to differentiate to osteogenic, chondrogenic and adipogenic cell lineages with similar potency as BM-derived MSC [17]. MSCl cells also had the capability to support the growth of undifferentiated human ESC via acting as feeder layer considered as an important MSC-associated property. Cells with potent self renewing capacity were also used as an *in vitro* model of human MSC to obtain high numbers of well characterized cells and for analyzing the immune suppressive potential and the mode of action in human primary monocyte-derived DC with both inflammatory and tolerogenic potential [19].

MSC are known to modulate immune responses via acting on multiple cell types such as DC, natural killer cells (NK), T- and B-lymphocytes [8,20] and were shown to suppress the differentiation, activation, migration and antigen presenting functions of conventional DC [20], a cell population essential for linking innate and adaptive immune mechanisms through presenting antigenic peptides to naive T-lymphocytes. This series of events can induce cell expansion and polarization of effector T-lymphocytes to Th1, Th2, Th17 and Treg directions [21]. It has also been demonstrated that BM-derived MSC can shift the differentiation of naive $CD4^+$ T-cells to an anti-inflammatory Th2 direction [22] underpinning the relevance of this type of regulation for utilization in clinical settings, exemplified by bone marrow transplantation, contact allergy and autoimmune disorders [23]. Conventional DC continuously binding and internalizing antigens use a wide spectrum of pattern recognition receptors (PRR). Upon sensing endogenous and exogenous danger signals DC become activated and migrate into the draining lymph nodes where they present their antigenic cargo to naïve T-lymphocytes. Retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated protein 5 (MDA5) are cytoplasmic PRR expressed by a wide array of cell types and play essential roles in recognizing and eliminating viruses. DC activated by the specific

ligands (ssRNA, dsRNA or DNA) of the RIG-I receptors results in the secretion of pro-inflammatory cytokines and chemokines [24]. RIG-I is also expressed and is functional in MSC and has an important role in supporting cell survival indicating its possible contribution to the regulation of MSC functional activities [25]. Human MSC express several TLR, which are involved in the regulation of cell migration and the production of immune modulatory factors. The functional properties of human MSC was suggested to depend on the type of TLR as TLR4-primed MSC gave rise to inflammatory MSC1, while TLR3-induced MSC to suppressive MSC2 cell populations [26].

Encounter of conventional DC with naïve T-lymphocytes depends on the timely migration of activated DC to the draining lymph nodes [27,28] driven by the CCR7 chemokine receptor expressed by stimulated DC, as well as by the expression of the CCR7 chemokine receptor ligands CCL19 and/or CCL21 [28]. This receptor-ligand interaction up-regulates the expression of matrix metalloproteinases (MMP), responsible for the degradation and remodelling of the extracellular matrix upon cell migration [29], while the proteolytic activity of MMP is regulated by the tissue inhibitors of MMP enzymes (TIMP). Maintaining the balance between these opposing activities MMP and TIMP are crucial for preventing uncontrolled enzymatic degradation of the extracellular matrix known to contribute to the initiation of inflammation, autoimmune disorders and cancer metastasis [30].

In a previous work we have characterized the expression patterns of MMP enzymes and TIMP inhibitors in monocytes and monocyte-derived DC subpopulations [31] and discovered that the chemokine driven migration of activated DC is regulated by the expression of the voltage gated sodium channel Nav1.7 [32]. Based on these findings in this study we aimed to investigate the effects of MSCl cells on DC activation triggered by ligand-specific RIG-I stimulation. Our results demonstrated for the first time that MSCl cells are able to modify the activation status of DC, the secretion level of cytokines and chemokines as well as the

outcome of DC-mediated T-cell polarization suggesting its profound impact on the outcome of DC functionality. We also propose that MSCl cells could be used as a feasible model for human *in vitro* studies by offering means for developing novel MSC-based strategies to design directed immune suppressive cellular therapies.

MATERIALS AND METHODS

Generation of monocyte-derived dendritic cells

Monocytes were separated from peripheral blood mononuclear cells (PBMC) of healthy blood donors drawn at the Regional Blood Center of the Hungarian National Blood Transfusion Service in accordance with the written approval of the Director of the National Blood Transfusion Service according to the directives of the European Union. PBMC were separated by Ficoll Pacque Plus (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) gradient centrifugation followed by positive selection of $CD14^+$ monocytes by using anti-CD14 coated magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). Monocytes were plated at $2x10^6$ cell/ml concentration in RPMI (Hyclone, South Logan, Utah) supplemented by10% FCS (Gibco, Paisley, Scotland) and 1% antibiotic/antimycotic solution (Hyclone, South Logan, Utah) in the presence of 100 ng/ml IL-4 and 75 ng/ml GM-CSF (Peprotech EC, London, UK) added on days 0 and 2.

Generation of mesenchymal stromal cell-like cells

Mesenchymal stromal cell-like cells derived from the human embryonic stem cell lines HUES9 and HUES1 were kindly provided by Douglas Melton, HHMI. MSCl cells were used according to the ethical permission 6681/2012/EHR. The cells were cultured on mitotically inactivated mouse embryonic fibroblast (MEF) to form embryonic bodies (EB) followed by trypsinization to obtain single cell cultures, which were further cultured on gelatin covered 10

cm plates in DMEM containing 10% FBS [18]. Cells of the confluent cultures exhibited fibroblast like morphology and were further characterized in a collaborative study with the laboratory of Balázs Sarkadi, Membrane Research Group of the Hungarian Academy of Sciences, Semmelweis University and National Blood Service, Budapest, Hungary [17]. MSCl cell passages in opto-mechanical-treated polystyrene flasks (TPP, Trasadingen, Switzerland) provided coherent cell layers in the presence of L-glutamine, 10% FCS and 1% anti-mycoticum/anti-bioticum solution (Hyclone, South Logan, Utah) in low glucose DMEM. (Hyclone, South Logan, Utah). The cultured MSCl cells were used after 10 passages.

Co-cultures of DC and MSCl cells

MSCl cells were collected by using 0.05 –0.02% Trypsin/EDTA solution in Dulbecco's PBS (DPBS), washed and cultured at a cell density of $4x10^5$ cells in opto-mechanical treated six well plates for optimal cell growth on a 8.9 cm^2/well area (TPP, Trasadingen, Switzerland). To achieve confluent cell layers the MSCl cells were cultured in 2.5 ml RPMI supplemented with 10% FCS containing 1% antimycotic/antibiotic solution (Hyclone, South Logan, Utah) for minimum 6 hours. $2x10^6$ freshly isolated monocytes were placed directly on the top of the adherent MSCl cells and the co-cultured DC were differentiated in the presence of GM-CSF and IL-4 at the same concentration as used for DC differentiation.

Activation of DC by inflammatory stimuli

On day 5 of *in vitro* DC differentiation the cells were activated by different inflammatory stimuli in the presence or absence of MSCl cells. These involved inflammatory cocktail containing 10 ng/ml TNF-α, 5 ng/ml IL-1β, 75 ng/ml GM-CSF, 20 ng/ml IL-6 and 1 μg/ml PGE_2 (Peprotech EC, London, UK), the RIG-I ligand poly(I:C) used at 25 μg/ml, or with 1 μg/ml 5'ppp-dsRNA introduced by the Lyovec transfection reagent (Invivogen, San Diego,

CA, USA). 24h after activation DC expressing CD209/DC-SIGN on the cell surface were separated by magnetic beads (Miltenyi Biotec).

Phenotypic characterization of DC differentiated in the presence or absence of MSCl cells

The phenotypic characterization of resting and activated DC was performed by flow cytomerty on day 6 of *in vitro* cell differentiation and was compared to cells co-cultured with MSCl cells (BD Biosciences, Franklin Lakes, NJ, USA), and the data were analyzed by the FlowJo software (Tree Star, Ashland, OR, USA). MSCl cells were identified by the expression of CD105, whereas DC were captured by CD209/DC-SIGN (BioLegend, San Diego, CA, USA). To measure changes in the expression levels of the CD80, CD83, CD86 and CD1a cell surface markers fluorochrome-labeled antibodies were used along with the respective isotype matched control antibodies (BD Pharmingen, San Diego, CA, USA).

RNA isolation, cDNA synthesis and quantitative RT-PCR

Total RNA was isolated by using TRIzol reagent (MRC, Cincinnati, OH USA). 2 μg of RNA was reverse-transcribed at 37°C for 120 minutes using the High Capacity cDNA Archive Kit (Appied Biosystems, Foster City, CA) and oligo-(dT) primers (Promega, Madison, WI, USA). Quantitative real-time PCR was performed by using gene-specific TaqMan assays (Applied Biosystems, Foster City, CA, USA), DreamTaq DNA polymerase (Fermentas St. Leon-Rot, Germany) in a final volume of 12.5 μl and in ABI StepOnePlus real-time PCR instrument (Applied Biosystems). The housekeeping gene h36B4 was used for data normalization. Cycle thresholds were determined by using the StepOne Software v2.1 (Applied Biosystems).

Cytokine measurements

Concentration of the pro-inflammatory cytokines IL-1β, TNF-α, IL-6, the chemokines CXCL8 and CXCL10, and that of the T-cell polarizing cytokines IL-12, IL-10 and IFNγ was measured in the supernatants of cell cultures by ELISA kits (BD Biosciences) following the manufacturer's instructions. Optical densities were determined by microplate reader (Biotek, Winooski, VT, USA).

ELISPOT assay

Activated DC previously co-cultured or not with MSCl cells were cultured with allogeneic T-cells in serum-free RPMI medium for 3 days at 37°C. The number of IFNγ secreting T-cells was detected by the avidin-HRP system (NatuTec, GmbH, Germany) and the results were analyzed by the ImmunoScan plate Reader (CTL, Shaker Heights OH, USA).

Migration assays

Migration of the differentiated DC co-cultured or not with MSCl cells was tested in a Transwell system of 6.5 mm diameter and 5μm pore size (Corning Inc., Glendale, Arizona, USA). DCs were co-cultured with MSCl cells for 6 days and were activated by PolyI:C or 5'ppp-RNA for 24h followed by the separation of DCs from the MSCl cells by using CD209/DC-SIGN magnetic beads. 10^6 isolated DC were added to the upper chamber of the transwell plate and the migration of DC was measured in the presence or absence of 200ng/ml CCL19 chemokine placed to the lower chamber. After 24h incubation at 37° the migrated cells from the lower chamber were collected and counted by flow cytometry using poly-styrene beads as controls (Fluka St. Gallen, Switzerland).

Statistical analysis

Statistical analyses were performed by the one-way ANOVA with Bonferroni post-hoc test using the GraphPad Prism v.6 software (GraphPad Software Inc. La Jolla, CA, USA). Differences were considered to be statistically significant at $*P < 0.05$; $**P < 0.01$; $***P < 0.005$.

RESULTS

MSCl cells inhibit the differentiation and activation of monocyte-derived dendritic cells

Freshly isolated $CD14^+$ monocytes were cultured in the presence of GM-CSF and IL-4 for 5 days to generate competent DC used as control. Another fraction of DC was differentiated at similar conditions together with MSCl cells added at 5:1 monocyte to MSCl ratio present throughout the differentiation process. On day 5 both the control DC and the DC co-cultured with MSCl cells were activated with poly(I:C), 5'pppRNA or with an inflammatory cytokine cocktail containing GM-CSF, IL-1β, TNF-α, IL-6 and PGE2. On day 6 the activated DC were separated from the MSCl cells by the positive selection of CD209/DC-SIGN expressing cells and their proportion was measured in both the control and the MSCl-DC co-cultures. This procedure resulted in cell populations with >90±5% DC in both the *in vitro* differentiated and the MSCl co-cultured cells indicating complete DC differentiation in both cases (data not shown). Cell surface expression of the CD1a (**Figure 1A**) and CD83 (**Figure 1B**) membrane proteins, used as indicators of DC activation, showed significant decrease when the DC were co-cultured with MSCl cells irrespective of the mode of activation induced by poly(I:C), 5'pppRNA or the inflammatory cocktail. Interestingly, stimulation of cells in the DC-MSCl co-cultures by the specific RIG-I ligand 5'pppRNA resulted in significant up-regulation of the co-stimulatory molecules CD80 (**Figure 1C**) and CD86 (**Figure 1D**) on the DC surface as compared to DC cultured without MSCl cells, whereas the presence of MSCl cells did not affect the expression levels of these markers in DC stimulated by the inflammatory cocktail or

by poly(I:C) (**Figure 1 C, D**). These results indicated the potential of MSCl cells to modulate DC activation and consequently its other functional activities.

MSCl cells modulate the cytokine secretion of activated DC

To further analyse the functional cross-talk of DC and MSCl cells the specific RIG-I ligands poly(I:C) and 5'pppRNA were added to the DC-MSCl co-cultures and the secretion levels of the pro-inflammatory cytokines IL-6 and TNF-α, the chemokines CXCL8 and CXCL10, as well as the T-lymphocyte polarizing cytokines IL-12 and IL-10 were measured in the supernatants of the activated DC, MSCl cells and DC-MSCl co-cultures. We found that both RIG-I ligands could up-regulate the secretion of IL-6 (**Figure 2A**), CXCL8 (**Figure 2C**) and IL-10 (**Figure 2E**) in the DC-MSCl co-cultures as compared to activated DC and MSCl cells cultured separately. Although IL-10 was present in the supernatants of both resting and activated DC at low but measurable levels, we could not detect IL-10 in the supernatants of activated MSCl cells (**Figure 2E**). However, the enhanced level of IL-10 in the supernatant of the co-cultured and stimulated cells suggest that IL-10 could contribute to the anti-inflammatory effects of MSCl cells presumably via inhibiting the secretion of pro-inflammatory cytokines.

Our results also revealed that the concomitant secretion of TNF-α (**Figure 2B**) and CXCL10 (**Figure 2D**) did not induce a synergistic inflammatory effect but could efficiently be inhibited by MSCl cells indicating their potent anti-inflammatory activity. In this experimental system the production of IL-12, a key Th1 polarizing cytokine was also dramatically decreased in the presence of MSCl cells (**Figure 2F**) likely owing to its potential to induce clonal expansion and differentiation of IFNγ-producing $CD4^+$ T-lymphocytes. Moreover, IL-12 was able to stimulate the production of other inflammatory cytokines such as TNF-α, IFNγ and the interferon-induced chemokine CXCL10 to multiply the inflammatory response.

In line with these results the production of IL-6 (**Figure 2A**) and CXCL8 (**Figure 2C**), both acting as important mediators of tissue repair and angiogenesis, were detected in the supernatants of DC co-cultured with MSCl cells at increased levels suggesting that they may contribute to create a local anti-inflammatory milieu in response to inflammatory signals. These observations also imply that beside direct cell-to-cell contacts cytokines and chemokines could also contribute to the regulatory functions of MSCl cells.

MSCl cells interfere with dendritic cell-mediated allogeneic T-lymphocyte activation and polarization

To further analyse the outcome of DC-mediated functional activities in the presence of MSCl cells we sought to measure the secretion of IFNγ directly by ELISA and also as a result of DC-mediated allogeneic T-lymphocyte polarization by using ELISPOT assays. Our results demonstrated that IFNγ secretion of DC could be induced by both poly(I:C) and 5'pppRNA, however the presence of MSCl cells decreased its secretion significantly (**Figure 3A**). The potential of poly(I:C) or 5'pppRNA-stimulated DC to drive allogeneic T-lymphocyte polarization confirmed that MSCl cells are able to interfere with this translational event through exerting potent anti-inflammatory effects on DC activation (**Figure 3B**). These results altogether demonstrate that DC, upon interacting with MSCl cells, can create an anti-inflammatory local environment.

The effect of MSCl cells on DC migration

Previous results demonstrated that efficient presentation of peptide antigens to naive T-lymphocytes critically depends on the migration of tissue resident DC to the draining lymph nodes [33-35]. As a novel finding our previous studies demonstrated that the low expression level of the Nav1.7 ion channel, in combination with the high expression of CCR7, is the pre-

requisite of DC migration from the site of inflammation to the lymph nodes [32]. Based on this information we used a transwell system to study the effects of MSCl cells on the migratory potential of DC in the presence or absence of MSCl cells. In this experimental setting the upper chamber contained resting DC or DC stimulated either with poly(I:C) or the inflammatory cytokine cocktail in the presence or absence of MSCl cells. The lower chamber was filled with fresh medium containing 0.5% BSA and the chemokine CCL19/MIP-3β, a strong inducer of activated DC migration guided by the membrane bound chemokine receptor CCR7. The upper and lower chambers were connected by a membrane of 5 μm pore size to allow chemokine driven migration of DC towards CCL19 gradients. The migrated cells were collected from the lower chamber and after 24h the number of migrating cells was counted by flow cytometry. In correlation with the level of CCR7 chemokine expression in the presence of MSCl cells a statistically significant decrease in the number of migratory cells was observed (**Figure 4A, B**).

Considering that the expression of matrix metalloproteinase enzymes (MMP) and their specific inhibitors (TIMP) play important roles in regulating DC migration, we also assessed the contribution of these enzymes to the regulation of MSCl cell-mediated inhibitory functions. Expression of the MMP9, MMP12, TIMP1 and TIMP2 genes, which could be induced by the interaction of the CCR7 receptor with its specific ligand CCL19, was monitored in both resting and activated DC in the presence or absence of MSCl cells. We showed that the expression of MMP9 was up-regulated (**Figure 4C**), whereas that of MMP12 was down regulated (**Figure 4D**). The relative gene expression levels of TIMP1 and TIMP2 were higher in $CD1a^-$ DC than in its inflammatory $CD1a^+$ counterpart [32] and could be associated with a DC phenotye similar to cells co-cultured with MSCl cells (**Figure 4E, F**).

Another regulatory mechanism that might be involved in regulating MSCl cell-related functions could be associated with the activity of the Nav1.7 ion channel. We have previously

shown that functionally active Nav1.7 is predominantly expressed in resting DC but its expression is decreased in parallel with DC activation thus providing a sensitive tool for correlating the state of DC activation to Nav1.7 channel activity [32]. When the expression of Nav1.7 was measured in activated DC without MSCl cells, the level of Nav1.7 expression decreased, even when the cells were stimulated by the inflammatory cocktail or by poly (I:C), but it was increased in DC suppressed by MSCl cells (**Figure 4G**). These changes are in good accordance with the low/undetectable numbers of inflammatory $CD1a^+$ DC detected in these cultures as compared to the tolerogenic $CD1a^-$ DC subset [36,37].

MSCl cells inhibit the expression of RIG-like receptor family members

Our results summarized in Figure 1 and 2 show that MSCl cells are potent inhibitors of DC functionality induced by the specific ligands poly(I:C) and 5'pppRNA of the cytosolic RIG-like receptors RIG-I, MDA5 and LGP-2 recognizing dsRNA, ssRNA or DNA [24]. Taking a step further we hypothesized that co-culturing DC with MSCl cells might have a direct inhibitory effect on the expression of intracellular RIG-I receptors. Indeed, our Q-PCR results revealed that mRNA expression of the RIG-I, MDA-5 and LGP-2 receptors belonging to the RLR family of cytosolic pattern recognition receptors is up-regulated upon stimulation by specific ligands but in the presence of MSCl cells this induction does not occur likely due to the inhibited expression of these receptors (**Figure 5A-C**) accompanied by the decreased production of IFNβ (**Figure 5D**). Based on these data we conclude that in the presence of MSCl cells the specific ligands of RIG-I could not be recognized by DC due to the down modulation of RLR receptor expression by MSCl cells.

DISCUSSION

The unique capability of MSC of different origin is to modulate the outcome of allogeneic bone marrow transplantation that has been pioneered by Le Blanc and her group in humans and was confirmed in several mice models [9,38]. These results also revealed the potential of MSC to exert multiple effects on other cell types through regulatory, anti-inflammatory and by stander effects and via targeting injured tissues as described in several diseases, such as grade IV severe acute graft versus host disease (GVHD) of the gut and liver [6] and steroid resistant severe acute GVHD [39]. The utility of MSC also involves the prevention of transplanted skin graft rejection [40], treatment of osteogenesis imperfecta by mismatched allogeneic liver-derived MSC in immune competent fetus [41], autoimmune encephalomyelitis [42], diabetes mellitus [43] and collagen-induced arthritis [44]. Further studies also demonstrated that the anti-inflammatory cytokine environment created by the transplanted MSC at the site of inflammation was able to improve the outcome of acute renal, neural and lung injury [42,45,46]. Thus in the past years MSC became clinically important cell types due to their regenerative potential that can be utilized in cell therapies aimed to treat inflammatory and autoimmune disorders or apply them for tissue engineering. This approach is also supported by the unique capability of MSC to bypass MHC compatibility for inducing immune suppression [47]. It is well established that MSCl cells can be stimulated by TLRs [26] and upon stimulation they migrate to damaged tissues driven by cytokines, chemokines and secreted extracellular matrix (ECM) proteins [48]. Beside the secretion of soluble factors the direct contact of MSC with immune cells seems to be essential for creating a supportive local milieu [49]. However, application of these cells for therapeutic interventions is limited due to the low number of MSC available. To overcome this restriction several groups explored new strategies for identifying cell lines with immune suppressive properties [50]. In a previous collaborative study we described the phenotypic, some functional as well as the differentiating capability of a cell line of embryonic origin with MSC-like phenotype and

compared its functional activities with BM-MSC and a human foreskin fibroblast cell line (HFF) [17]. This analysis demonstrated similar morphology, phenotype and functional attributes resembling BM-derived MSC and offered us to exploit the further functional characterization of these cells in terms of their immune suppressive potential. The present study provided evidence for the potent immune suppressive nature of the MSCl cell line characterized by its profound effects on multiple functional properties of resting and activated monocyte-derived DC. In a recent report the immune suppressive effects of human ESC-derived MA07 cells on CD83 expression and IL-12p70 secretion by IFNγ and LPS activated DC was observed that was associated with enhanced IL-2 induced expansion of regulatory T-cells [16].

Our results performed with human primary monocyte-derived DC also revealed that MSCl cells could affect the maturation, activation and a wide array of functional activities in a similar manner as human BM-MSC. As a consequence, the phenotype of DC was modified in the presence of MSCl cells resulting in decreased expression of the CD1a and CD83 activation markers on the surface of activated DC induced by an inflammatory cytokine cocktail or by specific ligands of the RIG-I receptors. A similar effect was described in LPS-induced activation of mice in the presence of BM-MSCs [51] and TNF-α induced stimulation of umbilical cord blood or bone marrow derived MSC [52]. In contrast, the expression of the CD80 and CD86 co-stimulatory molecules was up-regulated in the human DC-MSC co-cultures, while in mice the expression of these molecules was decreased in the presence of both murine and human BM-MSC [53,54] indicating species related differences in the inhibitory function of MSC.

The secretion of CXCL8 and IL-6 by MSC1 and CXCL10 by MSC2 cells suggested the induction of different cytokine combinations induced by these MSC subsets [26] similar to our results showing increased secretion of IL-6, CXCL8 and CXC10 by MSCl cells activated

by poly(I:C). However, no detectable changes could be observed when the RIG-I specific 5'pppRNA ligand was used for DC activation. In contrast, the activation of co-cultured DC and MSC resulted in dramatic changes in the secretion of cytokines and chemokines. Even though changes in cytokine secretion by DC have been demonstrated in the presence of MSC of various origins the mechanistic background of this regulation has not been explored.

It was also shown that MSC, isolated from mouse embryonic fibroblasts could induce the generation of IL-10 dependent regulatory DC via SOCS3 activation leading to increased secretion of anti-inflammatory IL-10 [55]. The cytokines IL-6 and CXCL8 have been considered as important mediators of tissue repair and angiogenesis. In LPS stimulated DC the presence of MSC increased the secretion of IL-6 and CXCL8 [56,57] and in response to poly(I:C) or 5'pppRNA the increased secretion of IL-6, IL-10 and CXCL8 was also detected opposing the significantly decreased levels of IL-12 and TNF-α in the supernatant of the DC-MSCl co-cultures. These results suggest that the contact and/or the communication of cells and soluble factors may trigger anti-inflammatory and/or healing mechanisms that shift the balance of the immune response to tissue regeneration and changes in cytokine and chemokine secretion.

The CCL19 chemokine is expressed in the thymus and lymph nodes and its secretion is essential for the migration of $CCR7^{high}$ DC to the draining lymph nodes where they interact with naive T-lymphocytes [33]. However, the expression of CCR7 and the migration of DC derived from the DC-MSCl co-cultures exhibited significantly decreased migration as compared to DC cultured in the absence of MSCl cells. One of the mechanisms involved in this complex regulation was discovered recently [32] showing the contribution of the voltage gated membrane channel Nav1.7, which regulates intracellular Ca^{2+} concentration in DC and also acts as a master regulator of the cell cycle. High intracellular Ca^{2+} concentration was shown to be essential for DC activation and was associated with down regulated Nav1.7

expression. As anticipated, the expression of the voltage gated sodium channel Nav1.7 was also dramatically down modulated and could be used as a sensitive indicator of the DC activation state [32]. On the contrary, Nav1.7 expression was maintained in the presence of MSCl cells, which may indicate either dysregulation of the $Ca2^{+}$ signal accompanying DC activation or an alternative pathway for the regulation of Nav1.7 expression. Either way, MSCl cells interfere with DC activation thereby identifying this sodium channel as a novel marker of suppressed DC. Overall, these data indicate that in the presence of MSCl cells the generation of DC results in 'semi-activated', $Nav1.7^{high}$ and $CCR7^{low}$ cells, which express high amounts of active MMP9 enzyme and also TIMP with inhibitory potential to induce dramatically reduced migratory potential of DCs.

Up-regulation of CXCL8 chemokine was shown to support the expression of MMP-2 and MMP-9 in trophoblast cells [58]. The balance of MMP and TIMP is also known to have an impact on cell migration, homeostasis, survival and other functional activities of immune cells via regulating the cleavage of extracellular matrix components (ECM) to generate soluble cytokines, chemokines and growth factors together with their matching receptors [59]. Considering that the regulation of the MMP-TIMP axis is also involved in the triggering of signal transduction pathways connected to DC functions, we provoked DC migration with an inflammatory cocktail or with poly(I:C) and could increase the expression level of MMP-9 and MMP-12 in DC accompanied by decreased TIMP-1 and TIMP-2 expression showing a tight control of the inflammatory response [31]. However, in the presence of MSCl cells, the expression level of TIMP was dramatically up-regulated and resulted in inhibited DC migration via blocking ECM degradation. More importantly, these effects could also modify the regulatory capacity of MMP by the secretion and proteolytic cleavage of cytokines and chemokines. Based on these results we conclude that MSCl cells are able to modulate the expression of key molecules involved in DC migration. Moreover, the presence of MSCl cells

may also have an impact on the level and activity of secreted cytokines and chemokines, and can affect the expression of their receptors for fine tuning DC activities in the actual inflammatory environment.

In vivo studies have revealed the inhibition of antigen-specific effector T-cell functions in the course of mouse allograft rejection [40], in graft versus host disease [60], autoimmune encephalomyelitis [42] and collagen induced arthritis [44]. In a murine model DC conditioned by allogeneic MSC could inhibit MHC class I and II expression and antigen presentation by DC while decreased CD69 expression on $CD8^+$ T-cells. In a human system, the presence of activated DC co-cultured with BM-MSC was shown to decrease the secretion of IFNγ by T-cells [61]. In the present study, the inhibited secretion of IFNγ could be detected by a sensitive ELISPOT assay. In this experimental setting human allogeneic T-lymphocytes, primed by activated DC and co-cultured with MSCl cells were able to down regulate T-cell polarization to the Th1 direction to a similar extent as DC co-cultured with BM-MSC. In our human *in vitro* studies MSC also could alter DC functions significantly, and together with our previous studies confirmed the potential of MSCl cells to exert inhibitory signals on antigen-specific T-cell responses.

In this contex however, the expression of RIG-like receptors has not been investigated. We hypothesized that the functional changes induced by the RLR ligand poly(I:C) are the consequence of decreased RLR expression. Our results indeed demonstrated that in the presence of MSCl cells the expression of RIG-I, MDA-5 and LGP-2 in DC was dramatically impaired. Referring to the previously described partially activated DC with tolerogenic and immune suppressive properties [62] MSCl cells may assist the maintenance of DC in a ’semi-mature’ suppressive state [63]. Our results obtained in a human system suggest that MSCl cells with unlimited proliferating capacity could be harnessed as a reliable model for inhibiting DC activation by MSC-mediated immune suppression.

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DISCLOSURE STATEMENT

The authors declare no competing financial interest.

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