IL-1B Primed Mesenchymal Stromal Cells Moderate Hemorrhagic Shock Induced Organ Injuries

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Research

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Abstract

Background

Organ damages following hemorrhagic shock (HS) have been partly attributed to an immunological dysfunction. The current challenge in the management of HS patients is to prevent organ injury-induced morbidity and mortality which currently has not etiological treatment available. Mesenchymal stromal cells (MSC) are used in clinical cell therapy for immunomodulation and tissue repair. *In vitro* priming is often used to improve the immunomodulation efficiency of MSC before administration.

Objective

Assess the effect of naive MSC (MSCn) or interleukin (IL)-1 β primed (MSCp) treatment in a context of HS-induced organ injury.

Methods

Rats underwent fixed pressure HS and were treated with allogenic MSCn or MSCp. Liver and kidney injuries were evaluated 6h later by histological and biochemical analysis. Whole blood was collected to measure leukocytes phenotypes. Then, *in vitro* characterization of MSCn or MSCp was carried out.

Results

Plasma Creatinine, blood urea nitrogen and Cystatin C were decrease by MSCp infusion as well as kidney injury molecule (KIM)-1 on histological kidney sections. Transaminases, GGT and liver histology were normalized by MSCp. Systemic cytokines (IL-6 and IL-10) as well as CD80, 86 and PD-1/PDL-1 axis were decreased by MSCp on monocytes and T lymphocytes. *In vitro*, MSCp showed higher level of secreted immunomodulatory molecules than MSCn.

Conclusion

An early administration of MSCp moderates HS-induced kidney and liver injury. IL-1 β priming improves MSC efficiency by promoting their immunomodulatory activity. These data provide proof of concept that MSCp could be a therapeutic tool to prevent the appearance of organs injury following HS.

Background

Hemorrhagic shock (HS) is an absolute hypovolemia leading to circulatory failure and inadequate perfusion of tissues. Deaths from hemorrhage represents a world health problem, estimated at 1.9 million deaths per year worldwide, 1.5 million of which result from physical trauma (1). Moreover, in military practice, bleeding is the second leading cause of death after head injury (2). 25% of high-risk polytrauma patients developed post-injury complications ending up with multiple organ dysfunction syndrome (MODS) (3). During the last decade, many advances have been made in early-phase management

strategies, more injured patients survive and reach critical care units. Post-hemorrhagic resuscitation improves tissue perfusion, but does not resolve ischemia-reperfusion related damage and activation of the systemic inflammatory response. Indeed, organ injury following trauma-hemorrhage has been attributed to an acquired immunological dysfunction (4, 5) with an imbalance between pro-inflammatory and anti-inflammatory counter-regulatory mechanisms. An excessive pro-inflammatory response (Systemic Inflammatory Response Syndrome, SIRS) induced by danger-associated molecular patterns (DAMPs) is characterized by local and systemic release of pro-inflammatory cytokines, complement factors, coagulation proteins as well as hormonal mediators and excessive activation of innate cells. In parallel, anti-inflammatory mediators are produced, immune cells become anergic, initiating the compensatory anti-inflammatory response syndrome (CARS) (6, 7). This systemic response aggravates ischemic organ damage. Modulating the immune response could be a potential therapeutic strategy for preventing the complication of trauma-HS as it is in sepsis (8).

Mesenchymal stromal cells (MSC) are multipotent adult stem cells and progenitors isolated from tissues of mesodermal origin such as bone marrow (9). MSC are defined by the International Society for Cellular Therapy consortium as plastic adherent, expressing positive and negative hematopoietic surface markers, and able to differentiate towards osteoblasts, adipocytes, and chondroblasts (10). MSC are used in clinical cell therapy for immunomodulation and tissue repair, mediated by direct cellular contact or by paracrine activity (11). Several evidences of MSC's therapeutic activity in other SIRS-associated pathology have already been described (12). Moreover, these cells are highly sensitive to their environment and modifying their culture conditions (priming), for example with inflammatory cytokines, can improve their immunomodulatory efficiency (13). In several studies, *in vitro* IL-1 β priming was used as a tool to maximize MSC's immunomodulation (14, 15). Taken together, there are strong arguments to propose that MSC could protect against HS-induced organ injury, however, to our knowledge, no study evaluated neither naive MSC nor primed-MSC therapy in HS models.

The objective of this study was to prevent the occurrence of organ injuries in animals undergoing HS by modulating immune system. For this purpose, early administrations of naive MSC (MSCn) or IL-1 β -primed (MSCp) have been evaluated.

Methods

MSC isolation and culture

Bone marrow were isolated from femurs and tibias of 8 Sprague-Dawley's rats. MSC were isolated by plastic adhesion, amplified up to 80–90% confluence in a complete medium containing MEM alpha (Biological Industries) supplemented with 10% fetal calf serum (FCS, HyClone) + 1% penicillin streptomycin (Gibco) and 1 ng/mL of bFGF (Peprotech), then pooled at the same ratio for each donor. The cells were frozen in MEM α + 10% Albumin (Vialebex, LFB) + 1% penicillin streptomycin + 10% Dimethylsulfoxide (Sigma Aldrich) and finally preserved in liquid nitrogen. For *in vivo* experiments, MSC were thawed and seeded at 3000 to 5000 cells/cm². Medium was changed one day after thawing. When

MSC reached reached 90 to 100% of confluence, they were harvested using trypsin-EDTA (Gibco) and suspended in a sterile syringe at a concentration of $2 \cdot 10^6$ cells/mL of Ringer Lactate solution (Fresinus).

Then, we verified that rat bone marrow MSC fulfill the minimal criteria defining MSC (10). First, their capacity to adhere to plastic and proliferate were confirmed. Rat MSC expressed the classical surface markers CD29, CD73, CD90, and CD105 and were negative for CD11b, CD45, and HLA-DR. Third, these MSC could differentiate into osteoblasts, chondroblasts and adipocytes *in vitro*. In addition, formulation (Ringer Lactate suspension) and storage (+ 4 °C) have been set up to optimize the best cell viability.

MSC priming

When MSC reached 80% of confluence the medium was changed and replaced by complete medium + 5 ng/mL rat IL-1 β (Peprotech) for 24 h. Then, primed MSC were washed twice, harvested and suspended as described above.

Animal Model

All experimental protocols, procedures and endpoints criteria were approved by the institutional animal committee of Paris University (French Ministry of Research number CEEALV/#9498).

A total of 64 Sprague-Dawley male rats (Janvier Labs) weighting 392 ± 5 g, were included in this protocol. The anesthesia was carried out by spontaneous mask ventilation with isoflurane (Iso-Vet, Piramal Healthcare). A subcutaneous injection of buprenorphine (50 μ g/kg) and 100 μ L of local anesthetic: Lidocaine hydrochloride (Xylocaine, Aguettant) 2.8 mg/mL + Levobupivacaine Chlorhydrate (Chirocaine, Abbvie) 1.4 mg/mL were performed on the incision site. These injections were repeated before the animals were awakened at the same dose. Both carotid artery and jugular vein were catheterized for bleeding the animals (controlled hemorrhage) and for administration of fluid resuscitation, treatment and blood re-transfusion. After surgery, an injection of 300 μ L heparin Choay was carried out (100 UI/mL, Sanofi). Further details are provided in the supplemental data – Methods.

Experimental protocol

46 Rats were allocated to four groups as described below:

Sham Group

Rats underwent surgery without bleeding, fluid resuscitation and blood re-transfusion. They received 0.9% saline (2 mL/h) after anesthesia.

Hemorrhage Shock Group (HS)

Blood was withdrawn to reach a 35 ± 2 mmHg MAP and maintained for 90 minutes. At T0H, Ringer Lactate infusion started (2 mL/kg/min) equal to twice the volume of spoiled blood. After 1 hour (T1H), blood withdrawan in heparinized (25 UI) syringes was re-transfused.

Treated Groups with naive MSC (MSCn) or IL-1 β -primed MSC (MSCp): At T0H after hemorrhagic shock, MCSs were slowly injected intravenously into the flow of Ringer Lactate (10^6 cells). Blood withdrawal was re-transfused at T1H.

For all animals, the catheters were removed at T2H, the skin sutured and then the isoflurane was stopped. At the time of sacrifice (T6H), samples of blood and organs (kidney, liver) were taken.

Plasma analysis

Whole blood collected from the aorta at T6H on heparinized syringe was centrifuged at 2600 g during 12 min. Plasma concentrations of cytokines: IL-1 β , IL-6 and IL-10 (Duoset, R&D) as well as Cystatin C (Quantikine R&D), were measured in duplicates using sandwich ELISA essay according to the manufacturer's instructions.

Plasma biochemistry analyses were performed using a Cobas 6000 automated analyzer in Percy military hospital (Clamart, France). Liver function was assessed by plasma levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and gamma-glutamyl transferase (GGT). Kidney function was assessed by the plasma level of blood urea nitrogen (BUN) as well as the plasma levels of creatinine.

Histological and Immunohistochemistry

The organs were fixed in 4% buffered formaldehyde, and embedded in paraffin. Sections were then cut into 4 μ m slices for Hematoxylin-Eosin-Safran (HES) (liver) and Periodic acid-Schiff (PAS) (kidney) staining. Slides were scanned and examined on CaseViewer software by two blind investigators. Kidney Tubular injury score (tubular dilation, tubular cast formation and tubular necrosis) (16) was quantified in the external medulla. Liver injury score (congestion, vacuolization and necrosis of hepatocytes) (17), was quantified on 30 randomly selected fields. For kidney injury molecule-1 (KIM-1), sections were labeled with goat anti-rat KIM-1 antibody at 1 μ g/mL concentration (R&D). Slides were scanned and 5 randomly selected fields of the kidney external medulla were analysis with Fiji software. Further details are provided in the supplemental data – Methods.

Cytometry analysis

For phenotype characterization of white blood cells, a multi-parameter analysis was carried out by flow cytometry (Navios, Beckman Coulter®). Whole blood was collected in tube field with cellular antigen stabilization solution (Transfix, Cliniscience) and kept at 4 °C up to 14 days. Red blood cells were lysed with Macs lysing solution. To minimize non-specific antibody binding, after centrifugation, cells were incubated in a buffer containing PBS, 2% human albumin (LFB) and 2 μ g/mL polyvalent human immunoglobulin (R&D Systems) before staining. Cells were then incubated with fluorescent monoclonal antibodies: CD8a (OX8, eBioscience), CD4 (OX35, eBioscience), CD3 (1F4, BioRad), CD80 (3H5, BioRad), CD279 or PD-1 (KLH, Bioss), CD274 or PD-L1 (KLH, Bioss), CD11b/c (REA, Miltenyi), CD28 (REA, Miltenyi), CD45 5REA, Miltenyi), CD86 (REA, Miltenyi) and MHC2 (REA, Miltenyi), at a saturating concentration for 20 minutes at + 4 °C. Isotype antibodies, non-stain cells and fluorescence minus one were used as

controls. The FlowJo software was used to set up gating and analyze positivity frequencies of the makers of interest.

Statistical analyses

All data are presented as median $\pm$ interquartile range and statistical analyses were performed on the Prism 7 Graphpad software. Mann-Whitney statistical analysis was used to test the effect of our hemorrhaging model versus Sham. Dunn's test (adjust for multiplicity) were used to compare the effects of the treatments (MSCn and MSCp) to the HS group. The significance level was defined as a p value < 0.05.

Results

For details on characteristics of animal model see Supplementary Results, Fig. S1, Legends, and Table S1.

MSCp attenuated hepatic injury after HS

Hemorrhagic shock resulted in liver injury visible at T6H (Fig. 1). Plasma levels of AST, ALT and GGT significantly increased in the HS group compared to the Sham group. The evaluation of HS hepatic histological sections also revealed evidence of congestion, vacuolization and hepatocyte necrosis. Injection of MSCp significantly decreased AST levels (334 [736] vs 111 [119] UI/L for HS and MSCp respectively) and GGT levels (4.00 [8.20] vs 3.00 [0.00] UI/L for HS and MSCp respectively) and reduced the total score for histological lesions in the liver compared to the untreated group (HS). On the other hand, MSCn treatment did not significantly prevent hepatocellular injury.

MSCp attenuated kidney injury and dysfunction after HS

HS resulted in an acute kidney injury (AKI) defined as a significant increase in plasma creatinine, urea (BUN), and Cystatin C levels compared to the Sham group (Fig. 2A). Furthermore, rats undergoing HS showed typical but scattered acute tubular necrosis in the outer medulla of kidney section evaluated by the presence of dilatation with epithelial cells necrosis at the proximal tubules, sometimes associated with the presence of cast in the lumen of these tubules (Fig. 2B-C). Similarly, KIM-1 expression was up-regulated after HS (Fig. 2D).

The administration of MSCn had no significant effect on AKI plasma markers compared to the HS group. However, MSCp administration significantly improved renal function, with lower levels of creatinine (68.5 [49.5] vs 36.0 [31.0] μ mol/L for HS and MSCp respectively) and Cystatin C (2.54 [1.92] 10^6 vs 1.92 [0.64] 10^6 pg/mL for HS and MSCp respectively) compared to the HS group. BUN remained non-significantly different between the MSCp and HS groups. Moreover, the administration of MSC (naive or primed) tended to improve the scoring of tubular necrosis (Fig. 2C). KIM-1 mRNA level and histochemistry staining were lower after in MSCp group (Fig. 2D).

MSCp reduced plasma cytokines concentration

Because HS induced a systemic inflammatory response, we measured plasma concentrations of the IL-1 β , IL-6 and IL-10 cytokines. At T6h, circulating levels of IL-6 and IL-10 in HS animals were increased compared to Sham rats. MSCn administration induced no difference between groups, while MSCp decreased all cytokines concentration (IL-6: 241 [3822] vs 128 [28] pg/mL for HS vs MSCp and IL-10: 98.8 [245] vs 52.3 [13.5] pg/mL for HS vs MSCp) (Fig. 3A). IL-1 β was undetectable in all animals except 4 HS and 2 MSCn (data not shown).

MSCp decreased co-receptors expression on monocytes, granulocytes or T lymphocytes

The monocyte population was identified as CD45 + CD11b/c + and low granularity whereas granulocytes were CD45 + CD11b/c + and high granularity (Fig. 3B). HS induced a minor change in monocytes phenotype, CD80 and PD-L1 were higher after HS than in Sham animals. Monocytes antigen presentation co-stimulation receptors CD80 and CD86 were strongly decreased by MSCp infusion (CD80: 4.52 [3.07] vs 0.84 [2.93] % positive cells in HS and MSCp respectively; CD86: 6.95 [11.0] vs 0 [2.89] % positive cells in HS and MSCp respectively). The inhibition marker PDL-1 was also decreased by MSCp (PDL-1: 14.4 [16.8] % vs 0.32 [1.53] % in HS and MSCp respectively). MHC II was not modulated on monocytes by any protocol (Fig. 3B). PD-1, PDL-1's receptor expressed on T lymphocytes CD8+, showed a similar decrease after MSCp infusion (PD-1 T lymphocytes CD8+, 84.7 [25.6] % vs 30.2 [28.5] % in HS and MSCp respectively). The activation marker CD28 on T lymphocytes showed a trend to decrease after MSCp infusion (Fig. 3C). The same results were found in granulocytes (data not show).

Discussion

In this report we showed that IL-1 β -primed MSC attenuated HS-induced early organ injury and dysfunction and reduced the SIRS/CARS syndrome as shown by a decrease in plasma cytokine concentrations and phenotypic activation of circulating immune cells.

The fixed pressure hemorrhagic shock model established was extremely severe as demonstrated by lactate level (18) and led to a very high mortality at 24 h with serious and irreversible organ damages. Therefore, we decided to choose the analysis time at T6H post-hemorrhage to avoided unbalance in groups due to early mortality and to allow the exploration of the driver mechanisms leading to organ injury after HS. Furthermore, at T6H we observed kidney and liver injuries as previously reported by Rönne et al. in a similar model (19). However, neither ventilation parameters nor lung histological scoring were affected by HS contrary to what has been shown by others (20). It can be argued that these authors have a more severe model of HS. Another hypothesis, as proposed previously, could be that the blood flow has been redistributed at the expense of the splanchnic, skeletal muscles and skin circulations (21) in order to preserve the circulation in the vital organs including the lungs.

In this study, MSCp infusion attenuated the early AKI and liver injury. MSCp administration tended to moderate tubular cell necrosis and significantly decrease KIM-1 in tubulars cells, a biomarker of renal

proximal tubule injury (22). MSCp also attenuated hepatocytes vacuolization and normalized transaminase plasmatic levels markers of liver injury. It was previously demonstrated that MSC therapy is effective in reducing acute kidney injury (AKI) or liver injury in diverse experimental models of isolated organ damage including ischemia/reperfusion (I/R) or hepatotoxic and nephrotoxic drug (23, 24). MSC are also currently evaluated in clinical trials for the treatment of acute kidney injury (9 registered) and liver injury (4 registered) (<https://clinicaltrials.gov>). Here we show for the first time attenuation of both liver and kidney damage in the context of HS. Nowadays, the driving mechanisms by which MSC moderate organ injury are still in debate. This could be either a direct effect of MSC, through antioxidant or trophic factors (25, 26). Or it could be an indirect, immunomodulatory effect of MSC, through a down regulation of inflammation notably by reducing systemic cytokines (20, 27).

Systemic IL-6 and IL-10 cytokines were early up-regulated in SIRS/CARS pathology (28). In this context, IL-6 is usually correlated with mortality and can be used as biomarker to identify patients at risk of developing MODS after trauma (30). We observed that HS induced an up-regulation of these pro and anti-inflammatory cytokines. MSCp infusion decreased IL-6 and IL-10 plasma levels. Granulocytes are important mediators of inflammation-induced injury (30) as well as monocytes that also contribute to both innate and adaptive immune suppression (31, 32). Moreover, a body of recent evidence demonstrates that under pathological or inflammatory conditions, granulocytes can acquire the function of antigen presenting cells. An increase in CD80 on monocytes and both CD80 and CD86 costimulatory receptors on granulocytes was observed in our study in contrast to previous observations after trauma or sepsis (33, 34). After MSCp infusion, innate cells showed a lower expression of CD80 and CD86 compared to HS alone, suggesting a reduced capacity to present the antigen to T Lymphocytes. Moreover, the regulation of the PD-1 / PD-L1 axis is a very promising pathway in trauma (35) as it is in sepsis. In a polymicrobial sepsis model, PD-1 knockout in mice increases survival rate (8). The present study showed an increase of PD-1 and PDL-1 on T Lymphocytes CD8 + and monocytes/granulocytes upon HS as reported in human (36) and MSCp decreased their expression. Therefore, MSCp therapy decreased both activation and inhibition markers on monocytes, granulocytes and T lymphocytes and the plasmatic expression of pro and anti-inflammatory cytokines. Altogether, our results suggest a return to homeostasis induced by MSCp in a dysregulated immune context.

In this report, IL-1 β -primed MSC infusion is necessary to obtain a therapeutic effect. IL-1 β pro-inflammatory priming enhance MSC's therapeutic activity through pro and anti-inflammatory mediators. A study of Amann et al. had already shown that the stimulation of MSC with IL-1 β or sera from polytrauma patients tended towards an increase in pro-inflammatory, pro-angiogenic factors (IL6, VEGFA) and chemokines (CXCL1, CCL2) in their supernatant. Their data also revealed that priming MSC with IL-1 β may improve the therapeutic effect of MSC by induction of cell adhesion molecules and anti-inflammatory and anti-fibrotic molecules (ICAM1, MMP1, MMP10, IL1RN, TNFAIP6, VEGFA) (37). Therefore, MSC seem to have a role in the orchestration of the inflammatory response by allowing the activation of immune cells in the inflammatory environment during the first phases (38, 39). Then, a switch to an inhibitory role would be set up to avoid prolonged tissue damage. The mechanisms involved could be an attraction of immune cells via chemokines and a communication through bioactives

molecules toward homeostasis state. The mechanism of the efficacy of MSC therapy remains a challenging question and requires further investigation.

Conclusion

This work demonstrates that the early administration of rat IL-1 β -primed bone marrow MSC could moderate HS induced AKI and liver injury in a context of dysregulated systemic inflammation. These works further indicate that IL-1 β priming is required to achieve a therapeutic efficiency of MSC. Our hypothesis is that early administration of MSCp could be able to modulate organ injury through attenuation of a complex immune dysfunction. Further studies are necessary to evaluate this therapeutic product in a context of traumatic hemorrhagic shock closer to the pathophysiological conditions encountered in the clinic.

Abbreviations

HS: hemorrhagic shock; MSC: Mesenchymal stromal cells; MSCn: naive MSC; MSCp: interleukin (IL)-1 β primed; KIM-1: kidney injury molecule-1; MODS: multiple organ dysfunction syndrome; SIRS: Systemic Inflammatory Response Syndrome; DAMPs: danger-associated molecular patterns; CARS: compensatory anti-inflammatory response syndrome; FCS: fetal calf serum; AST: aspartate aminotransferase; ALT: alanine aminotransferase; gamma-glutamyl transferase (GGT); BUN: blood urea nitrogen; HES: Hematoxylin-Eosin-Safran; PAS: Periodic acid-Schiff; AKI: acute kidney injury; I/R: ischemia/reperfusion;

Declarations

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Authors' contributions

C.A. and N.B. designed and conducted the study, analyzed the data, and wrote the manuscript; M.G. conducted in vitro the studies and analyzed the data; C.C. conducted an in vitro experiment included in the manuscript and analyzed the data; E.V. participated in the design of the study the study and and proofreading of the manuscript; S.B. participated in the design of the study the study and and

proofreading of the manuscript and J.P. designed the study, analyzed the data and wrote the manuscript. The authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article. These data are available from: Juliette.peltzer@inserm.fr.

Ethics approval and consent to participate

All experimental procedures and were performed in accordance with the European Community Council Directive (2010/63/UE) for the care and use of laboratory animals. Procedures on animals were authorized by the Ministère de l'Education Nationale, de l'Enseignement Supérieur et de la Recherche after approbation by the National Committee for Ethics in Animal Experimentation (CEEALV/#9498).

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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Figures

Figure 1

Hepatic injury (A) Plasma AST, ALT and GGT levels (n= 10, 14, 10 and 12 in Sham, HS, MSCn and MSCp groups respectively). (B) The liver was collected and stained by HES. Representative pictures are presented for every experimental group. (C) Liver injury scores (n= 9, 14, 10 and 12 in Sham, HS, MSCn and MSCp groups respectively). Data are expressed as median and interquartile range in the four experimental groups. Mann-Whitney statistical analysis was used to test the effect of our hemorrhaging

model versus Sham, # $p < 0.05$, ### $p < 0.005$ or #### $p < 0.001$. Dunn's test (with adjustment for multiplicity) was used to compare the effects of the treatments (MSCn and MSCp) to the HS group, * $p < 0.05$ or ** $p < 0.01$. HS: Hemorrhagic Shock, MSCn or MSCp: naive or primed Mesenchymal Stromal Cells, AST: Aspartate Aminotransferase, ALT: Alanine Aminotransferase, GGT: Gamma-Glutamyl Transferase, HES: Hematoxylin-Eosin-Safran.

Figure 2

kidney injury and dysfunction (A) Plasma levels of Creatinine, BUN and Cystatin C (n= 10, 14, 10 and 12 in Sham, HS, MSCn and MSCp groups respectively for Creatinine and BUN; n= 10, 14, 10 and 11 in Sham, HS, MSCn and MSCp groups respectively for Cystatin C). (B) The kidney was harvested and stained with PAS and immunohistochemistry for KIM-1. Representative images of the tubules of the renal external medullary pictures are presented for each experimental group. (C) Tubular injury scores (n= 10, 14, 10 and 12 in Sham, HS, MSCn and MSCp groups respectively). (D) KIM-1 quantification by immunohistochemistry and whole kidney mRNA (n= 10, 11, 10 and 12 in Sham, HS, MSCn and MSCp groups respectively for KIM-1 immunohistochemistry; n= 10, 14, 10 and 12 in Sham, HS, MSCn and MSCp groups respectively for KIM-1 mRNA). Data are expressed as median and interquartile range in the four experimental groups. Mann-Whitney statistical analysis was used to test the effect of our hemorrhaging model versus Sham, ### p <0.005. or #### p<0.001. Dunn's test (with adjustment for multiplicity) was used to compare the effects of the treatments (MSCn and MSCp) to the HS group, * p value <0.05 or ** p<0.01. HS: Hemorrhagic Shock, MSCn or MSCp: naive or primed Mesenchymal Stromal Cells, BUN: Blood Urea Nitrogen, PAS: Periodic Acid Schiff, KIM-1: Kidney Injury Molecule-1.

Figure 3

Systemic inflammation (A) Circulating concentrations of anti and pro-inflammatory cytokines IL-6 and IL-10 (n= 10, 13, 10 and 12 in Sham, HS, MSCn and MSCp groups respectively). (B) At the top, representative histograms of flow cytometry gating strategies. Following identification of single cells, the debris were eliminated. The CD11b/c+ CD45+ population were selected and then, monocytes or granulocytes were separated upon their side squatter and CD45 characteristics. Below, frequency of monocytes parents'

positives cells were expressed for CD80, CD86, MHC II and PD-L1 markers (n= 6, 7, 3 and 9 in Sham, HS, MSCn and MSCp groups respectively). (C) At the top, representative histograms of flow cytometry gating strategies. Following identification of single cells, the debris were eliminated. The lymphocyte population were selected and then, CD4+ or CD8a+ cells were separated. Below, frequency of monocytes parents' positives cells were expressed for CD28 and PD-1 markers (n= 6, 7, 3 and 5 in Sham, HS, MSCn and MSCp groups respectively for CD28+ and CD4+; n= 6, 7, 3 and 6 in Sham, HS, MSCn and MSCp groups respectively for CD8a+). Datas are expressed with interquartile range. Mann-Whitney statistical analysis was used to test the effect of our hemorrhaging model versus Sham, # p <0.05, ## p <0.01. Dunn's test (with adjustment for multiplicity) was used to compare the effects of the treatments (MSCn and MSCp) to the HS group, * p value <0.05 or ** p<0.01. HS: hemorrhagic shock, MSCn or MSCp: naive or primed Mesenchymal Stromal Cells, IL-6: Interleukin-6, IL-10: Interleukin-10, FSC-A: Forward Scatter-Area, FSC-H: Forward Scatter-Height, SSC-A: Side Scatter-Area, PD-L1: Program Death- Ligand 1, PD-1: Program Death-1, MHC II: Major Histocompatibility Complex II.

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