A Proinflammatory Secretome Mediates the Impaired Immunopotency of Human Mesenchymal Stromal Cells in Elderly Patients with Atherosclerosis

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ABSTRACT

Inflammation plays a pivotal role in the initiation and progression of atherosclerosis (ATH). Due to their potent immunomodulatory properties, mesenchymal stromal cells (MSCs) are evaluated as therapeutic tools in ATH and other chronic inflammatory disorders. Aging reduces MSCs immunopotency potentially limiting their therapeutic utility. The mechanisms that mediate the effect of age on MSCs immune-regulatory function remain elusive and are the focus of this study. Human adipose tissue-derived MSCs were isolated from patients undergoing coronary artery bypass graft surgery. MSCs:CD4^{+}T-cell suppression, a readout of MSCs' immunopotency, was assessed in allogeneic coculture systems. MSCs from elderly subjects were found to exhibit a diminished capacity to suppress the proliferation of activated T cells. Soluble factors and, to a lesser extent, direct cell-cell contact mechanisms mediated the MSCs:T-cell suppression. Elderly MSCs exhibited a proinflammatory secretome with increased levels of interleukin-6 (IL-6), IL-8/CXCL8, and monocyte chemoattractant protein-1 (MCP-1/CCL2). Neutralization of these factors enhanced the immunomodulatory function of elderly MSCs. In summary, our data reveal that in contrast to young MSCs, MSCs from elderly individuals with ATH secrete high levels of IL-6, IL-8/CXCL8 and MCP-1/CCL2 which mediate their reduced immunopotency. Consequently, strategies aimed at targeting proinflammatory cytokines/chemokines produced by MSCs could enhance the efficacy of autologous cell-based therapies in the elderly. STEM CELLS TRANSLATIONAL MEDICINE *2017;6:1132–1140*

SIGNIFICANCE STATEMENT

This study provides novel insights into the functional characterization of adipose tissue derived human mesenchymal stromal cells (MSCs). Our data suggest that MSCs from elderly patients with atherosclerosis have reduced immunopotency and secrete senescence associated inflammatory cytokines. The neutralization of IL-6, IL-8 and MCP-1 improves the defective immunomodulatory function of elderly MSCs. This work emphasizes the relevance of appropriate donor selection for MSCs based therapies and the potential for modulating the MSCs secretome as a way to enhance their therapeutic benefit. The integration of this knowledge into clinical trial design could enhance the efficacy of MSCs therapy.

INTRODUCTION

Atherosclerosis (ATH) is a complex chronic inflammatory disease involving aberrant immune responses resulting in the development of atheromatous plaques within the walls of the coronary, cerebrovascular, and peripheral arteries. The complications of ATH (e.g., myocardial infarction, stroke) are the leading cause of mortality worldwide accounting for 16.7 million deaths each year [1, 2].

The immune system plays a crucial role in the development and progression of atherosclerotic plaques. Activated T-cells, at the site of the atherosclerotic lesion, are key players in plaque progression and instability [3]. Indeed, the use of an anti-CD3 antibody resulted in the reduction of T-cells in the plaques and regression of established lesions in murine models of ATH [4, 5]. Further, the lipid-lowering agents statins exert immunomodulatory properties through the inhibition of T cell activation contributing to plaque stabilization [6, 7]. Due to the evidence supporting the role of inflammation in the etiology and pathophysiology of ATH, ongoing large-scale placebo-controlled clinical trials are evaluating the clinical efficacy of anti-inflammatory strategies for the treatment of ATH. Among them are the Canakinumab Antiinflammatory Thrombosis Outcomes Study-CANTOS, which is assessing the relevance of

interleukin-1β inhibition in ATH prevention, and the Cardiovascular Inflammation Reduction Trial (CIRT), which is evaluating the effect of low-dose methotrexate in patients with a high prevalence of subclinical vascular inflammation) [8, 9]. While awaiting the results of these studies, it is critical to assess alternative anti-inflammatory strategies for plaque stabilization.

Mesenchymal stromal cells (MSCs) possess a strong ability to migrate to inflammatory sites, where they serve as potent modulators of immune responses with a net tolerogenic effect [10–13]. Because of their immunoregulatory capacity, MSCs are being tested in clinical studies as cellular therapies for a variety of inflammatory conditions. In fact, preclinical studies have shown that adoptively transferred MSCs can prevent allograft rejection via modulation of immune responses [14, 15] and can improve various autoimmune diseases [16–18]. Similarly to statins, MSCs have recently been shown to exhibit multifactorial and pleiotropic therapeutic potential. Indeed, injection of MSCs in a murine model of ATH reduced plaque progression and dyslipidemia, ultimately promoting plaque stabilization and preventing its rupture with subsequent atherothrombosis [19].

Although MSCs-based therapies are a promising strategy for immunomodulation, previous work from our group and others have revealed that aging is independently linked to reduced MSCs immunomodulatory function potentially limiting their therapeutic effects [20, 21]. This is especially problematic considering the prevalence of ATH among elderly individuals and the potential advantages of using autologous MSCs [22]. The causes of the age-associated reduction of MSCs immunoregulatory capacity remain undefined. The aim of this study was to explore the mechanisms underlying the reduced immunomodulatory capacity of aged human MSCs from atherosclerotic patients, and the impact of their modulation in restoring MSCs function. The data from this study may potentially provide insights into how the immunomodulatory efficacy of aged MSCs can be enhanced both *in vivo* and *ex vivo* for therapeutic application. Further, our results may unveil a mechanistic link between the age-induced decline in MSCs immunomodulatory function and the increased frequency of inflammatory diseases (e.g., ATH) associated with age.

MATERIALS AND METHODS

Study Subjects

The McGill University Health Center Ethics Review Board approved the study, and participants provided written informed consent. Subcutaneous ($n = 28$) and pericardial ($n = 8$) adipose tissue was obtained from consecutive patients undergoing elective coronary artery bypass graft surgery. Exclusion criteria were a history of systemic autoimmune disease, cancer and acute or chronic infections.

Isolation of MSCs

Subcutaneous and pericardial adipose tissue (1–4 g) were washed extensively with phosphate-buffered saline (PBS), minced with surgical scissors and digested with 0.05% collagenase (Sigma-Aldrich Corporation, St. Louis, MO, USA) dissolved in Hank's balanced salt solution (Invitrogen, Waltham, MA, USA). Following the neutralization of collagenase, the sample was centrifuged at 2,000 rpm for 5 minutes and the supernatant was discarded. The pellet was resuspended in complete medium (CM) (1.0 g/l glucose, with L-glutamine and sodium pyruvate Dulbecco's modified Eagle's medium (DMEM) (Wisent Biotechnologies, St. Bruno, QC,

Canada), supplemented with 10% MSCs qualified fetal bovine serum (FBS) and 1% penicillin / streptomycin (10,000 unit/ml Penicillin, 10,000 mg/ml Streptomycin—Life technologies, Waltham, MA, USA). Digested tissue was cultured under standard conditions (5% carbon dioxide; 37°C) in 75-cm^2 tissue culture flasks (1 gram of tissue per flask). Two days after isolation, nonadherent cells were washed off and CM was added. Subsequently, at 80% confluency, MSCs were trypsinized and subcultured at a density of 5,000 cells per cm^2 [23].

MSCs Characterization

Immunophenotypic characterization of MSCs was performed according to criteria established by the International Society for Cellular Therapy [24] by multiparametric flow cytometry (BD LSRII; Becton Dickinson Co, Mountain View, CA, USA). Passage 2 MSCs were treated with Fc receptor blocking reagent and stained with the following fluorochrome-conjugated monoclonal antibodies (BD Biosciences, Mississauga, ON, Canada)): fluorescein isothiocyanate-conjugated anti-CD90 and anti-CD45; phycoerythrin (PE)-conjugated anti-CD73; allophycocyanin (APC)-conjugated anti-CD34, anti-CD19 and anti-HLA-DR; peridinin chlorophyll -conjugated anti-CD105, anti-CD44, and anti-CD14. Nonspecific staining was determined by incubation of similar cell aliquots with isotype controls. Data was analyzed with FlowJo software v9.7.2 (FlowJo, LLC, Ashland, OR, USA). In all samples, CD44, CD73, CD105, and CD90 expression was more than 95% while CD45, CD34, CD19, CD14, and HLA-DR expression was less than 5% (Supporting Information Fig. 1A).

Multilineage Differentiation Assays

At passage 3, MSCs were plated in 24-well plates at a density of 5,000 cells per cm^2. At ∼90% confluence, cells were incubated in one of the three differentiation mediums for 3 weeks as per the manufacture's protocol (StemPro Adipogenesis, Osteogenesis, Chondrogenesis Differentiation Kit, Waltham, MA USA). Cells were then fixed with 4% formaldehyde and stained with alizarin red S (Sigma-Aldrich) and oil red O (Sigma-Aldrich) to assess osteogenic and adipogenic differentiation, respectively. For chondrocyte differentiation, MSC micromass cultures were prepared as detailed in the StemPro Chondrogenesis Differentiation Kit. OCT mounting, cryostat sectioning and stains (Alcian blue and Safranin O) were performed by the Histopathology Platform at the MUHC-RI. (Supporting Information Fig. 1B).

Peripheral Blood Mononuclear Cell Isolation, Carboxyfluorescein Succinimidyl Ester Fluorescent Dye Labeling, and Activation

Peripheral blood mononuclear cell (PBMCs) were separated by Ficoll-Hypaque density gradient centrifugation (FICOLL 400*- Sigma-Aldrich) and cultured in 10% FBS RPMI (Wisent Biotechnologies) medium overnight to deplete monocytes. The efficacy of monocyte depletion (95%) was verified by flow cytometry. To assess the effect of MSCs on suppressing monocyte-depleted PBMCs proliferation, PBMCs were labeled with 10 uM carboxyfluorescein succinimidyl ester (CFSE) (Sigma), stimulated with anti-CD3/CD28 beads (1 bead per cell) (Dynabeads Human T-Activator CD3/CD28, Life Technologies) [25] and cultured for 4 days with MSCs.

Cocultures

The capacity of MSCs to suppress proliferative responses of activated CD4$^+$ and CD8$^+$ T-cells was assessed in a 4-day allogeneic coculture system (i.e., MSCs from different ATH donors were
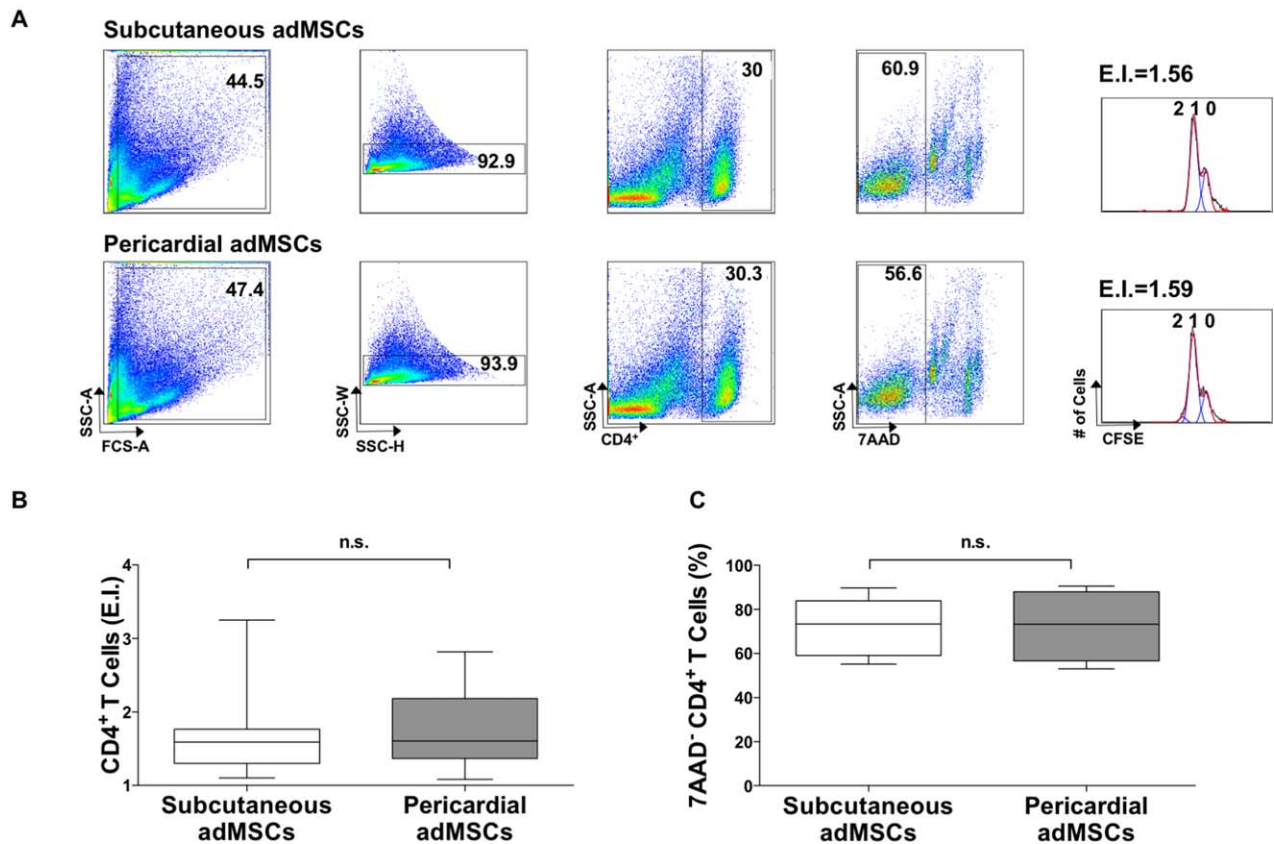
Figure 1. MSCs from pericardial and subcutaneous adipose tissue equally suppress T-cell proliferation. **(A)**: Representative example of a flow cytometry proliferation analysis of monocyte depleted peripheral blood mononuclear cells in coculture with subcutaneous or pericardial MSCs. MSCs from subcutaneous and pericardial fat have similar ability to suppress activated T-cells' proliferation **(B)** and to support T-cell viability **(C)** ($n = 8$). Abbreviations: 7 AAD, 7-aminoactinomycin D; adMSCs, adipose tissue-derived MSCs; EI, expansion index; CFSE, carboxyfluorescein succinimidyl ester; FSC-A: forward scatter area; SSC-A: side scatter area; SSC-H: side scatter height; SSC-W: side scatter width.

cultured with monocyte depleted PBMCs obtained from a single unrelated healthy donor [26]. MSCs were plated at 75×10^3 cells per well in flat-bottom 24-well plates (Corning, Corning, NY, USA) and cultured overnight. Activated monocyte-depleted CFSE-stained PBMCs (6×10^5 cells) were then cultured for 4 days with MSCs either in cell-cell contact-dependent (direct cocultures) or -independent conditions (transwell cultures) (MSCs:PBMCs ratio 1:8). In the later, MSCs and T-cells were separated by a 0.4 micrometer pore size membrane (Millipore, Etobicoke, ON, Canada). At day 4, cells were stained with CD8-PE, CD4-APC and with the cell viability marker 7-aminoactinomycin D (7AAD). T cell proliferation was calculated with the Proliferation Platform of the FlowJo software and expressed as Expansion Index (EI). EI determines the fold-expansion of the overall culture and is calculated based on the following formula,

$$\frac{\sum_0^i N_i}{\sum_0^i \frac{N_i}{2^i}}$$

where i is the generation number, and N_i is the number of events in generation i [27].

Flow Cytometry Analysis for γH2AX

Passage 4 MSCs were fixed in cytofix solution for 10 minutes followed by permeabilization for 30 minutes in 0.5% Triton X-100 (Sigma cat#93443) in PBS. Subsequently, cells were incubated in blocking solution [1% BSA, IgG free, protease free, 4% normal donkey serum (Jackson ImmunoResearch, West Grove, PA, USA: cat#001-000-162; Sigma cat#D966)] for 60 minutes prior to incubation with γH2AX antibodies overnight at 4°C. Cells were then washed with PBS and analyzed by flow cytometry (FACS). Background staining was determined by incubation of similar cells without any antibodies. Data was analyzed with FlowJo software v9.7.2.

Flow Cytometry Analysis of Reactive Oxygen Species

Intracellular reactive oxygen species (ROS) was determined with 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA). Passage 4 MSCs were trypsinized and stained with DCFDA (10 μM; Sigma) in PBS at 37°C for 30 minutes. Fluorescence intensity was measured by FACS and data was analyzed with FlowJo software v9.7.2.

Cytokine Array and Enzyme-Linked Immunosorbent Assays

MSCs were plated in 6-well plates at a density of 1×10^5 cells per well in 2 ml CM. Cells were cultured for 4 days and supernatants were collected and frozen at -80°C for both cytokine arrays and enzyme-linked immunosorbent assays (ELISA). Secreted levels of cytokines and chemokines in MSCs supernatants were screened with the R&D Systems Human Cytokine Array (Minneapolis, MN,

USA) and the multispot electrochemiluminescence immunoassay V-Plex Pro inflammatory Panel (MesoScale Discovery, Rockville, MD, USA: IFN-γ, IL-10, IL-12p70, IL-13, IL-1β, IL-2, IL-4, IL-6, IL-8/CXCL8, TNF-α) according to the manufacturer's instructions. For the V-Plex inflammatory panel ratio heat plot analysis, the value of each individual cytokine was normalized to the average value of that cytokine in all adult MSCs samples ("control group"). Fold increase or decrease of individual cytokines compared to the control group are reported. When the concentration of a sample was under the limit of detection (determined by the standard curve) or undetectable, that value was replaced by the limit of detection value of the standard curve in order to generate a ratio. The factors that were differentially expressed between adult and elderly MSCs in the cytokine array but were not captured by the V-Plex were confirmed by ELISA (i.e., interleukin (IL)−6, IL-8/CXCL8, monocyte chemoattractant protein (MCP-1), (Life Technologies) and macrophage migration inhibitory factor (MIF) (R&D Systems).

In Vitro Inhibition of IL-6, IL-8/CXCL8, MCP-1/CCL2, and MIF

To evaluate the functional implications of IL-6, IL-8/CXCL8, MCP-1/CCL2, and MIF as mediators of the MSCs:CD4$^+$ T-cell suppression, neutralization assays were performed by adding anti-IL-6 (20 μg/ml) (Abcam, Toronto, ON, Canada), anti-IL-8/CXCL8 (10 μg/ml), anti- MCP-1/CCL2 (Abcam) (45 μg/ml) [28] monoclonal antibodies or a MIF antagonist (S,R)-3-(4-Hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid (ISO-1) (85 nn/ml) (Santa Cruz Biotechnology, Dallas, TX, USA) [29] at the time the cocultures were started.

Statistical Analysis

All analyses were performed using the GraphPad Prism software (Graph-Pad, San Diego, CA, USA). Wilcoxon matched-pairs signed rank test was used to assess differences in the in vitro inhibition assays, whereas Mann-Whitney test was used for the comparisons between the adult and elderly MSCs. All data are expressed as mean ± standard deviation. All hypotheses tests were two-sided and a p value of <.05 was considered statistically significant.

RESULTS

MSCs From Pericardial and Subcutaneous Adipose Tissue Equally Suppress T-Cell Proliferation

Understanding the immunological properties of MSCs is key to the development of cell therapies [30]. Studies directly comparing MSCs from different tissues have consistently shown that adipose derived MSCs (adMSCs) have stronger immunosuppressive capabilities than alternative sources. However it is not known whether pericardial and subcutaneous adMSCs possess similar functional properties [31]. Suppression of proliferative responses of anti-CD3/CD28-activated CD4$^+$T-cells was thus assessed in MSCs isolated from pericardial and subcutaneous adipose tissue. MSCs were obtained from the same subjects in order to prevent donor-specific differences including age, genetic background, and medications taken at the time of sample collection ($n = 8$, ages = 38–75). Pericardial and subcutaneous adMSCs fulfilled the criteria proposed by The International Society for Cellular Therapy for defining multipotent MSCs (i.e., plastic adherence, tri-lineage differentiation and expression of positive and negative surface markers) and expressed similar levels of reactive oxygen species (ROS, DCFDA) and double-strand DNA breaks (γH2AX), two
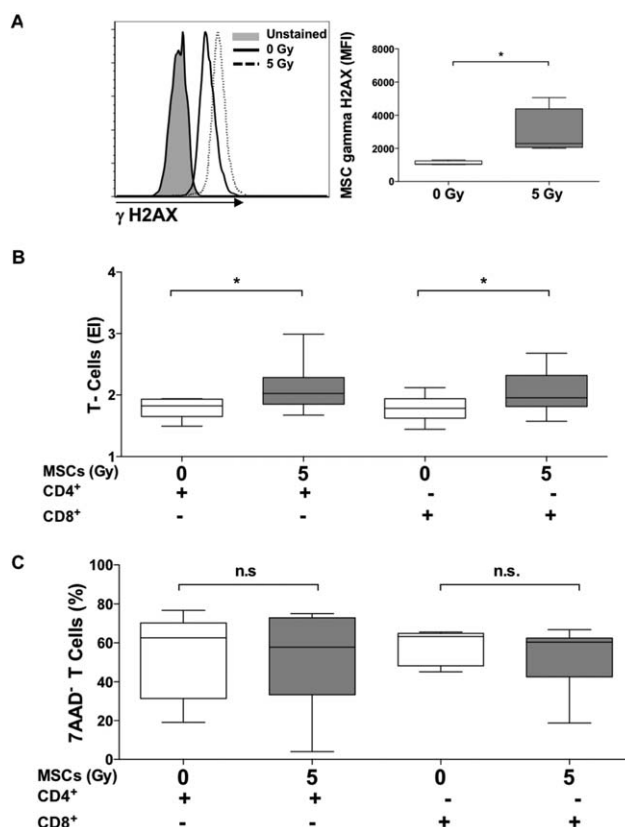

Figure 2. DNA damage impairs MSCs immunopotency. **(A)**: MSCs radiation (day 2 post-5 Gy) induces γH2AX phosphorylation reported as MFI (*, $p = .04$, $n = 4$). **(B)**: Irradiated MSCs have impaired CD4$^+$ and CD8$^+$T-cell suppressive ability (*, $p = .03$, $n = 6$). **(C)**: Irradiated MSCs do not affect CD4$^+$ and CD8$^+$ T cell viability (7AAD viability staining-FACS) ($n = 6$). Abbreviations: 7AAD, 7-aminoactinomycin D; EI, expansion index; Gy, gray unit; MFI, mean fluorescence intensity; MSCs, mesenchymal stromal cells.

hallmarks of cellular aging (Supporting Information Fig. 2). Subcutaneous and pericardial adMSCs had equal potency to suppress T cell proliferation (EI-CFSE) and similar viability (7AAD$^−$) at the end of the four day cocultures (Fig. 1). Although we cannot exclude the possibility of other functional differences between these two MSCs sources, our data suggests that the easily accessible subcutaneous adMSCs could be used as surrogates to estimate the T-cell suppressive effects of epicardial MSCs. On the other hand, the benefits reported in the use of subcutaneous adMSCs in subjects with acute myocardial infarction (APPOLO Trial; [32]) and chronic ischemic heart disease (PRECISE Trial; [33]) emphasize the relevance of quantifying and potentially optimizing the function of those cells for clinical use.

DNA Damage Reduces MSCs Immunopotency

Our group previously reported that irradiation-induced DNA damage leads to a cellular senescence phenotype in human adMSCs including the production of pro-inflammatory cytokines [34, 35]. To determine whether DNA damage would also affect the immunomodulatory properties of MSCs, we first treated MSCs with 5Gy gamma irradiation and then assessed for changes in immunopotency. As expected for this DNA damage marker, irradiation induced the phosphorylation of histone H2AX (γH2AX) in MSCs
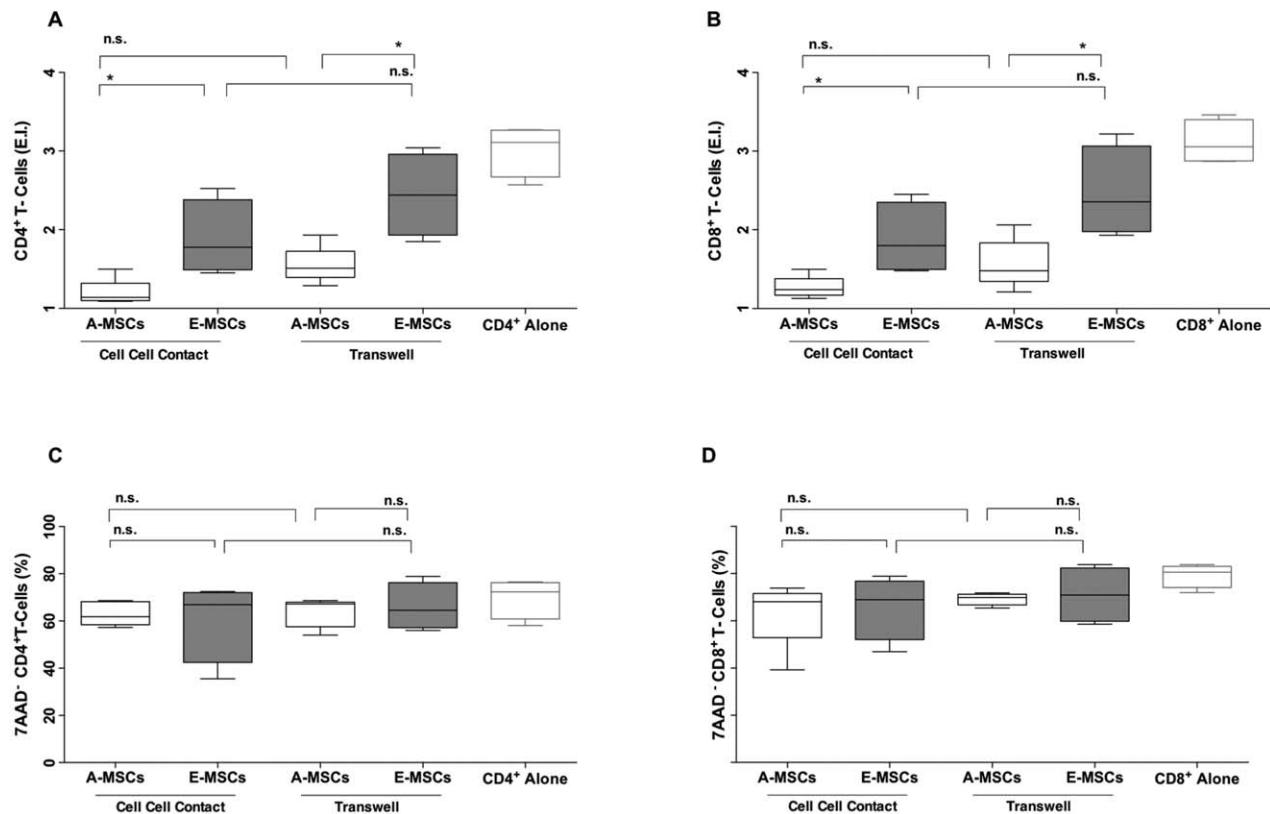
Figure 3. Soluble factors mediate the impaired immunopotency of elderly MSCs. MSCs immunopotency was assessed in cocultures either in direct contact with T lymphocytes (cell-cell Contact) or in a transwell system. Reduced suppressive effect of E-MSCs compared to A-MSCs on **(A)** CD4^{+} and **(B)** CD8^{+} T-lymphocyte proliferation in either direct contact (*, $p = .01$, A-MSCs $n = 5$, E-MSCs $n = 4$) or transwell (*, $p = .03$; *, $p = .05$, A-MSCs $n = 5$, E-MSCs $n = 4$) conditions. MSCs have equal ability to maintain **(C)** CD4^{+} and **(D)** CD8^{+} T cell viability (7AAD viability staining-FACS) either in direct contact or transwell conditions. Abbreviations: 7AAD, 7-aminoactinomycin D; A-MSCs, adult MSCs; E-MSCs, elderly MSCs.

(Fig. 2A), and also reduced their efficiency to suppress both CD4^{+} and CD8^{+} T-cell proliferation (Fig. 2B). It has been suggested that MSCs can induce apoptosis of T-cells [36], which could account for the impaired immunomodulatory function of irradiated MSCs. However, MSCs irradiation did not impact CD4^{+} and CD8^{+} T cell viability in coculture experiments (Fig. 2C).

Soluble Factors Mediate the Impaired Immunopotency of Elderly MSCs

The DNA damage theory of aging states that accumulation of DNA damage or chromosomal abnormalities over time can lead to cell dysfunction associated with cellular senescence [37, 38]. Given that ATH is an age-associated disease and in light of the above-described results linking MSCs DNA damage to their reduced immunosuppressive capacity, we assessed whether chronological aging in the context of ATH recapitulates hallmarks of DNA damage induced MSC senescence. Specifically, we compared the phenotype of MSCs from elderly ATH patients (E-MSCs; > 65 years old) to those of adult ATH patients (A- MSCs; <65 years old). E-MSCs not only had a larger cellular size (Supporting Information Fig. 3A) but also displayed ∼twofold increase in both γH2AX levels (Supporting Information Fig. 3B), a marker of DNA double strand breaks [39], and intracellular ROS levels (Supporting Information Fig 3C). We next conducted cell-cell contact dependent and independent (transwell) cocultures to assess the relevance of soluble factors as mediators of MSCs:T-cell suppression. Our results

indicate that the effect of T-cell suppression occurs in transwells but is enhanced by 20% when MSCs and T-cells are in direct contact (Fig. 3A, 3B). The suppressive ability of A-MSCs ($n = 5$, 55 ± 5.1) on both CD4^{+} and CD8^{+} T-cell proliferation was more effective than that of E-MSCs ($n = 4$, 74 ± 6.1), an effect that was not explained by differences in proliferation rates between A-MSCs and E-MSCs (Supporting Information Fig. 4) nor differences in MSCs-induced T-cell apoptosis (Fig. 3C, 3D). As a result, we conclude that (a) MSCs- suppression of T-cell proliferation is primarily mediated by secreted soluble factors, and (b) A-MSCs are superior to E-MSCs in inhibiting CD4^{+} and CD8^{+} T-cell proliferation.

Elderly MSCs Secrete Higher Levels of Senescence Associated Cytokines

It is now widely accepted that various factors secreted by MSCs (i.e., MSCs secretome) are responsible for their immunosuppressive function [40]. We hypothesized that relative to A-MSCs, E-MSCs may exhibit an altered secretome that would consequently account for their impaired immunomodulatory capacity. To test this, MSCs conditioned media was first profiled with human cytokine protein arrays. The expression of IL-6, IL-8/CXCL8, MCP-1/CCL2, and MIF was elevated in E-MSCs relative to A-MSCs (Supporting Information Fig. 5). Next we extended the analysis using a more sensitive and quantitative immunoassay (V-Plex). E-MSCs overall secreted higher levels of cytokines including IFN-γ, IL12p70, IL-13, IL-2, and IL-4 (Fig. 4A). Key factors of the
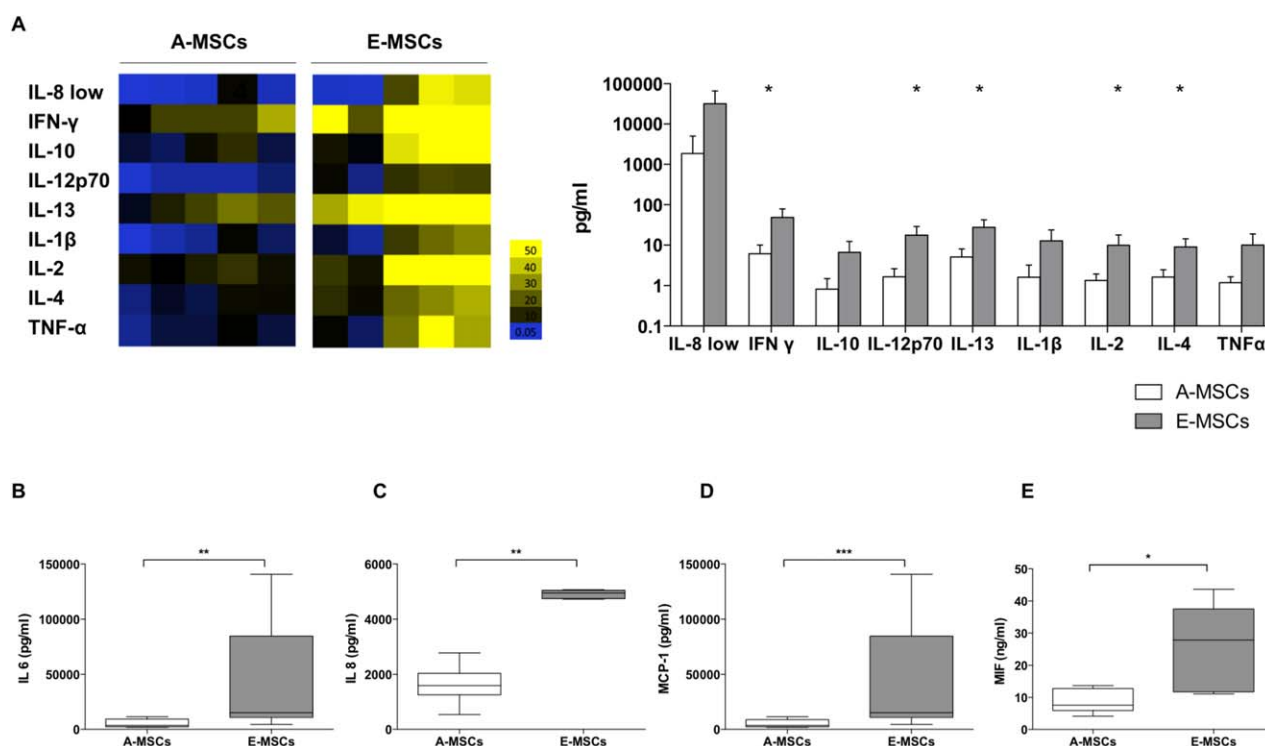
Figure 4. Elderly MSCs secrete higher levels of senescence associated cytokines. **(A)**: Baseline production of cytokines and chemokines by MSCs from adult and elderly individuals assessed by V-Plex assay. Data is reported as a ratio of secretion compared to the average of the A-MSCs groups. The color scale represents fold change ($n = 5$). **(B–E)**: Senescent associated cytokines and chemokines were confirmed by enzyme-linked immunosorbent assays. IL-6, IL-8/CXCL8 (**, $p < .01$; $n = 11$), MCP-1/CCL2 (***, $p < .001$, $n = 11$), MIF (*, $p = .01$ $n = 6$). Abbreviations: A-MSCs, adult MSCs; E-MSCs, elderly MSCs; MCP-1, monocyte chemoattractant protein-1; MIF, macrophage migration inhibitory factor.

senescence-associated secretome (i.e., IL-6, IL-8/CXCL8, MIF and MCP-1/CCL2) were tested in a larger number of samples by ELISA. Those results confirmed that E-MSCs secrete higher levels of IL-6, IL-8/CXCL8, MIF, and MCP-1/CCL2 (Fig. 4B–4E). A positive correlation between IL-6 and MCP-1/CCL2 levels assessed by ELISA (Supporting Information Fig. 6) was observed, which can relate to the fact that IL-6 is a potent inducer of MCP-1/CCL2 [41]. Next, antibody-mediated neutralization of IL-6, IL-8/CXCL8, and MCP-1/CCL2 and the use of a MIF antagonist was subsequently assessed in cocultures as a proof-of-concept for the role of these factors in the reduced immunomodulatory function of E-MSCs (Fig. 5). Indeed, neutralization of IL-6 (Fig. 5A, 5D), IL-8/CXCL8 (Fig. 5B, 5E), and MCP-1/CCL2 (Fig. 5C, 5F) significantly improved the E-MSCs immunomodulatory function, suggesting that these cytokines mediate the functional impairment of aged MSCs. In contrast, antagonizing MIF did not impact the MSCs immunomodulatory capacity (Supporting Information Fig. 7).

DISCUSSION

An enhanced understanding of the biology of MSCs has led to clinical trials testing their therapeutic effects in various conditions including cardiovascular diseases [32, 33]. Overall, these trials have demonstrated that MSCs-based therapies are promising; however, notable intratrial- and intertrial variations in therapeutic effectiveness were observed. These discrepancies have been attributed to a wide variety of factors including donor variance, tissue sources, epigenetic reprogramming and senescence

following expansion-cryopreservation, cell dose, timing of infusion, route of administration, and preactivated state of MSCs [42]. Furthermore, recent studies have shown that MSCs from different sources (i.e., bone marrow, adipose tissue and umbilical cord) display distinct differentiation tendencies, secrete unique paracrine factors and vary in their immunomodulatory capacity. Importantly, these studies consistently showed superior immunomodulatory function of adMSCs [31]. However, it is not clear if adipose tissue from different regions (i.e., pericardial and subcutaneous) differ in their immunomodulatory capacity. In this study, we first examined MSCs derived from pericardial tissue since cardiac stromal cells were previously suggested to exhibit better efficiency in cardiac repair capacity relative to their bone marrow counterparts [43]. Our results show that pericardial and subcutaneous adMSCs display comparable immunomodulatory capacities at least for the functional readouts used in this work (i.e., T cell proliferation and viability quantified by CFSE and 7AAD staining, respectively). These data do not exclude the possibility that differences may exist for other measures of immunomodulation and/or for the effect on other target immune cells. However, it is relevant to emphasize that T-cell suppression is regarded as a major mode of action of MSCs and the basis for their use in various human clinical trials [44].

Collectively, our data suggests that the easily accessible subcutaneous adMSCs could be used as a surrogate to estimate the T-cell suppressive effects of epicardial MSCs. Furthermore, results from human trials using subcutaneous adMSCs in subjects with acute myocardial infarction (APPOLO Trial; [32]) and chronic
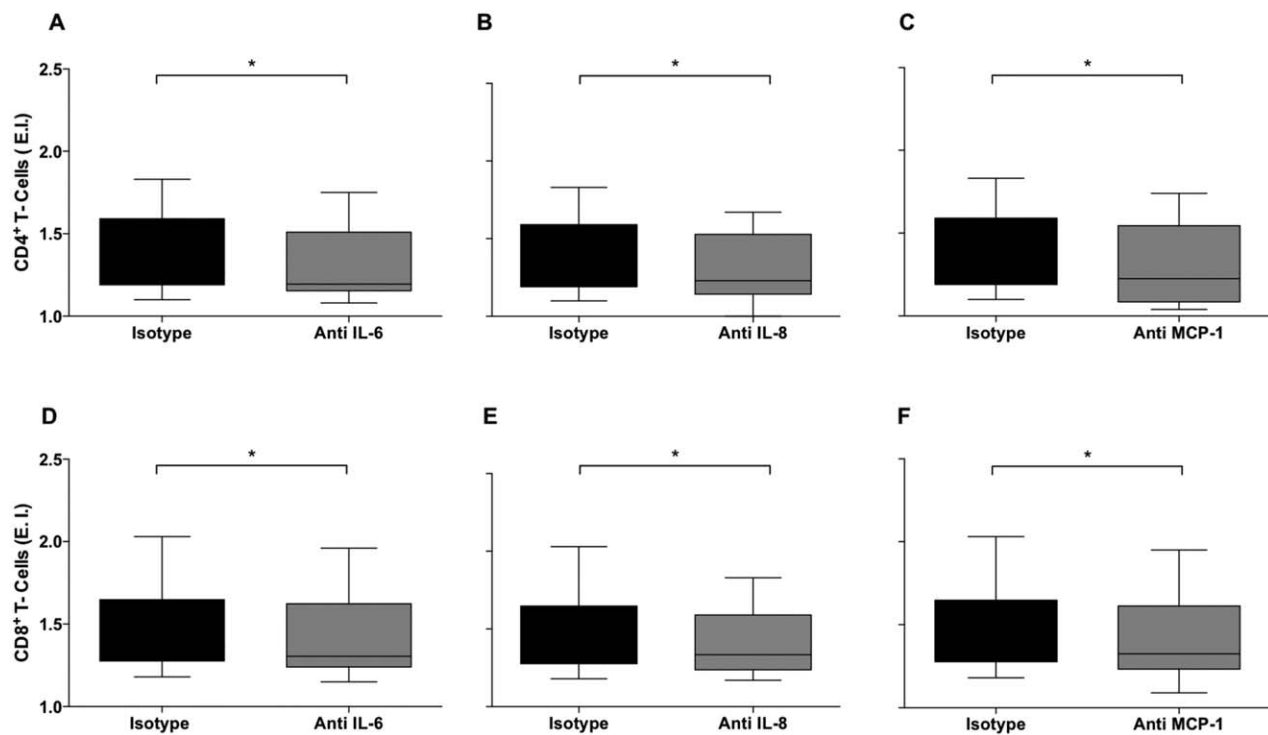
Figure 5. Antagonizing components of the senescence-associated secretory phenotype in cocultures enhances MSCs immunopotency. **(A)**: IL-6, **(B)**: IL-8/CXCL8, and **(C)**: MCP-1/CCL2 neutralization in MSCs:CD4^{+} T-cell cocultures improves MSCs immunopotency (*, $p = .03$, $n = 6$). Similarly, **(D)**: IL-6, **(E)**: IL-8/CXCL8, and **(F)**: MCP-1/CCL2 neutralization improves MSCs:CD8^{+}T-cell suppression (*, $p = .03$, $n = 6$). Abbreviations: EI, expansion index; IL-6, interleukin-6; IL-8, interleukin-8; MCP-1, monocyte chemoattractant protein-1.

ischemic heart disease (PRECISE Trial; [33]) have proved the safety of this source of MSCs as well as their therapeutic value.

To ensure maximal therapeutic efficacy, it is suggested that analysis of both senescent cell content and functionality of isolated MSCs be conducted prior to their use for transplantation [42]. Our data revealed that in the context of ATH, E-MSCs display cell senescence markers. These findings thus suggest a link between aging, MSCs senescence and their reduced immunomodulatory capacity in ATH. Understanding the effect of aging on MSCs is crucial to optimize their autologous use in the elderly, who are typically afflicted by cardiovascular diseases.

ATH is now considered a chronic inflammatory disease. Vascular inflammation in ATH is initiated in the adventitia and progresses toward to the intima [45]. MSCs have been isolated from all layers of the vasculature [46]; however, little is known about their role in the pathophysiology of ATH. MSCs secrete numerous factors (i.e., cytokines, chemokines and angiogenic molecules) that modulate the development of vascular disease. Our findings show that aging shifts the secretome profile of human atherosclerotic MSCs toward the expression of senescence-associated factors [38]. Importantly, antibody neutralization of those factors (IL-8/CXCL8, MCP-1/CCL2, and IL-6) enhanced the immunosuppressive capacity of E-MSCs, thus providing a direct functional association between the increased secretion of IL-8/CXCL8, MCP-1/CCL2, and IL-6 by E-MSCs and their impaired immunomodulatory efficacy.

Amongst numerous chemokines that have been associated with cardiovascular diseases, two that have been shown to have a consistent role in ATH are MCP-1/CCL2 and IL-8/CXCL8. MCP-1/CCL2 plays a crucial role in the initiation of atherosclerotic plaque

formation. Animal studies have shown that the absence of MCP-1/CCL2 limits the entry of monocytes and T-cells into the arterial intima and ultimately results in the inhibition of atherogenesis [47]. Moreover, MCP-1/CCL2 is linked to an increased risk of myocardial infarction and left ventricular heart failure [48]. Evidence from *in vitro* models, animal studies and case-control studies suggest a key role of IL-8/CXCL8 in the establishment and preservation of the inflammatory microenvironment of the insulted vascular wall contributing to ATH onset and progression (reviewed in [49]). Furthermore, increased IL-6 levels are also associated with atherosclerotic plaque development, plaque destabilization and increased risk of future cardiovascular events [50]. The increased secretion of MCP-1/CCL2, IL-8/CXCL8, and IL-6 by E-MSCs may therefore favor inflammation in the context of ATH directly, and indirectly via dampening the immunosuppressive efficacy of MSCs. Altogether, these findings suggest that in ATH, MSCs can undergo an age-dependent phenotypic switch from anti-inflammatory and atheroprotective to pro-inflammatory and atherogenic. Donor age should therefore be a primary consideration in studies assessing the therapeutic benefit of MSCs.

CONCLUSION

Collectively, our study provides novel insights into the characterization of adMSCs from subjects with ATH. Our data suggest that E-MSCs exhibit reduced immunomodulatory function and a heightened pro-inflammatory state. We also report that the modulation of IL-6, IL-8/CXCL8, and MCP-1/CCL2 enhances the T-cell

suppressive capacity of MSCs from elderly donors. Targeting these cytokines and chemokines may therefore be considered as a strategy to optimize the MSCs therapeutic efficacy in elderly individuals.

AUTHOR CONTRIBUTIONS

O.K.M.: collection, assembly, analysis and interpretation of data, manuscript writing; M.L.: analysis and interpretation of data; D.S.T.: provision of study material; S.N.: analysis and interpretation of data FR: data interpretation, manuscript writing; I.C.: conception and design, analysis and interpretation of data, manuscript writing, final approval of manuscript and financial support.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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