Differential MSC activation leads to distinct mononuclear leukocyte binding mechanisms

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Advances in the field of Multipotent Mesenchymal Stromal cell (MSC) biology have demonstrated that MSCs can improve disease outcome when ‘activated’ to exert immunomodulatory effects. However, the precise mechanisms modulating MSC-immune cells interactions remain largely elusive. In here, we activated MSC based on a recent polarization paradigm, in which MSCs can be polarized towards a pro- or anti-inflammatory phenotype depending on the Toll-like receptor stimulated, to dissect the mechanisms through which MSCs physically interact with and modulate leukocytes in this context. Our data show that MSCs activated through the Toll-like receptor (TLR) 4 pathway increased VCAM-1 and ICAM-1 dependent binding of leukocytes. On the other hand, TLR3 stimulation strongly increases leukocytes affinity to MSC comparatively, through the formation of cable-like hyaluronic acid structures. In addition, TLR4 activation elicited secretion of pro-inflammatory mediators by MSCs, whereas TLR3-activated MSCs displayed a milder pro-inflammatory phenotype, similar to inactivated MSCs. However, the differently activated MSCs maintained their ability to suppress leukocyte activation at similar levels in our in vitro model, and this immunomodulatory property was shown here to be partially mediated by prostaglandin. These results reinforce the concept that alternate activation profiles control MSC responses and may impact the therapeutic use of MSCs.

Advances in the Mesenchymal Stem cell (MSC) field have resulted in the concept that MSCs react dynamically to the surrounding microenvironment with potent immunomodulatory effects. Recent studies have shown that MSCs, like immune cells, can respond to different ‘danger’ signals that are generated at injured sites as result of inflammation, even in cases when inflammation occurs aseptically, as in the case of ischemia and autoimmune diseases. MSCs accomplish this feature by expressing different Toll-like receptors (TLR), whose activation leads to significant cellular changes on MSCs. Based on these observations, a recent MSC1/MSC2 polarization paradigm relying on TLR4/TLR3 priming has been suggested, in which MSC1s display a pro-inflammatory phenotype, with secretion of inflammatory mediators such as IL-6 and IL-8, while MSC2s secretes anti-inflammatory mediators like IP-10 and IL-1RA and can inhibit T lymphocyte proliferation through expression of PGE2 and IDO.

Noteworthy, many immunomodulatory factors expressed by MSCs, including PGE2, IDO, and NO require close proximity for effectiveness. Therefore it is not surprising that, when exposed to inflammatory cytokines, MSCs express known adhesion molecules such as VCAM-1 and ICAM-1, which enables MSCs to sequester and modulate immune cells. Interestingly, reports involving smooth muscle cells, another cell of mesenchymal origin, describe the deposition of Hyaluronic Acid (HA) structures on the cell body following TLR3 stimulation by Poly (I:C). Classically, leukocyte adhesion is mediated by interactions between integrins (CD11/CD18) expressed on leukocytes and adhesion molecules (such as VCAM-1 and ICAM-1). However, smooth muscle cells that accumulated HA formed cable-like structures that significantly increased smooth muscle cells adhesiveness towards mononuclear leukocytes, a process that was implicated in the progression of inflammatory bowel disease. Although functional myogenic differentiation of MSCs is thought to constitute a rare event, in here we sought to further determine the impact of MSC polarization on leukocyte-MSC interaction and inflammatory responses based on the hypothesis that activation of bone marrow-derived human MSCs with Poly (I:C) would lead to HA accumulation on MSCs.
Results

TLR3 activated MSCs bind more leukocytes than TLR4-a. Based on the hypothesis that the polarization regimens would differently affect MSC-leukocyte interactions, we investigated the effects of TLR4 or TLR3 activation on the adhesion properties of MSCs and leukocytes. It is known that upon activation with inflammatory cytokines (IL-1, TNF-α and IFN-γ), MSCs express adhesion molecules like ICAM-1 and VCAM-1 to increasingly bind leukocytes. However, the effects of TLR3 and TLR4 activation on the binding property of MSCs are currently unknown. To discern net changes in leukocyte adhesion, we activated adherent cultures of MSC with lipopolysaccharide (LPS) to activate TLR4 signaling (L-MSC), PIC to activate TLR3 pathway in MSC (P-MSC) or a media control (N-MSC). We then introduced non-adherent fluorescently-labeled leukocytes of various origins (PBMCs or monocytic leukemia cell lines THP-1 and U937) to the activated MSC and incubated the co-culture for 1 h at 4°C to prohibit any physiological changes initiated by cell-cell contact. Unbound cells were then washed away and the remaining mix of bound leukocytes and MSC were lysed and red fluorescence was used to count leukocytes compared to a standard curve.

Overall, binding of PBMCs, THP-1 and U937 cells to the P-MSCs was increased when compared to N-MSCs at 24 h after MSC activation (Fig. 1a–c). Among the cell types tested, U937 cells bound more efficiently to P-MSCs when compared to PBMCs or THP-1 cells (2 × 10⁵ versus 6 × 10⁴ and 6.3 × 10⁴ respectively) (Fig. 1a–c). Sufficient activation time was critical for strong U937 cell adhesion depended on activation time, as 6 h of activation did not significantly alter leukocyte binding within the MSC groups (data not shown). After 24 h of activation more U937 cells bound to L-MSC (1.01 × 10⁵) when compared to N-MSCs (4.42 × 10⁴), however, P-MSC bound most U937 cells (2.01 × 10⁵) (Fig. 1c). Therefore, all subsequent adhesion assays were performed using U937 cells and a 24 h activation period to study the effects of activation on MSC-leukocyte binding mechanics. Noteworthy, this effect was not due to changes in MSC proliferation or viability as a result of activation, as the number of MSCs remained constant across treatment groups (Fig. 1d).

Different MSC activation cues result in different binding mechanisms. Previous studies have shown that U937 cells bind to several cell types, including activated arterial and microvascular endothelial cells, using the adhesion molecule VCAM-1. In addition, a recent study has shown that only VCAM-1, but not L-selectin, ICAM-1 or VLA-4, was upregulated by TNF-α treatment in MSCs, and this effect has been associated with MSC’s ability to adhere to endothelial cells. Therefore, we sought to determine the role of adhesion molecules in the binding of U937 to activated MSC populations.

Our results show that MSCs activated by TLR4 increase surface VCAM-1 and ICAM-1 compared to TLR3. In a representative data set, flow cytometry analysis found that VCAM-1 was expressed in 16.5% of L-MSCs and 10% of P-MSCs, compared to 4% on N-MSCs (Fig. 2a). ICAM-1 was expressed on 35.2% on L-MSC and 19.4% in P-MSC, compared to 1.9% in N-MSCs (Fig. 2a). Similar changes were observed with two other MSC donors (data not shown). As expected, the presence of VCAM-1 and ICAM-1 binding inhibitors or a combination of both during the adhesion assay resulted in a significant reduction of U937 cell binding to L-MSC (Fig. 2b). The specific inhibition of P-selectin with KF 38789 had no significant effects on adhesion (data not shown). These treatments did not effect U937 binding to P-MSC (Fig. 2b). This strongly indicates that...
although VCAM-1 and ICAM-1 are involved in the U937 binding to L-MSCs, it is not predominantly responsible for the increased adhesion seen in P-MSCs.

Hyaluronic acid is responsible for the affinity of leukocytes to P-MSCs. As previously mentioned, smooth muscle cells can be activated specifically by TLR3 agonists to create HA cables with high affinity for mononuclear leukocytes. We investigated if HA plays a role in the adhesiveness of TLR3 stimulated MSC. MSCs activated with PIC were fixed and HA was visualized using fluorescently conjugated HA binding protein (HABP). Our results show that P-MSCs have a significant deposition of HA structures outside the cell in cable-like structures that span multiple cell lengths (Fig. 3a). A common pattern of high intensity, punctate, perinuclear staining is shared by N-MSCs, L-MSCs. This staining profile was highly consistent between MSCs derived from several different donors (Supplemental Fig. 1).

To visualize the interactions between HA and U937 cells, red fluorescently labeled U937 cells were added to activated MSC cultures, allowed to bind, and then stained for HA and visualized by fluorescence microscopy as above. As expected, fluorescent-labeled U937 cells were found often associated to HA structures on P-MSCs (Fig. 3b) whereas U937 primarily bound directly to MSC cell body in N-MSC and L-MSC in reduced numbers. Further characterization of the interaction between P-MSCs and U937 through HA revealed that this phenomenon is dependent on Poly (I:C) dose and time of exposure, and the presence of serum in culture media (Fig. 3c).

Deposition of HA on P-MSCs is not due to changes in HAS and TSG-6 gene expression. Stimuli such as growth factors and cytokines can regulate the expression of HA synthase (HAS) isoforms and TSG-6 in vitro. To address potential sources of the observed

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**Figure 2 | Activated MSCs utilize different leukocyte binding mechanisms.** MSCs (10^5 cells/well, n = 6) were activated, as before, in 24 well plates for 24 h. (a) Cells were harvested and stained with anti-ICAM-1 and anti VCAM-1 for detection of surface expression by flow cytometry. ICAM-1 and VCAM-1 expression and side scatter (SSC) were used to determine positive expression compared to unstained cells (not shown). Data are representative of 2 independent experiments with 3 MSC donors. (b) Fluorescent-labeled U937 (5 x 10^5 cells/well) were allowed to bind for 1 h at 4°C to activated MSCs. In some experiments, U937 cells were incubated with specific inhibitors of LFA-1 (50 μM) and VLA-4 (40 nM) prior to the binding phase for the inhibition of ICAM-1- and VCAM-1-mediated adhesion respectively. Plates were washed and quantification of bound cell was based on standard curves of known number of cells. Legends: C: no treatment; I: ICAM-1 binding inhibition, V: VCAM-1 binding inhibition. Values are mean ± SD. *p < 0.05, n.s. not significant, by ANOVA followed by Tukey’s test for multiple comparisons.
HA, we sought to determine if the different activation pathways altered expression of any of the three HAS or TSG-6, a known HA crosslinker expressed by MSCs. MSCs were activated and RNA collected at 8 h for assessment of TSG-6, and HAS 1, 2 and 3 gene expressions. Our results show that changes to HAS and TSG-6 expression do not correlate with the changes observed in HA between the activated MSC cultures. There was a slight decrease in the gene expression of HAS1 and HAS2, and no difference in TSG-6 expression on P-MSCs compared to N-MSCs (Fig. 4). A similar gene expression pattern between N- and P-MSCs was observed at 4 h and 18 h (data not shown). In L-MSC, expression of HAS1 and TSG-6 increased while HAS2 decreased (Fig. 4). HAS3 expression was not detected in any of the MSC samples (data not shown). Despite a recent study indicating that the ribosomal house-keeping gene RPL13A displayed superior stability when compared to beta-actin or GAPDH during MSCs differentiation, we did not observe significant changes on GAPDH expression when compared to RPL13A upon MSC activation (Supplemental Fig. 3). Collectively, these data indicate the deposition of HA is not due to changes in HAS and TSG-6 expression, suggesting another mechanism is responsible for the increased HA deposition from TLR3 activation.

**Despite altering secretome profile, different activators do not affect immunomodulatory capacity of MSCs.** Previous studies indicate MSCs have the ability to secrete several immune mediators depending on the activation of discrete pathways, often resulting in polarizing effects on immune responses. To further investigate how different activators could affect the ability of MSCs to modulate immune responses, we first measured changes to the MSC secretome following TLR activation using a cytokine detection array (Fig. 5a). Of interest, the concentration of a number of cytokines commonly considered pro-inflammatory (CXCL1, IL-6, IL-8, and CCL2) were increased when MSCs were activated with LPS. In contrast, after Poly (I:C) exposure, there was only a modest increase in IL-6 and MIF. Levels of CXCL1, IL-8 and CCL2 did not differ between N-MSCs and MSCs activated with Poly (I:C), confirming that TLR3 activation leads to a less inflammatory phenotype.

Finally, we compared the immunosuppressive properties of MSC among our activation regimens. Both MSC groups (L- and P-MSCs)
inhibited inflammatory responses of splenocytes exposed to LPS or ConcanavalinA in vitro comparable to the effects seen with N-MSCs (Fig. 5b and Supplemental Fig. 2). The anti-inflammatory effects were abrogated by the addition of Indomethacin (Fig. 5b and Supplemental Fig. 2), suggesting the involvement of PGE2 on the immunosuppressive effects of MSCs as previously described10,27, but not exclusively linked to an activation pathway tested here. Interestingly, when splenocytes were allowed to adhere to activated MSCs for 1 h at 4°C prior to activation, there was an enhanced anti-inflammatory effect (Fig. 5b). However, no difference was observed when N-, L- or P-MSC were treated with hyaluronidase (Fig. 5c), suggesting HA does not directly suppress inflammatory responses in this in vitro model. These data indicate that activated MSCs are similarly capable of modulating inflammatory responses despite differences in cytokine secretion profile through prostaglandin-related mechanisms and that HA does not play a role in this process.

Discussion

Our results demonstrate that MSCs respond differently to TLR3 and TLR4 activation. TLR3 pre-activation significantly increases the number of leukocytes that bind to MSCs, predominantly through interacting with HA that accumulates into extended structures.
around P-MSCs. Conversely, MSCs activated by TLR4 displayed punctate HA accumulation that did not associate with the smaller punctate HA accumulation that did not associate with the smaller

HA is a linear, uniformly repetitive polysaccharide that, unlike other glycosaminoglycans (GAGs) whose size is relatively smaller (15–20 KDa), is usually found as a megadalton molecule and extended lengths of 2–25 μm. The specific size of HA is determined during synthesis by which of the three transmembrane glycosyltransferase (HAS 1, 2 or 3) is expressed. HAS1 and HAS2 are reported to express high molecular mass HA (>500 KDa), whereas HAS3 is associated with the synthesis of short molecular weight forms of HA (<300 KDa)4. In our study, the deposit of HA structures observed here did not correlate with changes in the gene expression of HAS1, HAS2. Moreover, HAS3 expression levels could not be detected on MSCs by PCR in our study. A previous study reported expression of HAS1, HAS2 and HAS3 decreased as MSC passage number increased, vanishing when MSCs reached senescence45. This decrease in HAS expression correlated with a decreased in VCAM-1 expression, which was rescued by exogenous HA44. Unlike this observation, in our study low passage MSCs displayed very low levels of VCAM-1 expression. Upon activation, our MSCs had increased VCAM-1 and ICAM-1 expression, but no changes corresponding to HAS expression as described above. Future studies, such as knockdown and overexpression experiments, are needed to determine the specific mechanisms by which Poly (I:C) and LPS directly change MSC cell surface molecules and the surrounding ECM.

In addition, the presence of Inter-alpha inhibitor (IαI), a protease inhibitor present in the serum, has been implicated with the assembly of HA cables in smooth muscle cells46. In our study, MSCs activated with Poly (I:C) were able to assemble HA cable structures in the absence of serum. However, serum-free HA cables looked thinner (data not shown) and serum was an essential component for the binding of U937 to the P-MSCs. The variations between other reports and the results described here may reflect sampling problems in obtaining MSCs with bone marrow aspirates, intrinsic donor differences in MSCs, usage of different passage cells, or different growth conditions47,48.

The different activation responses by MSCs are also reflected in changes to their secretome, where TLR4 activation induce the expression of several “proinflammatory” cytokines while TLR3 activation results in a mild activation state similar to normal MSCs. Despite this difference, all the MSCs were capable of strongly activating TLR4, which results in a mild activation state similar to normal MSCs, usage of different passage cells, or different growth conditions47,48.

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Methods

Cell culture. We obtained frozen vials of pre-characterized human MSCs isolated from the bone marrow of healthy donors from the Center for the Preparation and Distribution of Adult Stem Cells (NIH/NCCR grant P40 RR 17447-06) - http://medicine.tamhs.edu/trm/msc-distribution.html. For Supplemental Figure 1, we also isolated MSCs from commercially available fresh bone marrow aspirates (ABCells, Emeryville, CA) according to the protocols previously established using density centrifugation and plastic adherence49. All cultures of MSCs were screened for typical MSC spindle-like morphology and growth kinetics. MSCs were expanded by thawing a frozen vial of 10^5 passage 2 or 3 cells, and planting at 200 cells/cm^2 in 225 cm^2 flasks with 50 ml of complete culture medium (CCM) that consisted of α-minimal essential medium (α-MEM; Life Technologies), and 2 mM L-glutamine (Life Technologies). MSCs were incubated with medium replaced every 2 days until about 70% confluent. The medium was discarded, the cultures were washed with phosphate-buffered saline (PBS, Life Technologies), and 2 mM L-glutamine (Life Technologies). MSCs were incubated with medium replaced every 2 days until about 70% confluent. The medium was discarded, the cultures were washed with phosphate-buffered saline (PBS, Life Technologies), and the adherent cells harvested with 0.25% trypsin/1 mM EDTA (Life Technologies) for 2–3 min at 37°C and resuspended with fresh media for subsequent experiments.

We isolated peripheral blood mononuclear cells following an IRB approved protocol (HSC-MS-10-0190) with informed consent. Briefly, blood from healthy donors was diluted 1 : 2 with PBS and layered on top of Ficoll-Paque and centrifuged at 400 g for 30 min at room temperature. The mononuclear cell layer was carefully collected and rinsed twice with PBS and PBMCs were suspended in complete RPMI media.
MSC activation. Unless otherwise noted, MSCs were treated with Poly (I:C) (R&D Systems, Minneapolis, MN, 20 μg/ml) or LPS (Invivogen, San Diego, CA, 250 ng/ml) or media alone as a negative control, which consisted of CCM with FBS concentration reduced to 2% (2%CCM), and incubated for 24 h prior to analysis, unless otherwise specified.

VCAM-1 and ICAM-1 expression on MSCs. We plated MSCs in 6-well plates at 104 cells per well and allowed to adhere for 6 h. MSC were activated in 2% CCM, harvested and labeled with mouse anti-human VCAM-1 (1: 100, clone 51-10C9, BD Biosciences, San Jose, CA) and mouse anti-human ICAM-1 antibodies (1: 100, clone HA58, Biolegend, CA) for 15 min at 4°C. Data was acquired on an LSRII flow cytometer and analyzed with BD FACSDiva software (BD Biosciences, San Jose, CA). Unstained cells were used to set a CD106− gate at the level of background fluorescence, and approximately ten thousand singlet cells were analyzed per sample.

MSC proliferation. We activated MSCs (5 × 104 cells/well) in 96 well plates in 2% CCM (dimethylthiourea (DMSO) + 2.5-diphenyl-tetrazolium bromide (MTT) assay (Life Technologies) was performed following manufacturer’s protocol. Briefly, the MTT reagent was added (10 μl per well) and the plate incubated for 4 h to allow for intracellular reduction of the soluble yellow MTT to the insoluble purple formazan dye. Detergent reagent was added to each well to solubilize the formazan dye prior to measuring the absorbance of each sample in a microplate reader at 570 nm.

Adhesion assay. MSCs (2 × 105 cells/well) were plated in 24-well plates (Corning, Corning, NY) in 2% CCM and allowed to adhere for at least 6 h and then activated as above. U937 cells were labeled with CellTracker Red CMPTX (Life Technologies) following manufacturer’s protocol. Briefly, U937 cells (2 × 105 cells/well) were resuspended in pre-warmed CellTracker dye working solution (10 μM in serum-free RPMI) and incubated at 37°C. After 45 min, cells were resuspended in complete RPMI medium. Following 30 min incubation at 37°C, cells were washed and resuspended at 105 cells/ml in complete RPMI medium. Fluorescent-labeled U937 cells (5 × 105 cells/well) were added to wells to be used as an internal control for setting gates (1 h at 4°C as previously described45), to determine the extent to which activated MSCs could bind leukocytes without any significant physiological changes to the cells upon contact. Next, the plate was washed with cold PBS for removal of unbound cells and remaining cells were lysed with CyQUANT® Cell lysis buffer (Life Technologies) and 100 μl removed for fluorescence measurement. The number of bound cells/well was calculated based on a standard curve generated with a serial dilution of known numbers of fluorescent-labeled U937.

Adhesion inhibitor assays. To inhibit VCAM-1- and ICAM-1-dependent adhesion, we incubated U937 cells with selective, high affinity inhibitors of the VCAM-1 ligand (Eli Lilly) or the ICAM-1 ligand LFA-1 (R&D Systems, Minneapolis, MN, 20 μM) for 10 min at 37°C, harvested and labeled with CellTracker Red CMPTX (Life Technologies) and assessed for adhesion.

Immunofluorescence. We grew MSCs on 4 or 8-well Lab-Tek slide chambers (Nalge Nunc Int, Roskilde, Denmark) in 2% CCM at 5 × 105 cells/well and allowed the cells to adhere for at least 6 h. MSCs were then activated with Poly (I:C) or LPS for 24 h and cells were fixed with PBS and fixed in an acid-formaldehyde-ethanol solution (3:7, v/v) formaldehyde-PBS, 70% ethanol and 5% glacial acetic acid, all v/v). HA was detected to adhere for at least 6 h. MSCs were then activated with Poly (I:C) or LPS, alone or in the presence of Indomethacin (10 μM), and incubated for 24 h in 96 well plates in the presence or absence of Indomethacin (1 μM, Sigma). In some experiments, splenocytes were allowed to adhere to MSCs 1 h at 4°C prior to LPS treatment. Culture supernatant was collected 18 h after LPS treatment and the samples analysed utilizing a TNF-α ELISA kit (Biolegend) following manufacturer’s protocol.

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