MITOCHONDRIAL DYSFUNCTION UNDERLIES THE PROINFLAMMATORY PHENOTYPE OF MULTIPOTENT MESENCHYMAL STROMAL CELLS FROM ATHEROSCLEROTIC INDIVIDUALS

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DEDICATION

I dedicate this work to my grandmother Ayse Yilmaz and my aunt Sevda Yilmaz…

ABSTRACT

Atherosclerotic (ATH) ischemic heart disease is the leading cause of mortality world - wide. Increasing evidence indicates that aging, and its associated chronic low-grade inflammatory state, contribute to ATH onset and progression. Specifically, immune activation through the increase in circulating pro-inflammatory cytokines, plays a central role in plaque destabilization and rupture resulting in acute coronary events (i.e. myocardial infarction (MI). Another evidence suggesting that ATH is associated with premature biological aging is that cells from the atherosclerotic plaque exhibit senescence hallmarks. While the mechanisms involved in cellular senescence are not fully understood, alterations in mitochondrial function largely contribute to this process. Dysfunctional mitochondria have reduced bioenergetical efficiency, generate high levels of reactive oxygen species (ROS), and are associated with increased pro-inflammatory cytokine secretion. Collectively, the evidence that normal vascular aging and ATH are associated with cellular senescence and increased ROS levels promoting a chronic inflammatory state, provide the rationale for current therapeutic efforts to prevent ATH progression through cytokine modulation.

Multipotent Mesenchymal Stromal cells (MSCs) reside in almost all tissues and migrate to inflammatory zones where they modulate immune responses. This is the basis for the therapeutic evaluation of MSCs in various conditions, including MI. The ability of MSCs to modify the microenvironment depends mainly on their activation and subsequent production of a variety of soluble molecules, known as the “MSC secretome”. This secretome has in general anti-inflammatory properties, however MSCs can switch to a pro-inflammatory phenotype (“MSCs polarization”).

Since redox imbalance is a crucial and non-cell specific mechanism involved in ATH, we hypothesized that in ATH-MSCs mitochondrial dysfunction results in increased intracellular ROS levels that in turn induce MSC polarization towards a pro-inflammatory phenotype. Further we posited that the modulation of the MSCs redox state would be a useful strategy to enhance the immunomodulatory function of ATH-MSCs.

My work indicates that mitochondrial dysfunction underlies the impaired immunomodulatory function of ATH-MSCs. Specifically, I showed that in ATH-MSCs dysfunctional mitochondria results in oxidative stress, and a glycolytic metabolic shift. As a result, ATH-MSCs have an altered secretome and are more susceptible to apoptosis. These data suggests that mitochondrial dysfunction is a core mechanism by which ATH alters the MSCs immunomodulatory capacity. Therefore, interventions aimed at restoring the ATH-MSC redox state may enhance their therapeutic efficiency through the modulation of their secretome.

RÉSUMÉ

La cardiopathie ischémique / L'athérosclérose (ATH) est la principale cause de mortalité mondiale. Un nombre croissant de preuves indiquent que le vieillissement, ainsi que le léger état inflammatoire chronique qui lui est associé, contribuerait à la manifestation de l'ATH et à sa progression. Plus spécifiquement, l'activation immunitaire, par l'augmentation des cytokines pro-inflammatoires circulantes, joue un rôle central dans la déstabilisation de la plaque et sa rupture, donnant lieu à l'apparition d'événements aigus coronariens (comme l'infarctus du myocarde). De plus, la présence de caractéristiques distinctives de sénescence dans les cellules de la plaque d'athérome est une autre indication suggérant l'association de l'ATH avec un vieillissement biologique prématuré. Alors que les mécanismes impliqués dans la sénescence cellulaire ne sont pas parfaitement compris, les altérations de la fonction mitochondriale contribuent en grande partie à ce processus. Les mitochondries dysfonctionnelles ont en effet un rendement bioénergétique réduit, génèrent de hauts taux de dérivés réactifs de l'oxygène (DRO) et sont associées à une augmentation de la sécrétion de cytokines pro-inflammatoires. De ce fait, la preuve que le vieillissement vasculaire normal et l'ATH soient associés à la sénescence cellulaire et à un taux élevé de DRO entrainant un état inflammatoire chronique, fournit une logique pour les efforts thérapeutiques actuels dans la prévention de la progression de l'ATH à travers la modulation des cytokines.

Les cellules souches mésenchymateuses multipotentes (CSMs) sont présentes dans presque tous les tissus et migrent vers les zones inflammatoires où elles modulent les réponses immunitaires. Ce constat sert de base à l'évaluation thérapeutique des CSMs dans diverses conditions, comme l'IM. Dans ce contexte, pour éviter le phénomène

d'immunogénicité, l'utilisation de CSMs autologues est préférable. La capacité des CSMs à modifier leur micro-environnement dépend principalement de leur activation et de la production inhérente d'une variété de molécules solubles, connue sous le nom de 'sécrétome des CSMs'. Ce sécrétome présente généralement des propriétés anti-inflammatoires; néanmoins, les CSMs peuvent passer à un phénotype pro-inflammatoire via un processus encore mal compris, connu sous le nom de 'polarisation des CSMs'.

Comme le déséquilibre de l'etat redox cellulaire est impliqué dans l'ATH, nous avons émis l'hypothèse suivante: le dysfonctionnement mitochondrial des CSMs provenant d'individus atteint de l'ATH (CSMs-ATH) résulte en une augmentation des taux de DRO qui, en retour, induisent la polarisation de ces CSMs vers un phénotype pro-inflammatoire. De plus, nous avons proposé que la modulation de l'état redox des CSMs serait une stratégie utile pour l'amélioration de la fonction immunomodulaire des CSMs-ATH.

Les contributions clé des travaux que j'ai conduits pour tester ces différentes hypothèses sont les suivantes: La dysfonction mitochondriale sous-tend la fonction immunomodulatrice altérée des ATH-MSC. Les mitochondries dysfonctionnelles conduisent au stress oxydatif et au déplacement métabolique glycolytique qui modifie ensuite la composition du sécrétome et augmente la susceptibilité à l'apoptose.

En conclusion, ces données suggèrent que l'altération de la fonction mitochondriale pourrait être l'un des principaux mécanismes par lesquels l'ATH altère la capacité immunomodulatrice des CSM. Les interventions visant à restaurer la fonction mitochondriale du CSM peuvent améliorer leur efficacité thérapeutique.

PREFACE

The work described in this thesis has been published as follows:

Chapter 2

Kizilay Mancini O, Shum-Tim D, Stochaj U, Correa JA, Colmegna I. Age, atherosclerosis and type 2 diabetes reduce human mesenchymal stromal cell-mediated T-cell suppression. Stem Cell Res Ther 2015;6:140.

https://www-ncbi-nlm-nih-gov.proxy3.library.mcgill.ca/pmc/articles/PMC4529693/

Chapter 3

Kizilay Mancini O, Lora M, Shum-Tim D, Nadeau S, Rodier F, Colmegna I. A proinflammatory secretome mediates the impaired immunopotency of human mesenchymal stromal cells in elderly patients with atherosclerosis. Stem Cells Transl Med 2017;6(4):1132-1140.

https://www-ncbi-nlm-nih-gov.proxy3.library.mcgill.ca/pmc/articles/PMC5442842/

Chapter 4

Kizilay Mancini O, Lora M, Cuillerier A, Shum-Tim D, Hamdy R, Burelle Y, Servant MJ, Stochaj U, Colmegna I. Mitochondrial oxidative stress reduces the immunopotency of mesenchymal stromal cells in adults with coronary artery disease. Circ Res. 2018; 19(2):255-266.

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CONTRIBUTION OF AUTHORS

Chapters 1 and 5: The work presented in chapters 1 and 5 was written by myself and reviewed and edited by Dr. Colmegna.

Chapter 2: The work presented in this chapter has been done with the collaboration of co- authors. In this work, I designed and performed all the experiments. I isolated MSCs from adipose tissue, performed FACS analysis for surface markers, and promoted the differentiation of MSCs into 3 lineages (adipose, osteoblast, chondrocyte). After confirming that all MSC samples fulfilled the minimal criteria proposed by the International Society for Cellular Therapy (ISCT), I performed, analyzed and interpreted MSC:T-cell suppression assays (i.e. classic mixed lymphocyte reaction-like assay). Under Dr. Colmegna's supervision, I prepared the figures, and wrote the first manuscript draft. Dr. Shum-Tim provided the studied samples (i.e. human adipose tissue from people requiring programmed cardiothoracic surgery). Dr. Stochaj provided critical input on the manuscript. Dr. Correa performed the statistical analysis and provided statistical descriptions for the manuscript. Dr. Colmegna supervised the design and execution of the experiments, assisted with their interpretation, edited and approved the final manuscript.

Chapter 3: The work completed in this chapter was performed with the contribution of co-authors. I isolated MSCs from adipose tissue and performed all the analysis to confirm they fulfill the ISCT minimal criteria. I designed and performed all the experiments except for those in Figure 4A and Supplementary Figure 1B. I have analyzed, interpreted the data and prepared all the figures. I wrote the manuscript under Dr. Colmegna's supervision. Dr. Lora provided the data for Supplementary Figure 1B, and reviewed the

manuscript. Dr. Shum-Tim provided the adipose tissue samples for MSCs isolation. Stephanie Nadeau performed the V-Plex assay shown in Figure 4A. Dr. Rodier supervised the V-plex assay data acquisition and review the manuscript. Dr. Colmegna supervised the design and execution of the experiments, reviewed, edited and approved the final manuscript.

Chapter 4: The work performed in this chapter was achieved with the contribution of co-authors. I isolated and characterized MSCs, designed and performed all the experiments with the exception of those presented in Figure 3C and supplementary Figure 4. In addition, I created the figures and wrote the manuscript. Dr. Lora performed the protein extraction for the WB analysis in supplementary Figure 4, and provided valuable technical expertise. Alexanne Cuillerier, performed the WB analysis shown in supplementary Figure 4. Dr. Shum-Tim and Dr. Hamdy provided adipose tissue samples from adult and pediatric donors respectively. Dr. Burelle and Dr. Servant reviewed the manuscript critically. Dr. Stochaj performed the immunohistochemistry experiments shown in Figure 3C and contributed to data interpretation and manuscript writing. Dr. Colmegna supervised the design and execution of the experiments, assisted with their interpretation, edited and approved the final manuscript.

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There comes in the end my dear family, firstly, my deepest gratitude goes to my mom and dad, who have kindly and patiently supported me throughout my entire education sacrificing their own dreams to give me a chance at achieving mine. Their unconditional love and support kept me going. They always believed in me even the times that I gave up on myself. Thank you for being such a wonderful parents and role models. The next in the list of my gratitude is my mother in law Antonietta Costantino for her generous support and constant encouragement especially within the past year by looking after her little granddaughter while I was working on my thesis. My aunt Selma Yilmaz who introduced me to research and opened her house when I started my journey at McGill. I will ever be grateful for her support and guidance. Last but not the least, no words suffice to express my indebtedness to my husband Arturo Mancini not only for his unfailing moral support, love and patience but also his intellectual input to my Ph.D. work as well as his editions/corrections of every manuscript that I wrote including this thesis. You make me a better person and I am thankful of every minute that I spent with you and blessed to have you as my husband. Also, the new addition to our family, our daughter Aria Sofia has been a bundle of joy and made us complete.

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LIST OF ABBREVIATIONS

ATH	Atherosclerosis
BMI	Body mass index
ECAR	Extracellular acidification rate
ECs	Endothelial cells
EGF	Epidermal growth factor
ET-1	Endothelin1
ETC	Electron transport chain
FGF	Fibroblast growth factors
GLUT	Glucose transporters
H_2O_2	Hydrogen peroxide
HGF	Hepatocyte growth factor
HIF 1α	Hypoxia-inducible factor 1-*alpha*
HL-DR	Human Leukocyte Antigen – antigen D Related
HLA-G	Human leukocyte antigen – G
HO-1	Heme oxygenase 1
HTN	Hypertension
ICAM-1	Intercellular adhesion molecule-1
IDO	Indoleamine 2,3-dioxygenase
IFNγ	Interferon gamma
IGF	Insulin-like growth factors
IL-1Ra	IL-1 receptor antagonist

IL-2R	Interleukin 2 receptor
IL-6	Interleukin-6
IL-8/CXCL8	Interleukin-8 /C-X-C motif chemokine ligand 8
LDL	Low-density lipoprotein
LFA-3	Leukocyte function-associated antigen-3
LIF	Leukocyte inhibitory factor
M-CSF	Monocyte colony stimulating factor
MAPK	Mitogen-activated protein kinases
MCP-1/CCL2	Monocyte chemoattractant protein-1 /Chemokine ligand 2
MHC	Major histocompatibility complex
MI	Myocardial infarction
MSCs	Multipotent mesenchymal stromal cells
NAC	N-acetyl-L-cysteine
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NO	Nitric oxide
NOX	NADPH oxidase
OCR	Oxygen consumption rate
OXPHOS	Oxidative phosphorylation
PBMCs	Peripheral blood mononuclear cells
PDGF	Platelet-derived growth factor
PGE_2	Prostaglandin-E2
ROS	Reactive oxygen species
SOD	Superoxide dismutase

STAT-1	Signal transducer and activator of transcription
T2DM	Type 2 diabetes mellitus
TCA	Tricarboxylic acid cycle
TCFA	Thin-cap fibroatheroma
TGF-β	Transforming growth factor β
TLRs	Toll-like receptors
TNFα	Tumor necrosis factor
TSDR	Treg-specific demethylated region
TSG-6	TNF-stimulate gene 6
VCAM-1	Vascular cell adhesion protein 1
VEGF	Vascular endothelial growth factor
VSMCs	Vascular smooth muscle cells

CHAPTER I: REVIEW OF THE LITERATURE

1.1. ATHEROSCLEROSIS AND CARDIOVASCULAR DISEASE

Due to improvements in lifestyle and healthcare, the world's elderly population (people >65 years old according to the World Health Organization (WHO) (1) is growing at a rapid rate and is expected to double by 2050 (2). Although all-cause mortality has decreased worldwide, cardiovascular diseases (CVD) due to atherosclerosis (ATH) remain the leading cause of death globally, even exceeding the deaths from all forms of cancer and chronic lower respiratory disease combined (3, 4). Approximately 17.7 million deaths (accounting for 31% of all deaths) in 2015 were due to CVD (i.e. coronary heart disease, cerebrovascular disease, peripheral artery disease, and aortic atherosclerosis and thoracic or abdominal aortic aneurysms). Moreover, CVD is no longer a disease of developed countries but has been also recognized as an important problem in developing countries. CVD places a huge burden on health care systems. The estimated global cost of CVD in 2010 was $863 billion (5). *As ATH is the main contributor to CVD, a detailed understanding of its pathophysiology is expected to translate into new interventions and therapies with the potential to greatly improve the lives of a large number of people worldwide, and reduce the societal economic burden of this disease.* For this reason, human ATH is the disease model that is the focus of my thesis.

1.1.1. Established risk factors for CVD

Although the specific cause of ATH is unknown, numerous studies have proven that a variety of factors, often acting in concert, are associated with an increased risk for

atherosclerotic plaques in coronary arteries and other arterial beds. The major risk factors for CVD can be classified into 2 broad categories, traditional and non-traditional. The traditional CVD risk factors are defined in Table 1. The non-traditional CVD risk factors, are novel conditions with the potential to augment clinical risk stratification by aiding in the prediction, identification, and assessment of atherosclerotic disease. Many individuals in the general population have one or more traditional risk factors for CVD, and over 90 percent of CVD events occur in individuals with at least one risk factor. Many of these risk factors are modifiable by specific preventive measures. The five leading modifiable risk factors (hypercholesterolemia, diabetes, hypertension, obesity, and smoking) are estimated to be responsible for more than half of cardiovascular mortality (6). On the other hand, the following nine potentially modifiable factors account for over 90 percent of the population-attributable risk of a first myocardial infarction (MI): smoking, dyslipidemia, hypertension (HTN), diabetes mellitus (DM), abdominal obesity, psychosocial factors, daily consumption of fruits and vegetables, regular alcohol consumption, and regular physical activity (7). In the following paragraphs, I will expand on concepts related to traditional risk factors that are relevant to my work, in particular to the variables included in the statistical models presented in Chapter 2 (8).

Non-modifiable factors	**Modifiable Factors**
Age: Male ≥45, female ≥55 or premature menopause without estrogen replacement therapy *Sex*: Male > risk *Ethnicity*: South Asians (Indians and Pakistanis) > risk of heart disease compared to Europeans *Family history* of premature coronary heart disease: Definite myocardial infarction or sudden death before age 55 in male first-degree relative, and before age 65 in female first-degree relative *Previous cardiovascular event*	*Obesity*: body mass index (BMI) greater than 30 kg/m^2 or BMI 25-29.9 kg/m^2 plus abdominal obesity *Smoking*: Current cigarette smoking *Hypertension*: Blood pressure >140/90 mmHg or use of an antihypertensive medication *Diabetes Mellitus*: Insulin resistance, hyperinsulinemia, and elevated blood glucose *Hyperlipidemia*: Total cholesterol >199 mg/dL Low Density Lipoprotein cholesterol >129 mg/dL High Density Lipoprotein cholesterol <35 mg/dL *Physical inactivity*

Table 1.1. Traditional cardiovascular risk factors; adapted from Grundy SM et al. (9).

1.1.1.1. Non-Modifiable Traditional CV Risk Factors: Implications to MSCs function

1.1.1.1.1. Age

In addition to being associated with numerus ATH risk factors, aging in and of itself is an independent risk factor for atherogenesis. Significant age-related increases in the incidence of ischemic heart disease and stroke, the two main clinical manifestations of ATH, have been demonstrated in various studies (10, 11).The link between aging and an increased ATH risk is further highlighted by the accelerated ATH observed in different premature aging syndromes (i.e., Hutchinson Gilford progeria, Werner syndrome) (12,13), as well as in radiation–induced premature aging studies (14).

Senescence markers, which are hallmarks of cellular aging are present in different cells of the plaque. Vascular smooth muscle cells (VSMCs), endothelial cells (ECs) and monocyte/macrophages from atherosclerotic plaques and aged (non-atherosclerotic) vessels have a high frequency of senescence-associated β galactosidase (SAβG) positivity and more nuclear and mitochondrial DNA damage than non-ATH and young donor's cells (15-17). Telomere lengths, another marker of biological age, are prematurely shortened in plaque VSMCs (18) and ECs (19); as well as in circulating leukocytes from individuals with ATH (20).

Senescent cells in the plaque may contribute to the atherosclerotic process through a variety of mechanisms. Among them, senescent ECs secrete high levels of endothelin-1 (ET-1) implicated in the development and progression of ATH and HTN; upregulate the expression of pro-inflammatory genes including vascular cell adhesion protein 1 (VCAM-

1) and Monocyte chemoattractant protein-1 /Chemokine ligand 2 (MCP-1); and are more susceptible to apoptosis (21-23). Altogether, senescent ECs have a pro-inflammatory and pro-atherogenic phenotype.

In my work, the age of the donor's MSCs (discussed in detail in section 1.2.) was the primary outcome variable. The contribution of donors' age to MSCs dysfunction was specifically assessed in Chapter 2 (8). In all subsequent experiments, cases and controls were age matched.

1.1.1.1.2. Sex

Observational studies have shown that males are at higher risk for CVD, and that they develop clinical manifestations almost a decade earlier compared to age-matched women (24, 25). Specifically, females have approximately 20 percent lower risk than males for all major cardiovascular endpoints including cardiovascular death (adjusted RR 0.83, 95% CI 0.75-0.92), MI (adjusted RR 0.78, 95% CI 0.68-0.89), and a combined endpoint of death, MI, stroke, and heart failure hospitalization (adjusted RR 0.81, 95% CI 0.76-0.87) (26). Although the precise cellular and molecular mechanisms underlying sex-specific differences in CVD risk remain elusive, a growing body of data suggest inherent sex-related differences in G–protein coupled estrogen receptor function, lipoprotein A metabolism, toll-like receptor (TLR) function and leucocyte-platelet aggregate marker [reviewed in (27)]. Further, pathological studies suggest that plaque composition and burden is higher in male compared to age-matched females. We assessed the impact of MSC's donor sex on MSCs functional differences by including this variable in the multivariate analysis presented in Chapter 2.

1.1.1.2. Modifiable Traditional CV Risk Factors: Potential confounders of MSCs function

1.1.1.2.1. Obesity

Obesity, defined as a body mass index (BMI) ≥ 30.0 kg/m^2, is an independent risk factor for the development of ATH and coronary artery disease (28-30). In addition, obesity is associated with HTN, insulin resistance and glucose intolerance, hypertriglyceridemia, reduced high density lipoprotein (HDL)-cholesterol, and low levels of adiponectin; all risk factors for ATH, CVD, and cardiovascular mortality. It is estimated that a 10-kg increase in body weight is associated with a 12 % increase risk in CVD (31). Obesity and ATH share numerous pathophysiological traits with inflammation serving as the common thread. Indeed, the current classification of ATH and obesity as chronic inflammatory disorders has significantly reshaped our understanding of the factors and mechanisms linking the two conditions. Similar to macrophage infiltration to the atherosclerotic plaque, macrophages also infiltrate adipose tissue in obese subjects and conjointly with surrounding adipocytes, secrete various factors that are directly or indirectly linked to the development and progression of ATH. Relative to non-obese subjects, obese individuals (particularly those with increased visceral adiposity) tend to display a pro-inflammatory profile characterized by high levels of circulating inflammatory markers [i.e., Tumor necrosis factor (TNF-alpha), interleukin (IL)-6, MCP-1/CCL2, and C-reactive protein (CRP)] (32, 33). Further, the adipose tissue from obese subjects secretes signaling proteins (i.e. adipokines) that promote endothelial dysfunction and VSMCs proliferation (e.g., leptin, heparin binding epidermal growth factor-like growth factor); and coagulation (plasminogen activator inhibitor-1) (34, 35). Other mechanisms potentially linking obesity

to an increased risk of ATH include alterations in the gut microbiota, impaired autophagy, and enhanced oxidative stress (36). Recent evidence suggest that obesity could alter the properties of MSCs, thus we collected the BMI of the MSC donors and included this confounder in the analysis (8, 37).

1.1.1.2.2. Smoking

Both smoking tobacco and passive smoking are risks for developing ATH. The incidence of MI is increased six fold in women and threefold in men who smoke at least 20 cigarettes per day compared with subjects who never smoked (38). The risk of MI is proportional to tobacco consumption in both men and women, and smoking accounts for 36 percent of the population-attributable risk of a first MI (7). Male smokers are 1.6-fold more likely than never-smokers to die from ischemic heart disease or stroke; in women, this risk doubles (39). Smoking promotes all phases of ATH, from early endothelial injury to plaque rupture and thrombosis, by triggering inflammation, thrombosis and the oxidation of low-density lipoprotein (LDL) cholesterol (40, 41). Indeed, smoking increases the peripheral blood leukocyte count by ~ 20-25% (42), the circulating levels of multiple inflammatory markers (i.e., CRP, IL-6, TNFα, MCP-1/CCL2, IL-8) (43-46), and the plasma concentrations of endothelial cell activation markers [i.e. Vascular cell adhesion molecule 1 (47, 48)]. Cigarette smoking has detrimental effects on MSC regenerative potential (49). These effects include; inhibition of MSC proliferation, migration and chondrocyte differentiation (50). Moreover, when MSCs are stimulated with cigarette smoke extract, their secretome was altered (51). Although cigarette smoking alters crucial functions of MSCs, its effect on MSCs immunopotency remains elusive. Therefore, tobacco use was a variable included in the multivariate analysis in Chapter 2 (8).

1.1.1.2.3. Hypertension

HTN is a well-established risk factor for adverse cardiovascular outcomes, accounting for 18 percent of the population-attributable risk of a first MI (7, 52). The best evidence for a causal role of HTN in cardiovascular events is their reduced frequency with antihypertensive therapy. Systolic blood pressure and isolated systolic HTN are major CVD risk factors at all ages and in both genders. The risk for both coronary disease and stroke increases progressively with incremental increases in blood pressure above 115/75 mmHg (53). In most studies [including ours (8)] and in clinical practice, patients who are actively taking antihypertensive medications are usually defined as having HTN regardless of their observed blood pressure.

The potential cellular and molecular mechanisms linking hypertension to ATH are numerous but mainly rooted in the production of reactive oxygen species (ROS) (54). In hypertension, altered mechanical forces (i.e., stretch), increased angiotensin II, sodium and catecholamines all stimulate the vascular production of ROS. In turn, ROS production augments endothelial permeability to LDL and promotes LDL oxidation in the subendothelium, which is then readily taken up by macrophages to give rise to atherogenic foam cells. Further, increased ROS levels promote the production of pro-inflammatory factors (cytokines, chemokines, adhesion molecules) by ECs. This ultimately creates a self-perpetuating pro-inflammatory environment at the lesion site [largely via activation of nuclear factor κB (NF-kB)]. ROS also oxidize and inactivate the anti-atherosclerotic actions of endothelium-derived nitric oxide (NO). Angiotensin II and catecholamines (i.e., epinephrine, norepinephrine) can also promote atherogenic

inflammation by directly engaging their respective G protein-coupled receptors on the various immune and non-immune cells involved in ATH (55-57).

Recent human studies found an association between essential HTN/left ventricular mass and increasing numbers of MSCs in peripheral blood. Those studies suggest that MSCs could be involved in the pathophysiology of cardiac hypertrophy and HTN. Further, angiotensin receptor blockers improve the regenerative capacity of MSCs in *in vivo* models of MI (58) and stroke (59). In Chapter 2, both HTN and anti-hypertensive medications were included as covariates in the multiple regression model (8).

1.1.1.2.4. Diabetes mellitus

Both type 1 and type 2 DM, as well as insulin resistance, and hyperinsulinemia are associated with an increased risk of atherosclerotic cardiovascular disease (i.e. 2- to 4-fold greater risk relative to non-diabetic subjects) (60, 61). In addition, diabetics have a greater burden of other atherogenic risk factors than non-diabetics, including HTN, obesity, increased total-to-HDL-cholesterol ratio, hypertriglyceridemia, and elevated plasma fibrinogen (62). A proposed mechanism by which prolonged hyperglycemia promotes atherogenesis involves the overproduction of ROS and ensuing oxidative stress. Hyperglycemia-induced overproduction of ROS by ECs mitochondria results in endothelial dysfunction and other pro-atherogenic molecular and biochemical responses (e.g., upregulation of pro-inflammatory factors and adhesion molecules involved in initial leukocyte recruitment; increased production of ET-1, resulting in vasoconstriction and platelet aggregation) [reviewed in (63)]. Additionally, hyperglycemia-induced glycation of LDL cholesterol was suggested to increase its atherogenic potential (64).

In *in vivo* models of type 2 DM, MSCs have reduced regenerative potential due to their decreased proliferative capacity and increased apoptosis rates. Increased levels of TNFα in the circulation underlie these defects, as blocking TNFα resulted in improved MSCs regenerative capacity (65) .

Oxidative stress has also been linked to the reduced multipotency of MSCs from diabetic mice. Transplantation of these MSCs into an ischemia model resulted in increased adipocyte differentiation and reduced neovascularization compared to WT. Moreover, pre-treatment of MSCs from diabetic mice with either *N*-acetylcysteine (NAC) or nicotinamide adenine dinucleotide phosphate-oxidase 4 (Nox4) siRNA rescued their impaired angiogenic properties (66). Comparative proteomic analysis of conditioned media from diabetic and control mice MSCs revealed different secretome composition, linked to altered angiogenesis of diabetic MSCs (67). In summary, there is a growing body of evidence suggesting a negative effect of DM on MSCs functions. Thus, we included this as a covariate in Chapter 2. Thereafter, we ensured a similar proportion of DM subjects in each comparison group in our subsequent papers (68, 69).

1.1.1.2.5. Hyperlipidemia

The prevalence of dyslipidemia is increased in patients with premature CVD, being as high as 75 to 85 percent compared with approximately 40 to 48 percent in age-matched controls without CVD (70). High triglyceride levels (>150 mg/dL), and a low HDL cholesterol (<40 mg/dL) increase the risk for CVD in both men and women (71). The Monitored Atherosclerosis Regression Study (MARS) showed a correlation between

triglyceride-rich lipoprotein levels and the rate of progression of mild/moderate (<50% stenosis) coronary lesions (72).

On the other hand, dyslipidemia impairs the regenerative capacity of MSCs (73) and inhibits their homing efficiency to the injured zone (74). Further, circulating plasma high density lipoprotein has a protective effect on MSCs by promoting their proliferation while reducing their apoptosis (75). The effects of statins on MSCs are dose dependent: while low doses improve MSCs survival and efficacy in an MI model (76), high doses induce apoptosis (77). Considering these data, in Chapter 2 we assessed the impact of dyslipidemia and the use of statins as predictors of MSCs immunopotency.

1.1.2. Current understanding of the ATH pathogenesis

Atherosclerosis as a chronic inflammatory process

Initially described as a disorder related to abnormal lipid metabolism and deposition, ATH is now considered a chronic inflammatory disease. The association between ATH and inflammation has been recognized since the mid 1800's, with the pathologists Rudolf Virchow and Carl von Rokitansky describing cellular inflammatory changes in atherosclerotic vessel walls. While von Rokitansky considered the inflammatory response to be a consequence of ATH, Virchow argued that inflammation played a primary causative role (78, 79).

Inflammatory processes are key to all ATH stages. Currently, the most widely-accepted model to explain ATH is the "inflammatory hypothesis of atherothrombosis", which stems from the "response to injury" model (80-82) and encompasses other hypotheses such as the 'response-to-retention' and 'oxidative modification' [reviewed in (83)]. (Figure 1.1.) According to the 'inflammatory hypothesis' the earliest event in atherogenesis is the injury

to the tunica intima of the endothelium in lesion-prone areas of the vasculature (e.g. vessel curvature and bifurcation points) (84). Such injury can be triggered by a number of insults, including direct physical trauma, noxious substances (e.g., ROS/free radicals from cigarette smoke and air pollution; hyperglycemia; hyperhomocysteinemia; hyperlipidemia/ hypercholesterolemia) and/or disturbed- turbulent blood flow (i.e. due to HTN).

Injured ECs secrete factors and present cell surface molecules that influence the behavior of other cells within the vessel wall (i.e., VSMCs, pericytes), and in the circulation (i.e., leukocytes, platelets). ECs injury results in a loss of selective permeability and barrier function with the accumulation of LDL in the subendothelial intimal space. The LDL is retained in the intima via its interaction with components of the extracellular matrix (i.e., proteoglycans; secreted lipolytic and lysosomal enzymes) (85, 86), and can be progressively oxidized (oxLDL) via chemical (i.e., ROS) and enzymatic (i.e., myeloperoxidase) processes (83, 87). OxLDL acts as a chemoattractant recruiting circulating monocytes and T lymphocytes to the arterial endothelium (88, 89). Those monocytes differentiate into macrophages and engulf oxLDL via their scavenger receptors [macrophage scavenger receptor 1, scavenger receptor for phosphatidylserine, oxidized lipoprotein (CXCL16) and CD36] and, through receptor-independent endocytocis (i.e., macropinocytocis and phagocytocis). This results in lipid-laden “foam cells” characteristic of ATH. Activated macrophages secrete a plethora of pro-inflammatory cytokines further propagating the pro-atherogenic inflammatory response (90, 91).

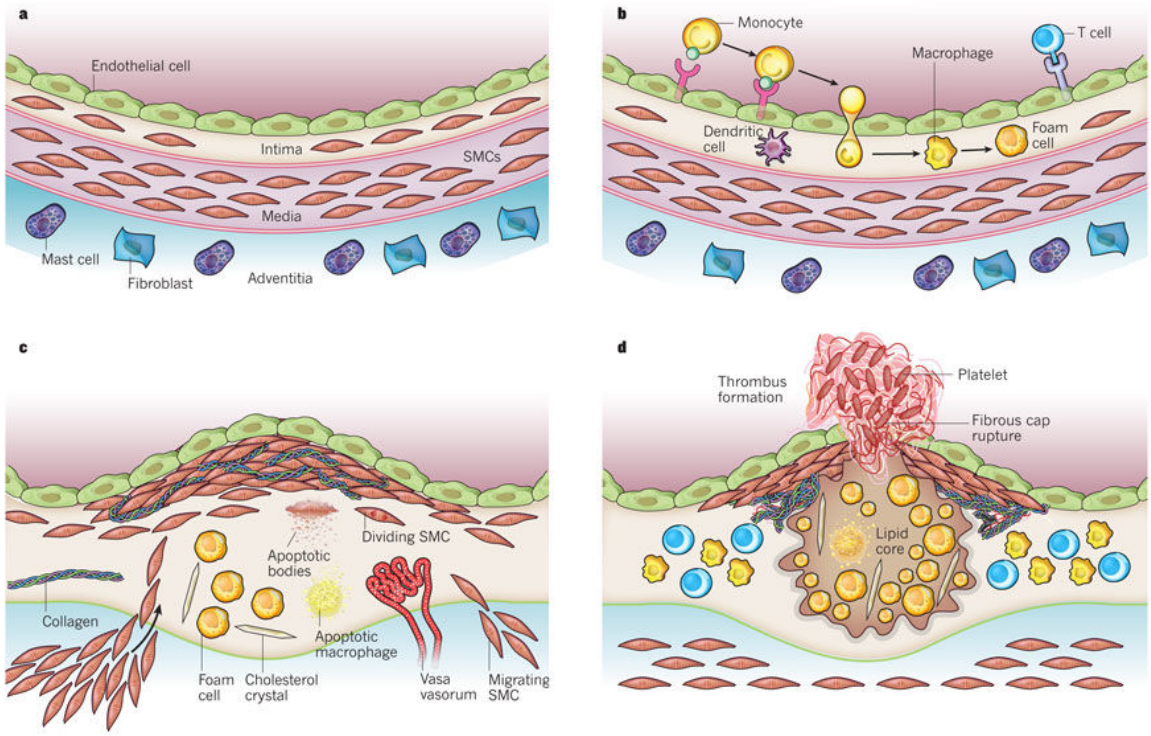


Figure 1.1. ATH: from plaque formation to vessel occlusion.

a- Three layers of a normal artery; tunica intima that contains a monolayer of ECs, VSMC; tunica media consisting of VSMCs surrounded by extracellular matrix; and tunica adventitia containing mast cells, nerve endings and microvessels. b- Initiation of the ATH plaque formation. Leukocytes from the blood stream adhere and migrate through the activated endothelial monolayer, macrophages mature and form foam cells c- VSMCs migrate from the media to the intima, they proliferate and produce extracellular matrix components including collagen, elastin and proteoglycans. The progression of the ATH plaque leads to apoptosis and necrosis of both VSMCs and macrophages with the accumulation of extracellular lipids derived from these cells creating a 'necrotic core'. d- The final stage of the ATH plaque is the formation of the fibrous cap. The figure was reproduced from Libby, P *et al* (2011), with the permission of the publisher (92).

OxLDL also propagates ECs injury and activates ECs to produce additional factors that perpetuate inflammation by recruiting monocytes and T lymphocytes to the arterial vessel wall (e.g., MCP-1/CCL-2, RANTES/CCL-5 and monocyte colony stimulating factor [M-CSF]). Injured ECs express cell surface adhesion molecules that promote leucocyte attachment to the endothelium among them VCAM-1 (93, 94). VCAM-1 confers selective and robust adhesion to lymphocytes, monocytes and eosinophils via their cell surface expression of the VCAM-1 counter-receptor, the $\alpha_4\beta_1$ integrin [also known as very late antigen 4 (VLA-4)] (95).

Collectively, this cascade of interconnected events increases the recruitment, adhesion and subendothelial infiltration of inflammatory cells to the initial injury site promoting the continued oxidation of LDL and the expansion of the atherosclerotic lesion.

Injured ECs also secrete a variety of growth factors and cytokines that induce a phenotypic switch of the VSMCs in the tunica intima and media, from a quiescent “contractile” phenotype to a “pro-inflammatory” phenotype. These pro-inflammatory VSMCs proliferate and migrate to the intima where they increase their production of extracellular matrix molecules (i.e., interstitial collagen and elastin) and give rise to the early fibroatheroma (96). A fibrous “cap” consisting of VSMCs in a proteoglycan-collagen matrix covers the fibroatheroma and is believed to promote plaque stabilization. This cap typically overlies an assemblage of foam cells, some of which die (by apoptosis and possibly autophagy) and release lipids that accumulate extracellularly (97). The inefficient clearance of dead cells also known as defective efferocytosis (efferocytosis: process by which dying/dead cells are removed by phagocytic cells) promotes the collection of cellular debris and extracellular lipids, forming a lipid-rich acellular necrotic core (98).

These fibroatheromatous plaques can lead to flow-limiting stenosis resulting in tissue ischemia. Progressively, the fibrous cap of the fibroatheroma weakens, resulting in a thin-cap fibroatheroma (TCFA) also known as ‘vulnerable plaque’ due to its high rupture risk. The majority of atherosclerotic subjects who suffer acute cardiovascular events (i.e., heart attack or stroke) do not have a significant plaque-mediated arterial blockage. Instead, rupture of a TCFA is the primary cause of acute vascular events in individuals with ATH (99). Upon breakage of the fibrous cap, the pro-coagulant contents of the necrotic core are exposed to coagulation proteins in the blood, triggering thrombosis. The resulting thrombi can obstruct blood flow at the site of plaque rupture; alternatively, thrombotic complications may occur distally (100).

In recent years, with the identification of various stem/progenitor cells in the vessel wall our understanding of repair and regeneration in the context of ATH progression is evolving. Further, clinical findings on the association of bone marrow failure and cardiovascular disease set the spotlight on MSC function as a key disease pathomechanism (101). MSCs, described as a pericyte or pericyte-like cells, have been suggested to have a perivascular origin (102-104). Few studies evaluated whether altered MSCs function contributes to ATH development. These studies suggest that MSCs residing in the vascular intima and adventitia through differentiation into osteoblast like cells could contribute to plaque calcification (105). Moreover, MSCs within the tunica media could transdifferentiate into VSMCs contributing to vascular remodeling in ATH (106). In addition, almost all of the immune cells (i.e., macrophages, T cells, B cells) implicated in ATH are targets of the MSCs’ immune modulatory effects. The specific mechanisms involved in MSCs immune modulation will be reviewed in section 1.2.1.1.

In the next section, I will review in-depth the role of $CD4^+$ T cells within the ATH plaque. The reason for focusing on this cell subset is that the functional readout that I chose to evaluate MSCs potency are allogeneic $CD4^+$ T-cells. By highlighting the key role of $CD4^+$ lymphocytes in plaque development, I intend to justify the potential relevance of my findings to atherogenesis.

1.1.2.1. Atherosclerosis: a T Cell–Driven Disease

Adaptive immunity plays a crucial role in human ATH (Figure 1.2.). T cells represent the largest adaptive immune system cell population in the ATH plaque, they are found in every stage of plaque development, and constitute 10% of all cells in human plaques. Almost 70% of T cells are $CD4^+$ with the remaining being $CD8^+$ (107).

The majority of $CD4^+$ T cells in the ATH plaque express Human Leukocyte Antigen – antigen D Related (HLA-DR) and interleukin 2 receptor (IL-2R; CD25) indicating they are activated (108). Moreover, most $CD4^+$ T cells in the plaque are Th1 and secrete high levels of pro-atherogenic cytokines including interferon gamma (IFNγ) and TNFα (109). Although Th2 cells and their related cytokines IL-4, IL-5, and IL-13 were also shown in the plaques, their role in disease progression is incompletely understood (110). Similarly, other T cell populations, such as Tregs, Th17 cells, and TCRγδ+ T cells, have been identified in lesions throughout the development of ATH. However, their pathogenic relevance remains to be proven [reviewed in (111)].

An oligoclonal expansion with a skewed pattern of the Complementarity-determining region 3 (CDR3) region in the T cell receptor (TCR) in ATH has been shown (112, 113), indicating the presence of activated T cells reacting to a particular antigenic stimulus. $ApoE^{-/-}$ mice lacking both T cells and B cells display reduced plaque formation compared

to immunocompetent ApoE$^{-/-}$ mice (114, 115). Moreover, adoptive transfer of LDL-specific T cells to immunodeficient ApoE$^{-/-}$ enhances the development of ATH. Similar to ApoE$^{-/-}$ immunodeficient mice, LDL$^{-/-}$ mice with lymphocyte-deficiency (Rag1$^{-/-}$) show significant delays in lesion initiation, formation and progression (116, 117). However, prolonged high-fat diet gradually lessens those differences (118), highlighting the relevance of immunometabolic interactions in the pathogenesis of ATH.

Most naive CD4^{+} T cells constitutively express CD28, that provides co-stimulatory signals required for the proliferation and survival of T cells upon antigen recognition (119, 120). A subset of CD4^{+} T cells that lack CD28 (CD4^{+}CD28^{null} T cells) has been identified in a number of chronic inflammatory diseases, including ATH (121). These cells have been shown to produce more pro-inflammatory cytokines (i.e. IFN-γ and TNFα) than conventional CD28^{+} T cells, both in the resting and activated state (122). Moreover, these cells express and release cytotoxic molecules such as perforin and granzyme B, that play an important role in their cytotoxic effects on ECs *in vitro* (123, 124). In addition, CD28^{null} T cells express reduced apoptotic molecules including Fas, Bim, and Bax. As a result, CD28^{null} T cells are resistant to apoptosis and expand in ATH patients correlating with a higher frequency of acute coronary syndromes and poorer outcomes (121). Further, CD28^{null} T cells preferentially accumulate in unstable lesions (125).

In a recent study, a subpopulation of CD4^{+} T cells expressing CXCR5^{+} was found to promote Signal transducer and activator of transcription (STAT)-1 and STAT3 phosphorylation in B cells that in turn secrete higher levels of IL-6 and IFN-γ (126). These

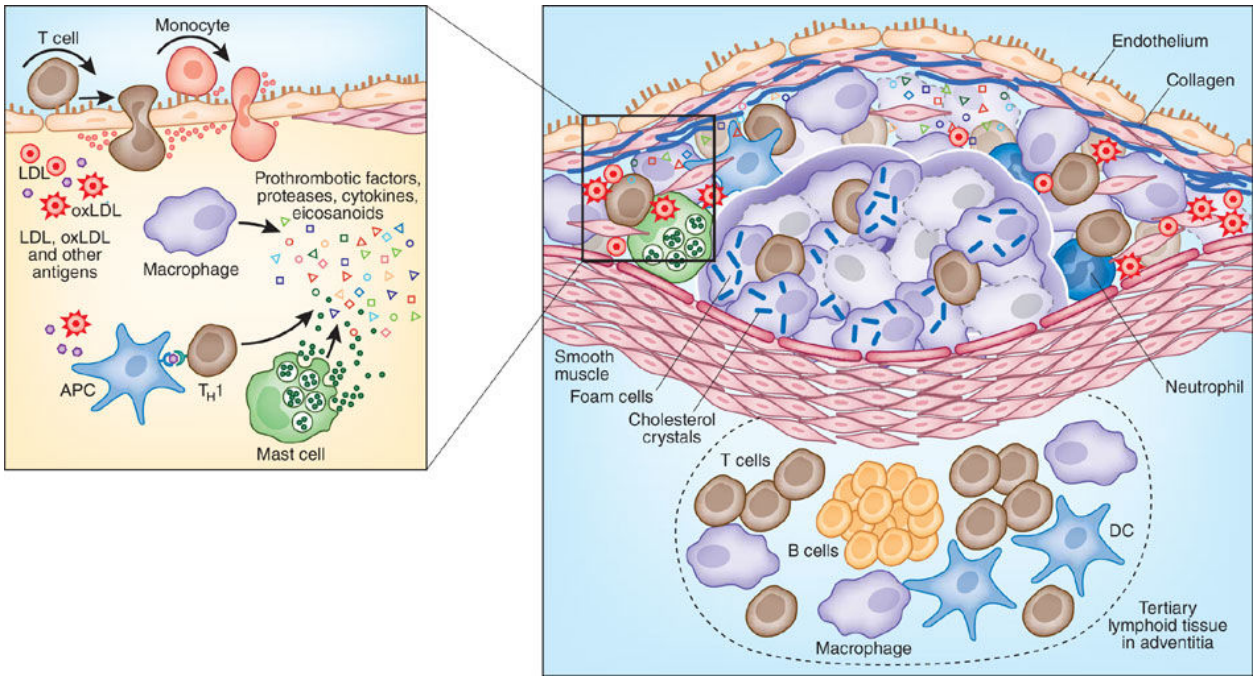


Figure 1.2. Immune cells promote the formation and progression of the ATH plaque. The atheroma consists of a necrotic lipid core made from apoptotic macrophages and VSMS and extracellular lipids. Cells of the innate and adaptive immune systems participate in the ATH plaque formation. Macrophages, T cells, mast cells and DCs are found in the plaque. These cells are organized as tertiary lymphoid structures located within the adventitia. Proliferating smooth muscle cells are present in the tunica media. The figure was reproduced from Hansson GK & Hermansson A (2011), with permission of the publisher (127).

findings indicate that T cells not only promote ATH themselves but also drive other immune cells towards a pro-atherogenic phenotype.

In summary, T cells are one of the main contributors of the inflammatory response in ATH. Targeting T cells in models of ATH significantly reduced the plaque burden. Moreover, clinical trials targeting inflammatory cytokines [interleukin-1β (CANTOS trial), IL-6 (ASSAIL-MI), and tumor necrosis factor-α (CIRT trial)] in ATH patients are ongoing (128). Although these trials will inform on the role of inflammation in ATH, considering the complexity and heterogeneity of this disease in humans, wide-ranging approaches may be required to modulate the immune-mediated events involved in its progression.

1.1.2.2. Role of other adaptive immune cells in atherosclerosis

Cytotoxic $CD8^+$ T cells are detected within the plaque even in the very early stages of the disease, and their frequency decrease as lesions progress. Treatment of $ApoE^{-/-}$ mice with an agonist of CD137, a receptor exhibiting costimulatory activity for activated T cells (primarily $CD8^+$ T cells), aggravated the inflammation increasing the $CD8^+$ T cell infiltration to the plaque (129). Contrarily, $CD8^+$ regulatory T cells were shown to limit ATH development by controlling the germinal center reaction in both secondary and tertiary lymphoid organs (130). In Chapter 2, the effect of age on the modulation of $CD8^+$ proliferation by MSCs was assessed (Supplementary Figure 2.2.)

B cells also participate in atherosclerotic plaque formation (107) and have been shown to exhibit both pro- and anti-atherogenic properties. B cell deficiency was found to be associated with increased lesion size (131), results that were corroborated by splenectomy and B cell transfer experiments (132). In addition, the increase mortality

due to ischemic heart disease in splenectomized patients supports an anti-atherogenic role of B cells in ATH (133). In contrast, mature B cell depletion using a CD20 (B cell specific surface marker)-specific monoclonal antibody induces a reduction in ATH in animal models (134, 135). Moreover, Rituximab treatment (anti-CD20 mAb) in patients with rheumatoid arthritis, was associated with a reduction in carotid ATH (136). Further, *in vivo* studies demonstrated auto antibody production triggered by oxLDL and the accumulation of these antibodies in advanced ATH lesions (137). My work did not assess the effect of ATH-MSCs on the function of B cells, and cells of the innate immune system implicated in atherogenesis.

1.1.3. Limitations of current ATH therapies: need for new strategies

Although there have been great advancements in the medical (e.g., lipid lowering agents HMG-CoA inhibitors, inhibitors of intestinal sterol absorption, bile acid-sequestering agents, proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitors PCSK9 antibodies), and interventional (e.g., angioplasty, drug coated stents) treatment of ATH, in many patients there is insufficient control of disease progression. This due to multiple factors such as inter-individual variations in statin responses (range 5–70%) (138, 139), and limited effects of lipid lowering therapies (reduces plaque size only up to 20-40%).

The realization of the critical role that inflammation plays in ATH plaque progression, justified new therapeutic approaches for this disease. Immunosuppressive drugs such as rapamycin reduce the frequency of re-stenosis by inhibiting the proliferation of VSMCs (140, 141). The results of ongoing trials using colchicine, methotrexate, anti-IL-1 and anti-IL-6 mAb as modulators of immune responses in primary and secondary CVD prevention can potentially change the management of ATH (142-144). Moreover, approaches that

mobilize and stimulate 'intrinsic' immune modulatory mechanisms thus slowing the progression of atherosclerotic cardiovascular disease are being tested (e.g. cellular therapy). In the next section, we will discuss the evidence of MSCs as potential therapeutic tools for ATH.

1.2. MULTIPOTENT MESENCHYMAL STROMAL CELLS

Multipotent mesenchymal stromal cells (MSCs), formerly known as mesenchymal stem cells (145), are a heterogeneous group of progenitor cells that play an important role in maintaining tissue hemostasis. MSCs were first isolated from bone marrow in the late 1960s by Friedenstein and described as "colony-forming unit fibroblasts" (146, 147). Subsequent studies showed that bone-marrow-derived stromal cells can be isolated from almost every tissue, the most common ones used for therapeutic purposes being bone marrow and adipose tissue. Due to varied tissue sources and methodologies of cell preparation, and the inability to compare and contrast studies from different groups, the International Society for Cellular Therapy (ISCT) has proposed minimal phenotypic and functional criteria for defining MSCs. According to the ISCT criteria, MSCs (148): must be plastic-adherent under standard culture conditions; must express positive markers (CD105, CD73 and CD90), and lack expression of hematopoietic markers (CD45, CD34, CD14 or CD11b, CD79α or CD19) and HLA class II; and under standard *in vitro* differentiating conditions, must differentiate into adipocytes, chondroblasts and osteoblasts.

1.2.1. MSCs function

MSCs have a broad range of functions, including: attenuation of tissue injury (stimulation of endogenous progenitor cells), inhibition of fibrotic remodeling, promotion of angiogenesis, reduction of oxidative stress, and modulation of the innate [i.e. dendritic cells (DCs), neutrophils, macrophages and natural killer (NK) cells] and adaptive (i.e. T and B cells) arms of the immune system with a net anti-inflammatory effect (reviewed in (149-154). MSCs exert these functions following activation (i.e. MSCs licensing), and through the secretion of paracrine factors capable of modulating the microenvironment and influencing the activity of resident cells (149). In fact, long term engraftment of MSCs is not necessary for their therapeutic effects, and the MSC's conditioned medium (CM) alone can reproduce these cells' effects (155). The MSC's secretome includes not only cytokines, growth factors, hormones, and other soluble factors but also extracellular vesicles with cargos including peptides, proteins, lipids, DNA, messenger RNA (mRNA), microRNA (miRNA), long noncoding RNA and even mitochondria (156). In the following section, I will expand on the immune-modulatory properties of MSCs as it is central to my work.

1.2.1.1. MSC Immunomodulation

MSCs play a central role in modulating immune responses by sensing inflammation, homing to affected tissues, and controlling local inflammatory effectors (157). MSCs have strong immunosuppressive properties through modulating both the innate and adaptive immune systems [reviewed in (150)]. Although the underlying mechanisms of MSC immunomodulation have yet to be fully elucidated, they are likely mediated by both

soluble factors and cell contact-dependent mechanisms. MSCs have been shown to regulate the adaptive and innate immune systems by suppressing T cell proliferation and dendritic cell maturation, inhibiting B cell activation and proliferation, reducing NK cell proliferation and cytotoxicity, and by promoting the generation of regulatory T cells.

"Licensing' or 'activation' is required for MSC to regulate other immune cells (158, 161). Licensing can occur in response to a variety of cytokines produced by immune effector cells as a consequence of antigen processing and activation. The combination of IFN-γ and TNF-α is considered as the optimal way to promote MSC licensing in vitro [reviewed in (158)]

In addition, MSCs sense their microenvironment recognizing endogenous (e.g. heat-shock proteins and RNA) and exogenous (e.g. bacterial products) danger signals through the expression of various TLRs. MSCs activated through TLR3 show enhanced immunosuppressive activity (defined as MSC1), whereas TLR4 priming confers a pro-inflammatory phenotype upon MSCs (defined as MSC2) associated with T-cell activation capability (159). Therefore, changes in the composition of the MSC's microenvironment impact their phenotype and function.

MSCs immunomodulation involves redundant mechanisms including: (**a**) secretion of anti-inflammatory factors (e.g. IL-6, IL-10, transforming growth factor β (TGF-β), prostaglandin-E2 (PGE_2), hepatocyte growth factor (HGF), Epidermal growth factor (EGF), Fibroblast growth factors (FGF), Platelet-derived growth factor (PDGF), Vascular endothelial growth factor (VEGF), insulin-like growth factors (IGF), stromal cell-derived factor 1 (SDF-1), leukocyte inhibitory factor (LIF), IL-1 receptor antagonist (IL-1Ra),

galectins, TNF-stimulate gene 6 (TSG-6), human leukocyte antigen-G (HLA-G) and indoleamine 2,3-dioxygenase (IDO) (160); (**b**) expression of cell surface molecules with immunosuppressive properties (e.g. programmed death ligand 1 [PD-L1], Fas ligand) (161); (**c**) secretion of chemokines that attract immune effector cells that will in turn be inhibited by MSCs (e.g. neutrophil chemo-attractant, IL-8, MCP-1/CCL2); (**d**) induction of other regulatory cells (i.e. DC to secrete IL-10, expand T regs, regulatory macrophages, regulatory B cells) (reviewed in (149) ; and (**e**) metabolic control of the immune system (e.g. indoleamine 2,3-dioxygenase (IDO) expression in MSCs inhibits lymphocyte proliferation) (162). These mechanisms lead to the suppression of T cell proliferation and dendritic cell maturation, inhibition of B cell activation and proliferation, reduction in NK cell proliferation and cytotoxicity, and generation of regulatory T cells. Conversely, there are some cytokines or chemokines secreted by MSCs such as IL-6, IL8 and MCP-1 known to promote an inflammatory response (68). The balance between anti- and pro-inflammatory factors in the MSC secretome determines the MSC immunomodulatory capacity.

In the next section, I will review in-depth the effects of MSCs on $CD4^+$ T cells. I will focus on the MSCs: T cell crosstalk, as the functional readout that I have used to evaluate MSCs immunopotency is the *in vitro* inhibition of T-cell expansion.

1.2.1.1.1. MSCs: T cell Crosstalk

T cells are the major cellular effectors of the adaptive immune response and play a central role in cellular-mediated immunity. Moreover, they are key players in atherosclerotic plaque formation - the disease model of my thesis- as discussed in section 1.1.2. Human

MSCs constitutively express VCAM-1, leukocyte function-associated antigen-3 (LFA-3), and major histocompatibility complex (MHC) class-I antigens that are essential for their communication with T cells. Upon (IFN)-γ licensing, MSCs also express intercellular adhesion molecule-1 (ICAM-1) and MHC class-II antigens.

MSCs inhibit T cell proliferation induced by different stimuli including mitogens (163), alloantigens, anti-CD3 and CD28 antibodies, or "professional" antigen presenting cells (APC) (164). The MSCs: T cell suppressive capacity is dose-dependent, and affects both naïve and memory $CD4^+$ and $CD8^+$ T cells. In addition, the MSCs immunopotency varies according to the tissue source. Compared to bone marrow and umbilical cord MSCs, adipose derived MSCs possess the strongest immunomodulatory potential (165). Lastly, the MSC's anti proliferative effects on T cells are reversible. Upon removal of MSCs, T lymphocytes respond to re-stimulation and proliferate (166).

Three fundamental mechanisms account for the effect of MSCs on T cells: (1) G0/G1 phase arrest due to cyclin D2 inhibition (167); (2) reduction in the IFN-γ production by activated $CD4^+$ T cells; and (3) induction of apoptosis in activated $CD4^+$T cells (168).

MSCs exert their inhibitory effect on T cells through both cell contact dependent and independent mechanisms. PGE_2, TGFβ, HGF, IDO and HLA-G5 were identified as important mediators in MSC inhibition T cell proliferation (165). Recent studies have shown that MSC-derived exosomes and microvesicles also exert immunomodulatory effects on T cells (169). The contribution of cell-cell contact dependent and independent mechanisms to MSCs immunomodulatory function on T cells were assessed in Chapter 3 (68)

1.2.1.1.1.1. MSCs: Th1 cells Crosstalk

MSCs have been shown to inhibit Th1 response by reducing their IFN-γ and TNFα secretion and increasing IL-4 secretion. Excessive levels of IFNγ and/or TNFα present under inflammatory conditions increase the expression of TGFβ by MSCs, which in turn prompts Th1 cells to increase their IL-10 secretion and reduce IFNγ production (170). In addition, MSCs stimulate Th1 cells to downregulate their IFNγ receptor (making them less responsive to IFNγ) and stimulate the expansion of T-bet$^+$ Th1 cells that co-express IFNγ and IL-10 (171).

1.2.1.1.1.2. MSCs: Th17 cells Crosstalk

MSCs have a dual effect on Th17 cells (172, 173) the nature of which depends on the CD4$^+$ T cell activation state. MSCs suppress Th17 cell expansion if MSCs are exposed to T cells at the time of their activation. However, once T cell activation has occurred, MSCs promote Th17 cells expansion. This is as a result of the increase in IL-6 levels produced by MSCs, a key mediator of Th17 cell differentiation, in the cultures (174).

1.2.1.1.1.3. MSCs: Treg cells Crosstalk

MSCs expand T cells that possess a methylated forkhead box P3 (FOXP3) gene with a Treg-specific demethylated region (TSDR) leading to an induction of Tregs (175). MSCs promote three major Treg subsets (**1**) IL-10$^+$ T regulatory 1 (Tr1), (**2**) Transforming growth factor (TGF)-β$^+$ T helper 3 (Th3), and (**3**) CD25$^+$FOXP3$^+$ natural Treg-like CD4$^+$ cells (176). FOXP3 is a downstream target for Notch signaling and activation of the Notch1 pathway in CD4$^+$ T cells induce Treg differentiation (177). These Tregs efficiently

suppress T-cell proliferation triggered by antigenic peptides. MSCs effect on Tregs requires both direct cell to cell contact, and secreted factors including PGE_2, TGF-β, HLA-G5 and heme oxygenase 1 (HO-1) (170, 176, 178). In addition, MSCs increase IL-10 secretion by Tregs and DCs, with DC-derived IL-10 in turn promoting the expansion of Tregs (179). There is also an indirect mechanism in which MSCs upregulate Fas ligand (FasL)/Fas-mediated apoptosis of T cells, thus leading ultimately to Treg induction. FAS regulates MCP-1 production by MSCs, then MCP-1 plays a crucial role in T cell recruitment, and subsequently expression of FASL by MSCs induce T cell apoptosis. These apoptotic T cells induce high levels of TGFβ production by macrophages leading to upregulation of Tregs and, ultimately to immune tolerance (180).

In vivo, MSCs induce Tregs ameliorating disease activity and promoting graft survival in transplant settings (165).

Finally, MSCs inhibit the proliferation and maturation of cytotoxic T lymphocytes (CTL) (181). They also downregulate the CD8 surface marker on CTLs via an indirect mechanism mediated by $CD14^+$ monocytes. Throughout this process, CD28 is downregulated on CTLs suggesting a switch from an effector-type (cytotoxic) to a regulatory-type (immunosuppressive) (182).

1.2.2. Effects of MSCs in the modulation of atherosclerosis

MSCs' powerful immunomodulatory capacity makes them a promising therapeutic tool in ATH. In experimental models of ATH, transplanting MSCs is atheroprotective due to a number of mechanisms that include: (**1**) increased secretion of anti-inflammatory cytokines (TGF-β and IL-10) and decreased circulating levels of pro-inflammatory cytokines [IFN-γ, TNFα, Matrix metallopeptidase (MMP)-1, IL-6 and CRP] (183, 184)

resulting in an overall reduced inflammatory state and a significant reduced differentiation of naive T cells; (**2**) reduction of oxLDL-induced changes in ECs and restoration of EC-dependent relaxation with inhibition of plaque formation (185); (**3**) modulation of the atherosclerotic plaque's composition with decrease in the number of macrophages and increase in intralesional Tregs (184, 186); (**4**) reduction in the number of overall apoptotic cells in the tunica media, lipid core and intima thus promoting plaque stability (183); (**5**) modulation of lipid metabolism by reducing the very low-density lipoprotein levels in plasma (187). Despite all these mechanisms, the long-term follow-up of *in vivo* studies using MSCs as ATH therapy, failed to show differences in the plaque burden of animals treated with MSCs (185). These data suggest that MSCs do not sustain their beneficial effect long-term. Further investigations should be directed at identifying the factors that could maintain the effects of MSCs; and defining the role of MSCs' in different stages of ATH.

1.2.3. Current status of MSCs based therapies

MSCs are one of the most commonly cell-based therapies under investigation for treating a variety of inflammatory human diseases (over 700 clinical trials listed at www.clinicaltrials.gov as of October 24th, 2017-using the key words: Mesenchymal stem cells / Mesenchymal stromal cells as searching strategy).

According to current evidence, MSC-based therapies are safe. However, the positive results seen in preclinical animal studies have not yet translated to phase II/III clinical trials. Donor variability, source of tissue, expansion-cryopreservation induced senescence, epigenetic reprogramming, cell dose, timing of infusion, route of administration, and pre-activated state of MSCs are some of the factors that account for the inconsistent

outcomes in MSCs clinical trials (188). The lack of standardization of the MSCs *ex vivo* expansion and cell manufacturing methods further contribute to inter-trial variability. MSC-based products generated for different clinical trials have differences in their phenotype and functional properties (189) that affect their immunomodulatory function and ultimately clinical outcomes. The MSCs inhibitory effect on $CD4^+$ T-cell proliferation (MSC immunopotency) is a key mechanism of action of MSCs. Prior to transplantation, *in vitro* assessment of immunopotency is suggested as a surrogate marker for MSCs clinical efficacy (190). A four-day co-culture of MSCs with anti CD3/CD28 activated PBMCs has been proposed as a reproducible assay to measure MSC-mediated T-cell suppression (190, 191). The limitations of this method include: the donor dependent intrinsic differences in responder PBMCs (192), and the use of unfractionated PBMCs with variable monocyte concentrations that subsequently would lead to different degrees of "MSCs' licensing" (148). To overcome these limitations in my work I used monocyte depleted PBMCs from a single PBMCs donor for all immunosuppression assays.

MSCs exert their therapeutic effects via secreted factors that are suggested to modulate inflammatory responses, and promote the proliferation and differentiation of tissue specific progenitor cells *in situ* (Figure 1.3.) (193). MSCs engraftment is very low after transplantation, and engrafted MSCs tend to be short-lived. A way to prolong the beneficial effects of MSCs could be achieved by improving their ability to survive after transplantation. One of the most important applications of MSC-based therapy is ischemic injuries including MI. Ischemic injuries are known to lack oxygen, carbon supply and pro-survival growth factors. Lack of these factors alter cellular metabolism and lead to cell

death. The ability to survive in an environment of deprived oxygen and nutrients (i.e. increased oxidative stress) significantly influences the efficacy of MSC-mediated tissue repair and regeneration. Thus, understanding the MSCs metabolism (baseline and post-cytokine priming) could inform ways to promote their survival and enhance their function.

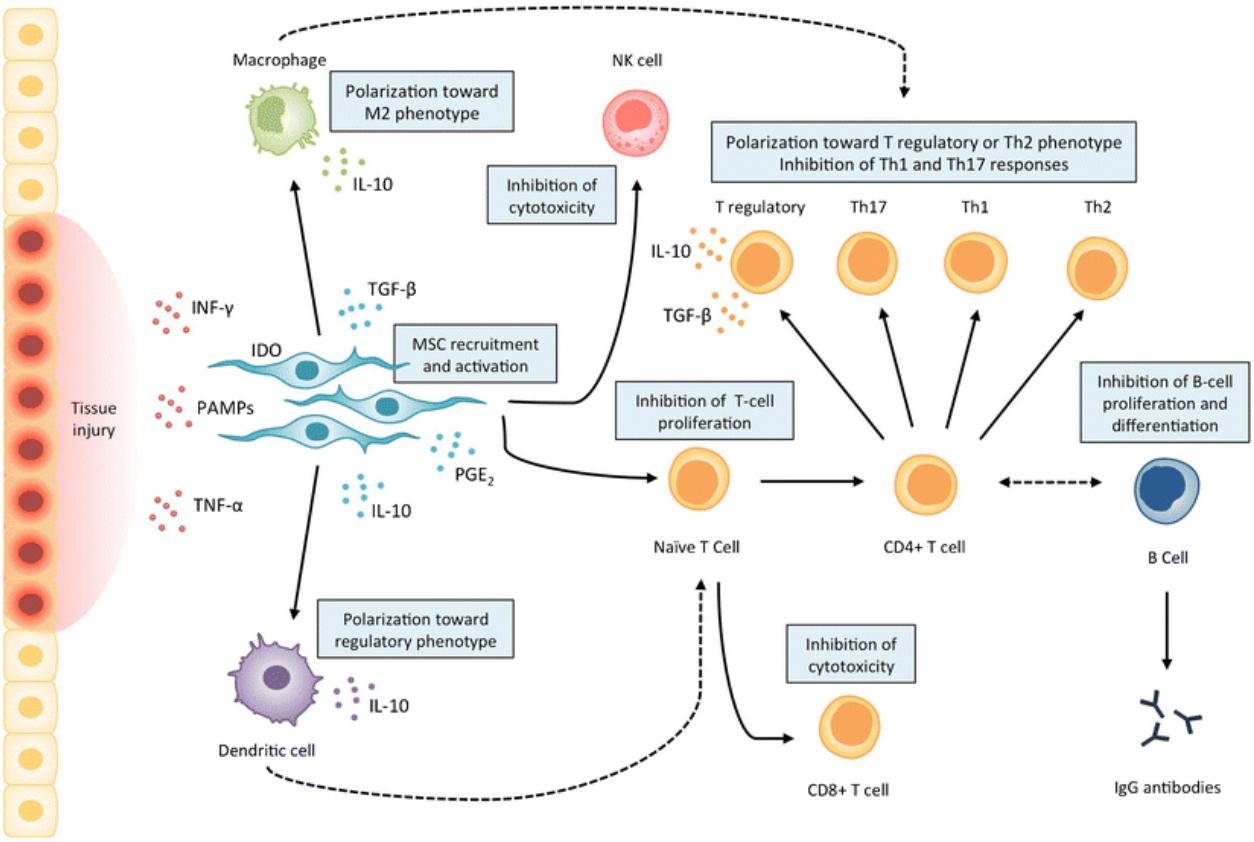


Figure 1.3. Mode of action of MSCs *in vivo*

MSCs migrate to injured tissues and are licensed by inflammatory stimuli in the microenvironment. Post-licensing MSCs interact with both adaptive and innate immune cells. They affect both the differentiation and proliferation of immune cells, promoting tolerogenic immune responses. The figure was reproduced from Brunel M, Herr F, & Durrbach A (2016), with the permission of the publisher (193).

Since my work is focused on ATH as a disease model and given the fact that microenvironment can modulate MSCs phenotype, in the next section, I will discuss the key concepts on cellular metabolism and oxidative stress in the context of ATH.

1.3. CELLULAR METABOLISM AND OXIDATIVE STRESS

Metabolism is a collection of chemical processes that take place in a living cell or organism that are essential for the maintenance of life. Although metabolism involves thousands of reactions and metabolites, it can be divided into three main processes (194): (**1**) Anabolism, defined as a synthesis of complex macromolecules from simple molecules, such as the formation of carbohydrates, proteins and fatty acids from their precursors; (**2**) Catabolism, that is the degradation of macromolecules into their smaller components to create energy; and the (**3**) elimination and recycling of waste products (195). The survival and proper function of cells is dependent on the balance between these metabolic mechanisms. The cellular dynamics are directly linked to their metabolic state. While quiescent cells are balanced between anabolic and catabolic metabolism; growing or proliferating cells shift their metabolism towards anabolic states (196).

1.3.1. Cellular Energy

The key energetic molecule of a cell is adenosine triphosphate (ATP). ATP is used as a cofactor in cellular processes, providing the necessary energy for enzymatic reactins. When hydrolyzed from its phosphoranhydride bonds, ATP releases 7.3kCAL (ADP + Pi), or 10.9kCAL (AMP + PPi) as energy (194). The amount of ATP is tightly controlled in cells, as the proper concentration levels are essential for cell viability (197). When ATP is hydrolyzed, it produces an inorganic phosphate (Pi) and ADP, which is then catalyzed by adenylate kinase to produce AMP and ATP. AMP plays an important role as an

intracellular signaling molecule to maintain the energy balance (198). The dynamic changes of the adenylate ratios within a cell (AMP:ADP:ATP) are influenced by different metabolic processes which will be discussed in the following sections.

1.3.2. Energy production: The choice between glycolysis and mitochondrial oxidative metabolism

Complex organisms coordinate their activity while ensuring the utilization of available energy in the most efficient manner. Energy is derived mostly from nutrients and to a lesser extent from the organism's own cells through autophagy (199).

The release of the energy stored in nutrients can be done by a series of complex processes, leading ultimately to ATP production. The initial step is the breakdown of macromolecules such as sugar, protein and fats into glucose, amino acids and glycerol/fatty acids, respectively. Then glycolysis and oxidative phosphorylation further metabolize these molecules to generate ATP (194). The amount of energy in the form of ATP that is produced by OXPHOS is much more compared to glycolysis (36 vs 2 ATP). Therefore, OXPHOS is the main path for energy production, while glycolysis is a default pathway. However, although OXPHOS is clearly more efficient for energy production, it carries a price: utilization of oxygen creates potentially toxic by-products, namely ROS (200). Furthermore, OXPHOS is a much more complex procedure thus more prone to errors.

1.3.2.1. Glycolysis

Glycolysis takes place in the cytosol and this pathway can be divided into three main stages. The first stage involves the conversion of glucose into fructose 1,6-bisphosphate,

the next stage is the cleavage of the fructose 1,6-bisphosphate into two interconvertible three-carbon molecules and the last stage is the ATP generation.

Metabolic alterations in ATH have been investigated in experimental models. A glycolytic shift with increased levels of glucose uptake was shown not only in atherosclerotic plaque resident cells, but also in stem and progenitor cells in these models (201, 202). Moreover, increased levels of aerobic glycolysis metabolites were found in the arteries of atherosclerotic animal models (203). Thus, the Warburg effect (i.e., a chronic shift in energy production from mitochondrial oxidative phosphorylation to aerobic glycolysis) that was originally described for cancer cells is also suggested to play a crucial role in ATH (204). Of note, the Warburg effect is partly attributed to mitochondrial dysfunction that results in enhanced glycolytic ATP production. Increased glycolysis is proven to promote VSMCs proliferation in ATH. More importantly, ATP generated from glycolysis is essential to meet the increased energy demands of VSMCs proliferation (205). These proliferating VSMCs within the vessel wall are known to be an important contributor to the development as well as the progression of ATH (206).

The glycolytic shift in ATH is also seen in other cells types, including macrophages. A metabolic shift towards glycolysis is an important feature of the M1-like pro-inflammatory macrophage phenotype, that promotes inflammatory responses in ATH progression (207). Plaque size can be reduced by inhibiting miR-33, which regulates aerobic glycolysis. In addition, increased aerobic glycolysis induces the transcription of IL-1β (208); IL-1β promotes the development of lipid plaques and subsequently destabilizes the plaques in $ApoE^{-/-}$ mice (209). Human studies also confirm these findings. Patients with

symptomatic ATH have a pro-inflammatory phenotype of circulating monocytes with increased expression of glycolytic enzymes (210).

Based on the above, glycolysis inhibition might alleviate the development of ATH by reducing inflammatory responses. Considering these data, we assessed in Chapter 4 wheather MSCs from ATH individuals shift their metabolism towards glycolysis if so what are the consequence of this switch on their secretory phenotype.

1.3.2.2. Electron Transport Chain

The electron transport chain (ETC) generates ATP for cellular functions. The mitochondrial respiratory chain consists of biochemically linked enzymes (complexes I, II, III, and IV) and two electron carriers (ubiquinone/coenzyme Q and cytochrome c). They create a proton gradient across the mitochondrial inner membrane that is used by F_1F_0-ATP synthase (complex V) to drive the synthesis of ATP (211). In our study, we used oligomycin, a complex V ATPase inhibitor, to induce mitochondrial dysfunction.

ETC complexes and mitochondrial dysfunction have been extensively studied in the development and progression of ATH [reviewed in (16)]. Both animal and human atherosclerotic plaques show increased mitochondrial DNA (mtDNA) damage. Furthermore, it has been postulated that mtDNA damage leads to a reduction in the expression of ETC complexes thus playing a direct causative role in the development of ATH (212). Targeting miR-33 have been shown to downregulate ABC transporters (ABCA1 and ABCG1) involved in cholesterol and HDL generation. Use of anti-miR-33 therapy increase mitochondrial respiration and ATP production, ultimately resulting in enhanced macrophage cholesterol efflux and attenuated ATH (213). In Chapter 4 we

evaluated the impact of ATH on MSCs' mitochondrial function furthermore, we investigated the link between mitochondrial dysfunction and MSCs immunopotency.

1.3.3. Reactive Oxygen Species

ROS family consists numerous small reactive ions and molecules that result from oxygen metabolism. Free radicals including superoxide ion ($O_2^{\bullet -}$) and hydroxyl radical ($OH^{\bullet -}$) are unstable and have short biological half-lives. Contrarily, nonradicals of ROS such as H_2O_2, singlet oxygen ($^1O_2$), peroxynitrite ($ONOO^-$) and hypochlorous acid (HOCl) are more stable (214). $O_2^{\bullet -}$ is the precursor of most ROS and is at extremely reactive radical with rapid spontaneous or enzymatic dismutation. Most of the $O_2^{\bullet -}$ is rapidly converted to H_2O_2 and mediates downstream cell signaling. The decomposition of H_2O_2 generates $OH^{\bullet -}$, which is a nonselective and irreversible reactive radical that is associated with oxidative damage (215). Under physiological conditions, intracellular ROS production and elimination are tightly controlled and maintained at a constant low level. While, modest amounts of ROS play a crucial physiological role in cellular signaling pathways, such as metabolism, growth, differentiation and death signaling, excessive ROS levels leads to tissue damage by inducing DNA strand breaks and various types of modification in DNA bases, proteins and lipids ultimately leading to cellular senescence and death (reviewed in (216). Oxidative stress has been implicated in several diseases, including ATH (217, 218). 2′-7′-Dichlorodihydrofluorescein diacetate (DCFDA) is the most commonly used method to detect intracellular ROS levels. DCFDA passively diffuses into cells and upon cleavage of the acetate groups by intracellular esterases and oxidation, the nonfluorescent H_2DCFDA is converted to the highly fluorescent 2',7'-dichlorofluorescein (DCF) which can be measured by flow cytometry (219).

In the next section I will discuss the important generators of intracellular ROS (Figure 1.4.)

1.3.3.1. Mitochondria

One of the major sources of ROS is the process of oxidative phosphorylation (OXPHOS). OXPHOS in the mitochondria generates limited physiological levels of superoxide, that is converted to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD). Experiments performed in isolated mitochondria showed that Complex I and III are the major sites of superoxide production of ETC. However, *in vivo*, various factors including the mitochondrial membrane potential are proposed to influence ROS generation (220). Indeed, a positive correlation between proton motive force and ROS production has been shown. Oxygen availability is also considered a regulator of mitochondrial ROS production. Yet, it is critical to emphasize that mitochondrial ROS also serve as key signaling molecules (221).

1.3.3.2. NADPH oxidases

The NADPH oxidase (NOX) family of enzymes is another important source of intracellular ROS production. Although NOX enzymes were first described in neutrophils as part of their essential role in host defense, it is now accepted that non-phagocytic cells also produce low levels of different NOX enzymes (222). There are seven human NOX homologues [NOX1–5, dual oxidase 1 (DUOX1) and (DUOX2)] that produce ROS for different purposes, ranging from host defense to signaling functions.

1.3.3.3. Other enzymes

Although mitochondria and the NOX family of enzymes are the best-characterized intracellular sources of ROS, there are many other sources of ROS production including xanthine oxidase, nitric oxide synthase, cyclooxygenases, cytochrome P450 enzymes and lipoxygenases. In addition to these enzymes, peroxisome and the endoplasmic reticulum are also known to produce ROS. The relative contribution of these additional sources of ROS varies for each cell types.

1.3.3.4. Antioxidants

Antioxidant systems exist to counter balance the harmful effect of excessive ROS in the organism. SOD, catalase and glutathione peroxidase (GPx) are the main antioxidant enzymes. SOD exists as three isoforms (SOD1, SOD2 and SOD3), all of which catalyze the dismutation of $O_2^{\bullet -}$ into H_2O_2 and oxygen (223). Catalase plays an important role in the elimination of H_2O_2 by decomposing H_2O_2 to oxygen and water. GPx reduces H_2O_2 to water and organic peroxides to their corresponding alcohols.

Other molecules that are known to react with ROS and exhibit antioxidant activity include vitamin C, vitamin E, uric acid, tripeptide GSH, phenolics, flavonoids and thiol compounds [reviewed in (224)].

There are several mitochondria targeted ROS scavengers. In our study we used MitoCP (a triphenylphosphonium moiety which targets the mitochondria, and a carboxy-proxyl structure with antioxidant properties); and MitoTempo [mitochondria-targeted superoxide dismutase mimetic, a compound composed by the antioxidant piperidine nitroxide

(TEMPO) and the lipophilic cation triphenylphosphonium, that allows passing through lipid bilayers thus accumulating in mitochondria] to reduce mitochondrial ROS in ATH MSCs. The balance between ROS generation and elimination is vital to redox homeostasis. While low levels of ROS play an essential role as second messengers in cellular functions [including cell renewal, cellular metabolism, proliferation and differentiation (225)], abnormal and excessive ROS accumulation results in oxidative stress and leads to irreversible cellular damage.

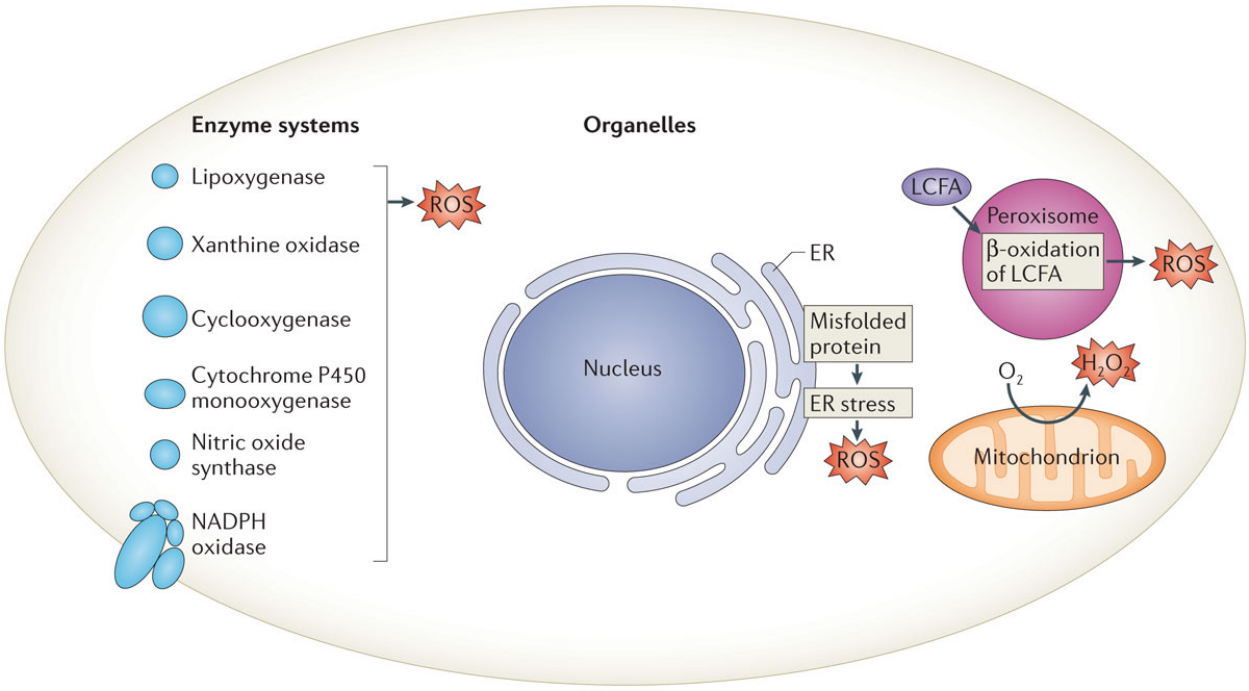


Figure 1.4. Intracellular sources of reactive oxygen species Organelles that can generate ROS include mitochondria, the endoplasmic reticulum and peroxisomes. Moreover, numerous enzymes, including oxidases and oxygenases, generate ROS during their enzymatic reactions. The figure was reproduced from Holmstrom KM & Finkel T (2014) with the permission of the publisher (226).

1.3.4. Implications of ROS in ATH

Animal models support the oxidative damage in both atherosclerotic plaque resident cells and circulating cells. Decreased GPx activity promotes atherogenesis, while reduction in superoxide generation attenuates ATH progression (16).

ROS production from the vessel resident cells (e.g., ECs, VSMCs) plays an important role in LDL oxidation as well as promoting inflammatory responses within the atherosclerotic plaques. Oxidative modification of LDL plays a causative role in both development and progression of ATH as discussed in section 1.1.2.

ROS cause the oxidation of polyunsaturated lipids, with resulting reaction by-products leading to the alteration of apolipoprotein (Apo) B-100 functions. Modified Apo B-100s are less efficient in removing LDL. In turn, this prolongs the exposure of LDL and ApoB-100s to ROS-mediated modification and ultimately further enhances the oxidation of LDLs (227). ROS are also implicated in oxidation of HDL by HOCl, in turn promoting atherogenesis by hampering the anti-atherogenic effect of HDL (228).

Increased oxidative stress and oxLDL levels results in enhanced macrophage production of macrophage colony-stimulating factor and MCP-1/CCL2. These cytokines subsequently attract more monocytes to the arterial wall and promote their differentiation into tissue macrophages within the atherosclerotic lesions (82). Furthermore, ROS were shown to activate NF-κB signaling, resulting in increased expression of MCP-1/CCL2, MMP9, VCAM-1 and pro-coagulant tissue factor in macrophages, ultimately leading to further accumulation of macrophages in vascular wall as well as foam cell formation (229).

Thrombosis is an important complication of ATH that results in arterial occlusion and ischemia. It has been proven that platelet activation plays a pivotal role in thrombus formation (230). A number of studies have suggested that excessive ROS levels indirectly affect platelet activation thus increasing the susceptibility to thrombotic events (231). Moreover, inhibiting NOX2 has been reported to decrease platelet adhesion and thus atherosclerotic plaque formation (232). In addition to observations from animal models of ATH, resident cells of human atherosclerotic plaques also have oxidative damage (233). VSMCs from atherosclerotic plaques display characteristics of oxidative stress including, poor proliferation, premature senescence both in culture and *in vivo* and extensive DNA damage, marked telomere shortening, and markers of senescence (234).

In summary, ROS mediated lipid oxidation is key to ATH pathogenesis, and increased ROS levels are present in the vessel wall at all stages of ATH (235). In Chapter 4 we investigated the features of oxidative stress in MSCs from ATH subjects.

1.3.5. ROS, Senescence and ATH

Cellular senescence is a process that leads to an irreversible cell cycle arrest. Senescent cells develop a distinct phenotype characterized by changes in their morphology (i.e., larger volume and cytoplasmic flattening), increased lysosomal enzymatic activity (SAβG) and content. Furthermore, senescent cells have an altered metabolism with excessive ROS production; and secretion of growth factors, extracellular matrix degrading proteins, and pro-inflammatory cytokines, collectively known as the senescence-associated secretory phenotype (SASP) [reviewed in (236)]. Mitochondrial dysfunction resulting in ROS accumulation has also been implicated in cellular senescence and aging (237, 238). The increased ROS levels in senescent cells are linked to an increased mitochondrial

mass, reduced mitochondrial membrane potential and defective antioxidant mechanisms. ROS have been shown to induce DNA damage and to accelerate telomere shortening, two known hallmarks of aging, that in turn activate the DNA damage response (DDR) (239, 240). Moreover, antioxidant treatment [e.g., N-acetyl cysteine (NAC)], prevents the cell-cycle arrest that is a hallmark of senescent cells. On the other hand, the inhibition of ETC complexes by either pharmacological agents [e.g., complex I by rotenone (241) , complex II by desferroxamine mesylate (242) , complex III by antimycin A (243) or genetic modifications [e.g., knockdown of the complex I assembly factor NDUFAF1 (244) and complex III activity associated mitochondrial Rieske iron-sulfur polypeptide (RISP) (241)] induce premature senescence. The induction of permanent growth arrest by the disruption of the ETC is explained by 2 mechanisms: 1- elevated ROS (resulting from increased electron leakage), and 2- decreased ATP production.

Features of senescence are detected in VSMCs, ECs and macrophages from atherosclerotic plaques. The evidence of cellular senescence in these cells includes their 1-Reduced proliferative capacity and prolonged population doubling time (245, 246); 2-Increased numbers of SAβG-positivity; 3- Telomeric shortening; 4-Increased nuclear and mitochondrial DNA damage [reviewed in (247)].

Although the mechanisms underlying cellular senescence in ATH are likely to be multiple and cumulative; the fact that oxidative stress induces DNA damage and subsequent activation of the DDR ultimately promoting senescence and apoptosis, provides evidence that excessive ROS may be critical in atherogenesis.

In summary, although there has been an enormous progression in the medical and interventional treatment of ATH, many patients still suffer from the complications of this

disease. MSCs represent a unique cell type with immunomodulatory functions, tissue-protective properties and multilineage differentiation potential. As a result of this, MSCs are actively being investigated as a “novel strategy” for tissue repair (i.e. immune modulation and suppression of dysregulated inflammatory responses), in ATH and its complications. To what extent ATH and its risk factors affect the functional potency thus limiting the therapeutic benefit of MSCs remains unknown. In my work, I characterized the function of MSC from patients with ATH and assessed the role of mitochondrial dysfunction as a culprit of the ATH-MSCs altered secretome and in vitro reduced immune suppressive ability.

Our findings open up the possibility that in atherosclerosis, dysfunctional MSCs could contribute to the state of chronic low grade inflammation promoting disease progression. The integration of this knowledge in the design of future trials could reduce the variability observed between reported studies on the benefit of MSCs treatment in a range of diseases.

Preface to Chapter 2

Atherosclerotic ischemic heart disease is the leading cause of death globally. Despite the success in treating ATH, preventing its complications remains a major challenge in the field. Since chronic low-grade systemic inflammation is a risk factor promoting accelerated ATH and its complications; targeting the immune system is a novel therapeutic approach to prevent ATH ischemic disease.

Due to their immunomodulatory properties, autologous transplantation of MSCs are evaluated as cell therapy for ATH and its associated complications (i.e., myocardial infarction and stroke). The reported therapeutic efficacy of autologous MSCs in ATH trials is highly variable. The discrepancy between studies can be attributed to several factors including: donor related intrinsic differences, variability of the source (i.e., BM, adipose tissue), expansion-cryopreservation induced senescence, number of cells and route of administration. The effect of donor age and age-associated conditions (i.e., type 2 diabetes mellitus (T2DM) and ATH) on MSCs immunomodulation is unknown. We hypothesized that age and age-associated chronic inflammatory conditions negatively impact the immunomodulatory capacity of MSC.

CHAPTER II: AGE, ATHEROSCLEROSIS AND TYPE 2 DIABETES REDUCE HUMAN MESENCHYMAL STROMAL CELL-MEDIATED T-CELL SUPPRESSION

AGE, ATHEROSCLEROSIS AND TYPE 2 DIABETES REDUCE HUMAN MESENCHYMAL STROMAL CELL-MEDIATED T-CELL SUPPRESSION

Ozge Kizilay Mancini, Dominique Shum-Tim, Ursula Stochaj, José A. Correa, Inés Colmegna

2.1. Abstract

Introduction: Atherosclerosis (ATH) is an age-associated, multifactorial process driven by immune activation and inflammation. Ongoing clinical trials aim to establish the role of mesenchymal stromal cells (MSCs) as therapeutic agents in ATH. The beneficial effects of MSCs derive from their immune-modulatory properties. Understanding the impact of aging and age-associated conditions (i.e. type 2 diabetes mellitus (T2DM) and ATH) on MSCs function is key to maximizing their therapeutic potency. The aim of this study was to assess the effect of chronological and biological aging on human MSCs–mediated $CD4^+$ T-cell suppression. Methods: Human MSCs were isolated from adipose tissue and the MSC:$CD4^+$T-cell suppression was assessed in a co-culture system. Results: MSCs from elderly donors (≥ 65 years) had significantly lower T-cell suppressive capacity compared to those from donors <65 years (p=0.003). Furthermore, MSCs from patients with ATH and T2DM were less efficient at suppressing T-cell proliferation (ATH p= 0.02, T2DM p=0.04 compared to non-disease controls). Sex and tobacco use did not impact the immunosuppressive capacity of MSCs. Conclusions: Advanced age, ATH and T2DM reduce the functional potency of MSCs. Optimizing the criteria for the selection of MSCs' donors could enhance the results of cell-based therapies.

2.2. Introduction

Atherosclerotic ischemic heart disease is the leading cause of death in developed countries. The prevalence, incidence, and severity of atherosclerosis (ATH) markedly increase with chronological age and in the context of age-associated chronic inflammatory conditions such as type 2 diabetes mellitus (T2DM) (248). Chronic inflammation is a key regulatory process that links multiple risk factors for ATH and its complications with altered arterial biology. In mature atherosclerotic lesions, immune responses mediated by $CD4^+$T-cells seem to be critical to accelerate atherogenesis and to promote plaque instability (249). This is supported by the correlation between increased circulating levels of activated $CD4^+$ and the extent of ATH in carotid and coronary arteries, and by the larger number of these cells in unstable plaques compared to those from patients with stable coronary artery disease (250, 251). *In vivo* studies also showed the arrest in the development and progression of ATH following T-cell targeted therapy (i.e. anti-CD3Ab) (252). The fundamental role of immune-activation in ATH provides the rationale for the evaluation of approaches to restore immune homeostasis as therapeutic interventions in ischemic heart disease. Among these strategies, the use of mesenchymal stromal cells (MSCs) showed promise in preclinical studies and most recently in patients with non-revascularizable ischemic myocardium [reviewed in (253)] (254). MSCs immunosuppressive and anti-inflammatory effects are key mechanisms underlying these cells' therapeutic effects (255). A critical aspect linked to the success of any type of cell therapy is the appropriate selection of donors, however the effect of donor's age and age-associated co-morbidities on human MSCs–mediated T-cell suppression remains undefined (256, 257). The aim of this study was to evaluate the

impact of chronological aging and of age-associated diseases, ATH and T2DM, on the immunomodulatory capacity of MSCs.

2.3. Methods

The McGill University Health Center Ethics Review Board approved the study and participants provided written informed consent. Subcutaneous adipose tissue was obtained from patients undergoing programmed cardiovascular surgery. **TABLE 2.1** summarizes the demographics and cardiovascular risk factors of the studied subjects. A full description of methods is provided as supplementary data. Briefly, MSCs were derived from adipose tissue and proven to meet the ISCT definition criteria (258). Freshly harvested, early passage (P4) MSCs were used in all assays. PBMCs were obtained from a single unrelated donor, monocyte depleted (<5% monocytes) (259), CFSE stained and activated with CD3/CD28 beads. MSCs-dependent $CD4^+$ T-cell suppression was assessed in co-cultures (190). Proliferation curves of live $CD4^+$ T-cells were plotted and the suppressive effect of MSCs on T-cells was established by comparing maximal proliferation (T-cells alone) versus proliferation in co-cultures (MSCs+T-cells) (**Sup Fig 2.1**). Wilcoxon's test was used for group comparisons, and multiple linear regression analysis to investigate the effects of age, ATH and T2DM on the mean MSC:$CD4^+$ T-cell suppression capacity, after adjusting for the covariates of interest. Assumptions of the regression model were investigated with graphical analysis of residuals. All analyses were performed using SAS version 9.2 (SAS Institute). All hypotheses tests were 2-sided and significance was set at the 0.05 level.

2.4. Results

2.4.1. MSCs from older donors are less efficient at suppressing T-cell proliferation

The immunomodulatory capacity of adult-MSCs (A-MSCs, <65 years-old, n=27) and elderly- MSCs (E-MSCs, ≥ 65 years-old, n=23) was examined by analyzing their ability to inhibit the proliferation of anti-CD3/CD28-activated $CD4^+$T-cells. The suppressive effect of A-MSCs and E-MSCs on $CD4^+$T-cell proliferation was dose dependent. At a MSC:T-cell 1:8 ratio (**FIGURE 2.1A**), A-MSCs (median 33.9%, IQR 6.8 - 46.0, n=27) inhibited activated $CD4^+$T-cells more effectively than E-MSCs (median 47.5%, IQR 35.6 - 58.0, n=23) ($p<0.003$). Similar results were obtained at MSC:T-cell ratios 1:14 and 1:20. A-MSCs at 1:14 ratio (median 50.1% IQR 37.6 - 62.2, n =17) had similar inhibitory capacity as E-MSCs at 1:8 ratio, highlighting the magnitude of the E-MSCs defect (**FIGURE 2.1B**). MSCs donor's age positively correlated with T-cell proliferation in both ATH and non-ATH groups (Pearson's r=0.4 and 0.7, respectively) (**FIGURE 2.1C**) indicating an age-associated decline in the MSCs immunomodulatory capacity. Similar defects of the E-MSCs suppressive ability were observed on CD8 T-cells (**Sup Fig 2.**). In a multiple linear regression model (**TABLE 2.2**), adjusting for covariates of interest, age had a significant effect in the reduction of MSC:$CD4^+$T-cell suppression ($p=0.02$), with increasing mean $CD4^+$ T-cell proliferation by 0.5% (95% CI 0.1, 1.0) for any 1-year increase in age.

2.4.2. ATH and T2DM reduce the immunomodulatory capacity of MSCs

The effect of biological aging on human MSCs–mediated T-cell suppression was tested by evaluating MSCs from donors with ATH and T2DM, diseases associated with chronic

inflammation and premature aging. MSCs from patients undergoing valve replacement surgery who had a normal pre-surgical coronary angiography (non-ATH subjects) (median 15.6%, IQR 9.9 - 42.5, n=9) had a higher ability to suppress activated $CD4^+$T-cells compared to MSCs from ATH age-matched controls (median 44.2, IQR 36.7 - 55.7, n=18) (**FIGURE 2.2A**). Moreover, in the age-matched ATH patients the diagnosis of T2DM further reduced the MSCs suppressive capacity (median 41.0, IQR 32.3 - 50.1 and 56.4, 40.8 - 74.2, for non-T2DM/T2DM, n=12, respectively) (**FIGURE 2.2B**). Unadjusted, age-matched comparisons of the MSCs function in a sample subset (n=7), suggested that the diagnosis of ATH and T2DM were associated with a reduction in the MSCs suppressive capacity (**FIGURE 2.2C**). In the multiple regression model (**TABLE 2.2**), the presence/absence of ATH and T2DM in MSCs donors had significant effects on their capacity to suppress proliferating $CD4^+$T-cells. Subjects with ATH had a higher mean percentage of proliferating $CD4^+$T-cells (decreased MSC:T-cell suppressive capacity) than those without ATH (mean difference 21.6 %, 95% CI: 36.1-37.1). Similarly, subjects with T2DM had a higher mean percentage of proliferating $CD4^+$T-cells (mean difference 14.4 %, 95%CI: 2.6-26.1). There was no statistically significant interaction neither between ATH and T2DM nor between each of them and age, which suggests that the effect of ATH is independent of T2DM and that these effects are also independent of age.

2.5. Discussion

The PRECISE trial proved the feasibility, safety and potential therapeutic benefit of the transendocardial administration of autologous adipose derived MSCs in no-option patients with ischemic cardiomyopathy (254). This study provided a proof of concept to test MSCs in larger (ongoing) clinical trials (253). However, variations in the improvement

of cardiac function following MSCs administration in preclinical trials emphasize the need to define determinants of the MSCs therapeutic efficiency that will inform the proper MSCs donor selection (254, 260). Previous data suggests that the aging process may unfavorably affect the functional activity of murine MSCs limiting their therapeutic potential (261). Delineating the effects of aging and age-associated conditions on MSCs function is critical since the vast majority of the population that would potentially benefit from the use of MSCs in the context of ATH are elderly individuals a significantly proportion of whom also have T2DM (262). Furthermore, defining the factors that impact MSCs function would allow the development of prediction models for the selection of the most therapeutically efficient cells for clinical application.

Recent evidence confirmed that early-passage (i.e., passage 2-4) and freshly harvested MSCs have better *in vitro* T-cell suppression capacity and are associated with more clinical benefits than late-passage and freeze/thawed MSCs (263). Our findings suggest that even when tested under “optimal” conditions [i.e. fresh-early passage MSCs co-cultured with monocyte depleted PBMCs from a single donor in a reproducible immunopotency assay (190)], MSCs from elderly subjects with ATH have impaired T-cell suppression strength compared to their adult counterparts. Consistent with that reported in previous studies (264, 265) this impaired function was not explained by differences in the MSCs proliferative capacity or phenotype. Similar to E-MSCs, MSCs from donors with ATH and T2DM have reduced MSCs–mediated T-cell suppression, and the coexistence of these chronic inflammatory conditions further impair MSCs function.

The overall efficacy of stem cell transplantation relies on the activity of donor cells and tissue environment. Our co-culture model is limited to MSC:PBMCs and does not fully

simulate all the components of the *in vivo* ischemic and inflammatory environment. However this system predicts the MSCs immunomodulatory potency, which in turn is the most relevant mechanism accounting for the MSCs' therapeutic effect (190). The finding that age, T2DM and ATH are associated with reduced MSCs-immunomodulatory function is in line with a recent report that although limited by a small sample size, suggested a reduced ability of MSCs from people over 60 years to ameliorate myocardial function compared to that from patients younger than 40 years (266). A reduction of MSCs-secreted angiogenic factors (264), an increased vulnerability to hypoxic injury (267) and higher levels of miR-335 are mechanims proposed to account for the reduced reparative activities of E-MSCs.

2.6. Conclusions

In summary, our results indicate that age and age-associated conditions (T2DM and ATH) decrease the immunomodulatory capacity of MSCs; highlighting the relevance of donor selection and the need for proper immunological characterization of MSCs. Understanding the interplay between aging, MSC function and their clinical implications remains the only rational path for the successful therapeutic use of MSCs'.

2.7. Competing interests

None

2.8. Authors' contributions

OKM carried out the experiments, analyzed and interpreted the data, and wrote the first manuscript draft. DST collected all human MSCs samples. US assisted in data interpretation. JC performed the statistical analysis, contributed to data interpretation and

provided all statistical descriptions for the manuscript. IC designed and coordinated the study, assisted in data interpretation, reviewed the manuscript critically, and has given final approval of the version to be published. All authors read and approved the final manuscript.

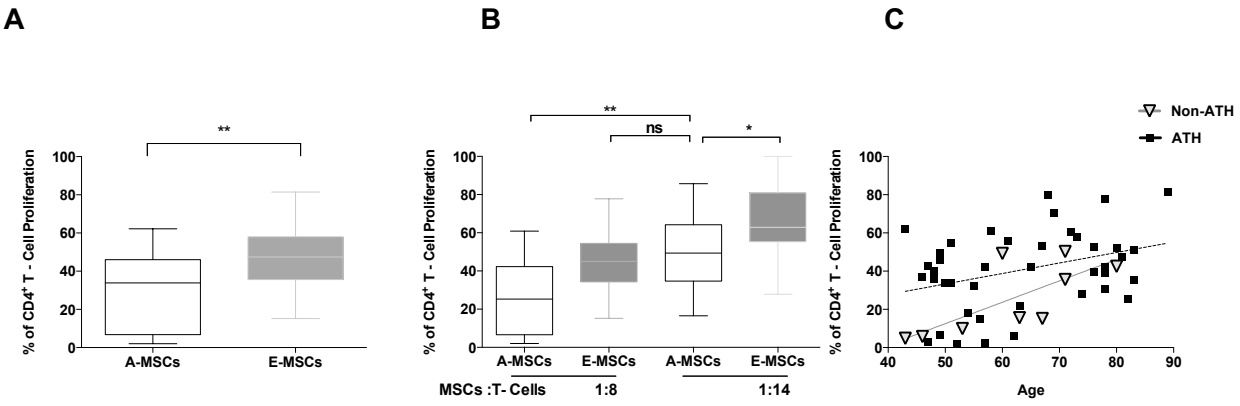


Figure 2.1. Age-associated decline in MSCs–mediated CD4+ T-cell suppression capacity.

(A) MSCs from elderly donors (E-MSCs, ≥ 65 years, n=23) are less efficient than those of adults (A-MSCs, < 65 years, n=27) to suppress $CD4^+$ T-cell proliferation at 1:8 MSC:$CD4^+$ T-cell ratio (**p=0.003). (B) The suppressive effect of MSCs on $CD4^+$ T-cells depends on the MSC:$CD4^+$ T-cell ratio (**p=0.004). Twice the number of E-MSCs are required to exert an effect similar to that of A-MSCs on $CD4^+$ T-cell suppression (p>0.9). (C) The effect of MSC-donors' age on the decline of $CD4^+$ T-cell suppression is observed in patients with atherosclerosis (ATH, n=18) (p=0.02, R^2=0,4) and without ATH (Non-ATH, n=9) (p=0.02, R^2=0,7).

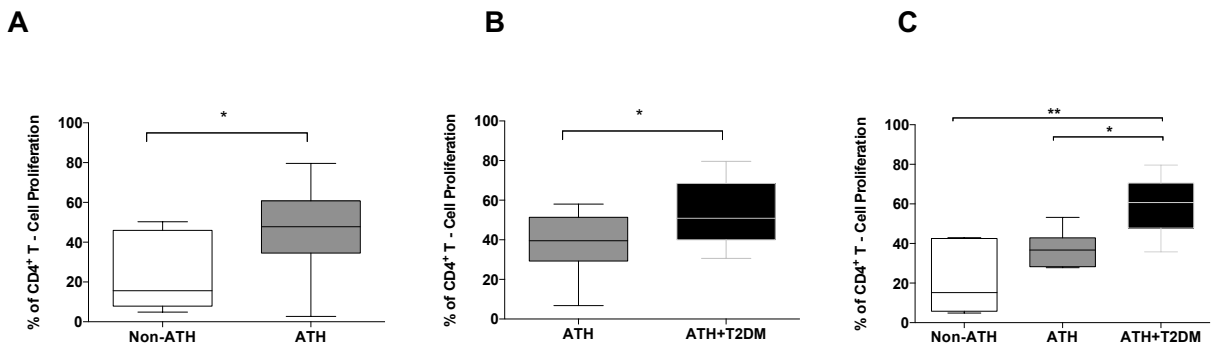
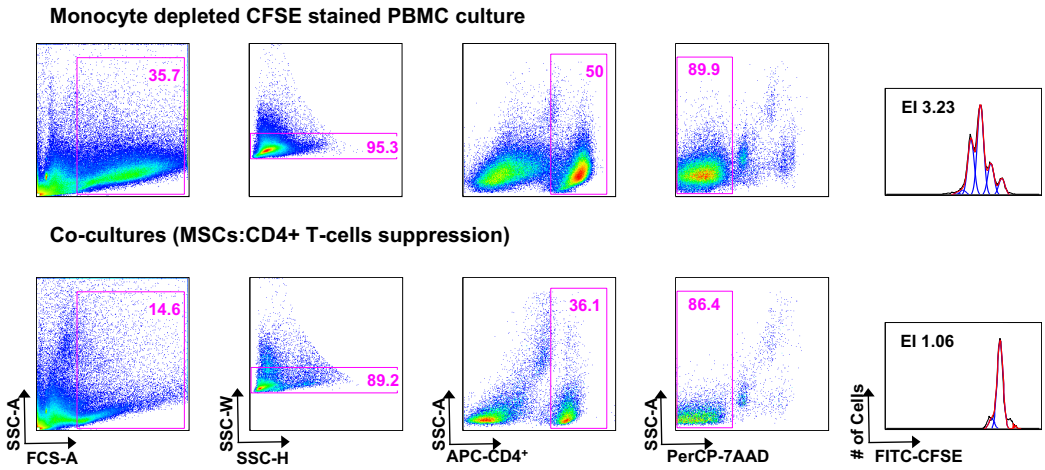
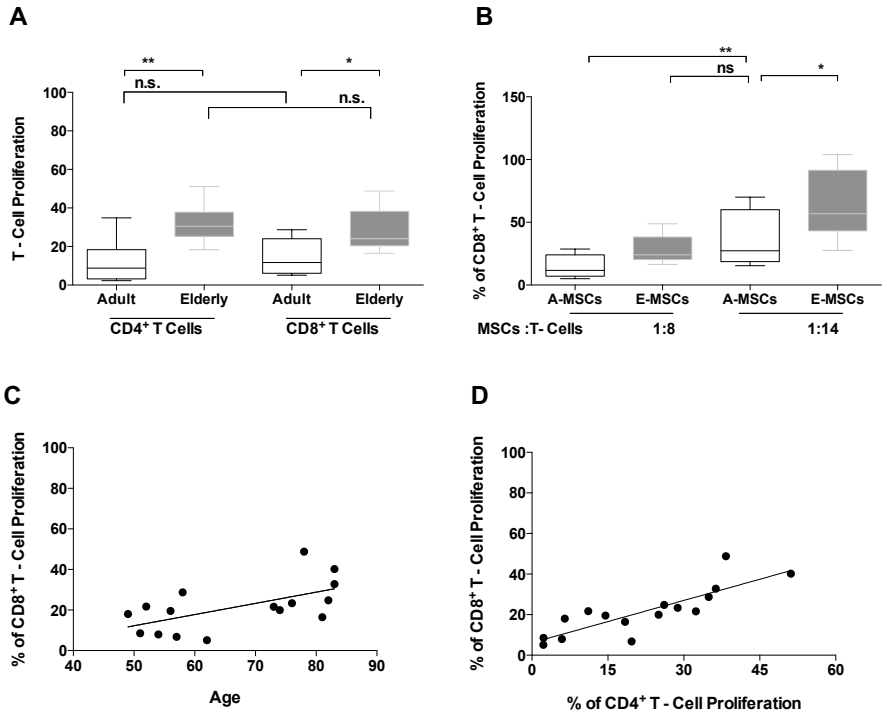


Figure 2.2. Reduced MSCs–mediated T-cell suppression capacity in patients with atherosclerosis and diabetes.

MSCs from patients with (A) atherosclerosis (ATH, n=18) (*p=0.02) have decreased capacity to suppress CD4+ T-cell proliferation at 1:8 MSC:CD4+ T-cell ratio compared to age-matched controls without atherosclerosis (Non-ATH, n=9). MSCs from patients with (B) ATH and type 2 diabetes (T2DM, n=12) have impaired suppressive capacity than age-matched ATH controls (*p=0.04). (C) Impaired MSCs' function in age-matched patients with chronic inflammatory diseases (Non- ATH<ATH<ATH+T2DM) (*p=0.02, **p=0.002).



Supplementary Figure 2.1. Gating strategy for the MSCs:T-cells suppression assay. The capacity of MSCs to suppress proliferative responses on activated CD4+T cells was assessed in a 4-day allogeneic co-culture system. A- PBMCs expanded for 4 days were used as controls ('maximal proliferation'). B- MSCs from different donors were co-cultured for 4 days with primary monocyte depleted PBMCs obtained from a single unrelated donor. At day 4 PBMCs were stained with 7-Aminoactinomycin D (7-AAD), and CD4-APC and flow cytometry was performed. The expansion index of 7AAD-CD4+ T-cells was calculated by FlowJo. The percentage of CD4+ T-cell proliferation was calculated according to the following formula: % of Proliferation = X-Control/Maximal Proliferation-Control x 100 where X = Expansion index of MSC-CD4+ T-cells co-culture for each sample, Control= Expansion index of non-stimulated CD4+ T-cells, and Maximal Proliferation = Expansion index of CD4+ T-cells stimulated with anti-CD3/CD28 beads in the absence of MSCs.



Supplementary Figure 2.2. Age associated reduction in MSCs–mediated CD8+ T-cell suppression

(A) The capacity of MSCs from adult donors (A-MSCs, < 65 years, n=8) to suppress CD8+ T-cells is higher than that of elderly donors (E-MSCs, ≥ 65 years, n=8) (*p=0.03; MSC:$CD8^+$ T-cell ratio of 1:8). (B) The E-MSCs:$CD8^+$ T-cells suppressive potency at 1:8 ratio is similar to that of A-MSCs at 1:14 ratio. (C) Age dependent decline in MSCs:$CD8^+$ T-cell suppression in ATH patients (p=0.01, R^2=0,35). (D) Correlation of the suppressive effect of MSCs on $CD4^+$ and $CD8^+$ T-cells (p< 0.0001, R^2=0,7, MSC:T-cell ratio of 1:8).

Table 2.1. Demographic Characteristics of the Study Subjects

	Surgical Procedure	
	Coronary Artery By-Pass Graft (ATH)* n =41	Valve Replacement (Non-ATH) n =9
Sex (Female:Male, n)	13:28	5:4
Age, mean (SD)	63.4 (13.3)	59.7 (14.8)
Ethnicity (Caucasian: Asian, n)	37:4	9:0
Body Mass Index, mean (SD)	29.1 (9.2)	27.3 (12.1)
Cardiovascular Risk Factors n (%)		
Tobacco	20 (49)	4 (44)
Hypertension	33 (80)	6 (66)
Hypercholesterolemia	33 (80)	4 (44)
Type II Diabetes	12 (29)	4 (44)
Medications n (%)		
Statins	33 (80)	5 (55)
ACE inhibitors†/ ARB‡	20 (49)	2 (22)
Beta blockers	26 (63)	2 (2)

*ATH-atherosclerosis; †ACE -angiotensin-converting enzyme;‡ARB angiotensin II receptor blockers

Table 2.2 Results of linear regression analysis of factors associated with MSC immunomodulatory function

Variable	Estimate	Standard Error	T	P value
Intercept	-5.62	16.28	-0.35	0.73
Presence of ATH	**21.61**	**7.68**	**2.82**	**0.01**
Presence of T2DM*	**14.37**	**5.80**	**2.48**	**0.02**
Age	**0.54**	**0.23**	**2.42**	**0.02**
Sex	2.58	5.90	0.44	0.66
Tobacco	-4.55	5.72	-0.80	0.43
LVD†	-1.67	6.76	-0.25	0.81
Statins	-9.00	6.96	-1.44	0.16
ACE inhibitors§ / ARB‖	-4.05	5.72	-0.71	0.48
Beta Blockers	-5.14	6.42	-0.80	0.43

*T2DM Type II diabetes mellitus; †LVD Left Ventricular Dysfunction, § Angiotensin-converting-enzyme inhibitor, || Angiotensin II receptor blockers

2.9. DETAILED MATERIAL AND METHODS

2.9.1. Study subjects

The McGill University Health Center Ethics Review Board approved the study and participants provided written informed consent. Subcutaneous adipose tissue was obtained from consecutive patients undergoing elective cardiovascular surgery (n=50). Samples from patients with atherosclerosis (ATH) requiring coronary artery bypass surgery (CABG) (n=41) were compared to those from patients without ATH (normal coronary angiogram) undergoing aortic valve replacement (n=9). Exclusion criteria for both groups were a history of systemic autoimmune diseases, cancer and acute or chronic infections. The demographic characteristics and cardiovascular risk factors of the patients included in the study are summarized in Table 1.

2.9.2. Isolation of Multipotent Mesenchymal Stromal Cells

Subcutaneous adipose tissue (1-4g) was washed extensively with phosphate-buffered saline (PBS), minced with surgical scissors and digested with 0.05% collagenase (Sigma-Aldrich Corporation, Missouri 63103 USA) dissolved in Hank's balanced salt solution (Invitrogen). Following the neutralization of the enzyme, the sample was centrifuged at 834 g for 5 min and the supernatant was discarded. The pellet was resuspended in complete medium (CM) (1.0g/L glucose, with L-glutamine & sodium pyruvate Dulbecco's modified Eagle's medium (DMEM) (Wisent Biotechnologies), supplemented with 10% MSC Qualified Fetal Bovine serum (FBS) 1% penicillin / streptomycin (10,000 unit / mL Penicillin, 10,000mg/mL Streptomycin - Life technologies). The cells were cultured under

standard conditions (5% carbon dioxide; 37°C) in 75-cm^2 tissue culture flasks (1gr of tissue/flask). Two days after isolation, non-adherent cells were washed off and CM was added. Subsequently, at 80% confluency mesenchymal stem cells (MSCs) were trypsinized and subcultured at a density of 5000 cells / cm^2 (268) .

2.9.3. Flow cytometric detection of cell surface receptors

Immunophenotypic characterization of MSCs was performed according to criteria established by the International Society for Cellular Therapy (258) by multiparametric flow cytometry (BD LSRII; Becton Dickinson Co, Mountain View, CA). Passage 2 MSCs were treated with Fc receptor blocking reagent and stained with the following flurochrome-conjugated monoclonal antibodies (BD Biosciences): fluorescein isothiocyanate (FITC)-conjugated anti-CD90 and anti-CD45; phycoerythrin (PE)-conjugated anti-CD73; allophycocyanin (APC)-conjugated anti-CD34, anti-CD19 and anti-HLA-DR; peridinin chlorophyll (PerCP)-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 9.7.2.

2.9.4. Multilineage differentiation assays

Passage 3 MSCs were plated in 24-well plates at a density of 5000 cells/cm^2. At ~90% confluence, cells were incubated in one of the three differentiation mediums for 3 weeks as per manufactures protocol (StemPro® Adipogenesis, osteogenesis, chondrogenesis Differentiation Kit). Cells were then fixed with 4% formaldehyde and stained with alizarin red S (Sigma-Aldrich), oil red O (Sigma-Aldrich) or safranin O (Sigma-Aldrich) to assess

osteogenic, adipogenic and chondrogenic differentiation, respectively.

2.9.5. Peripheral blood mononuclear cells (PBMC) isolation, carboxyfluorescein succinimidyl ester (CFSE) fluorescent dye labeling, and PBMC stimulation

PBMCs were freshly isolated for each experiment from the same donor (32 years old, healthy, female, no smoking). 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 (259). Monocyte-depleted PBMCs were stained with carboxyfluoroscein succinimidyl ester (CFSE) (Sigma) and stimulated with anti- CD3/CD28 beads (1 bead/cell) (Dynabeads® Human T-Activator CD3/CD28, life technologies) (269, 190).

2.9.6. Co-cultures

The capacity of MSCs to suppress proliferative responses on activated $CD4^+$T cells was assessed in a 4-day allogeneic co-culture system [MSCs from different donors exposed to the same unrelated primary monocyte depleted PBMCs (270)]. MSCs were plated at 25×10^3, 14×10^3, 10×10^3, cells per well in flat-bottomed 96-well plates (Corning) and cultured overnight. Following activation 2×10^5 monocyte depleted CFSE stained PBMCs were plated on MSCs (MSCs:PBMCs 1:8, 1:14, 1:20). PBMCs expanded for 4 days were used as controls (‘maximal proliferation’). At day 4 cells were stained with 7-Aminoactinomycin D (7-AAD), and CD4-APC. The Expansion Index of 7AAD-CD4+ cells was determined (FlowJo) and the percentage of $CD4^+$ T-cell proliferation was calculated

according to the following formula: % of Proliferation = X-Control/Maximal Proliferation-Control x 100 where X = Expansion index of MSC- $CD4^+$ T-cells co-culture for each sample, Control= Expansion index of $CD4^+$ T-cells without anti CD3/CD28 stimulation, and Maximal Proliferation = Expansion index of $CD4^+$ T cells stimulated by anti-CD3/CD28 beads in the absence of MSC.

2.9.7. Statistical Analysis

Descriptive statistics summarize all study variables. For categorical variables we report counts and percentages whereas for continuous variables we report means and standard deviations when the distribution of values is normal; otherwise we report medians, and inter-quartile range.

Multiple linear regression analysis was used to investigate the effect of age and premature aging-associated conditions (atherosclerosis-ATS and diabetes mellitus-T2DM) on the mean MSC:T-cell suppression capacity. The regression model included covariates reported to potentially influence the outcome: sex, tobacco and treatment (use of statins, angiotensin-converting-enzyme inhibitors ± angiotensin II receptor blockers and/or β-blockers). Age was modeled as a continuous variable whereas ATH, T2DM and other covariates of interest were modeled as binary variables (presence/absence). Interactions between age, ATH, T2DM and the covariates of interest were assessed. Assumptions of the regression model (randomness of errors, homogeneity of variance, normality, presence of outliers) were investigated with a graphical analysis of residuals. All hypotheses tests were 2-sided and performed at a significance level of 0.05. All analyses were performed using SAS version 9.3 (SAS Institute, Inc. Cary NC, USA).

Preface to Chapter 3

Knowing that ATH is a major contributor to altered MSC function, and that aging has an additive effect on their functional decline; we postulated that changes in the secretome composition could underlie the impaired function of MSCs from elderly ATH individuals. Understanding this is critical for appropriate donor selection for MSCs based therapies. In addition, the modulation of the MSCs secretome could be a strategy to enhance the MSCs therapeutic effects. Lastly, integrating this knowledge into clinical trial design could enhance the efficacy of MSCs therapy.

CHAPTER III: A PROINFLAMMATORY SECRETOME MEDIATES THE IMPAIRED IMMUNOPOTENCY OF HUMAN MESENCHYMAL STROMAL CELLS IN ELDERLY PATIENTS WITH ATHEROSCLEROSIS

A PROINFLAMMATORY SECRETOME MEDIATES THE IMPAIRED IMMUNOPOTENCY OF HUMAN MESENCHYMAL STROMAL CELLS IN ELDERLY PATIENTS WITH ATHEROSCLEROSIS

Ozge Kizilay Mancini, Dominique Shum-Tim, Stephanie Nadeau, Francis Rodier, Inés Colmegna

3.1. 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 co-culture 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 pro-inflammatory 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 pro-inflammatory

cytokines/chemokines produced by MSCs could enhance the efficacy of autologous cell-based therapies in the elderly.

3.2. 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 (39, 271).

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 (125). 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 (252, 272). Further, the lipid-lowering agent statins exert immunomodulatory properties through the inhibition of T cell activation contributing to plaque stabilization (273, 274). Due to the evidence supporting the role of inflammation in the etiology and pathophysiology of ATH, ongoing large-scale placebo-controlled clinical trials evaluate the clinical efficacy of anti-inflammatory strategies for the treatment of ATH. Among them the Canakinumab Antiinflammatory Thrombosis Outcomes Study-CANTOS assesses the relevance of interleukin-1β inhibition in ATH prevention; whereas the Cardiovascular Inflammation Reduction Trial (CIRT) evaluates the effect of low-dose methotrexate in patients with a high prevalence of subclinical vascular inflammation) (142, 143). 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 (275-278). 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 (279, 280) and can improve various autoimmune diseases (281-283). 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 (187). Although MSC-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 (8, 284). This is especially problematic considering the prevalence of ATH among elderly individuals and the potential advantages of using autologous MSCs (285). 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.

3.3. Material and Methods

3.3.1. 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.

3.3.2. Isolation of MSCs

Subcutaneous and pericardial adipose tissue (1-4g) were washed extensively with phosphate-buffered saline (PBS), minced with surgical scissors and digested with 0.05% collagenase (Sigma-Aldrich Corporation, Missouri 63103 USA) dissolved in Hank's balanced salt solution (Invitrogen). Following the neutralization of collagenase, the sample was centrifuged at 2000 rpm for 5 min and the supernatant was discarded. The pellet was resuspended in complete medium (CM) (1.0g/L glucose, with L-glutamine & sodium pyruvate Dulbecco's modified Eagle's medium (DMEM) (Wisent Biotechnologies), supplemented with 10% MSCs Qualified Fetal Bovine serum (FBS) 1% penicillin / streptomycin (10,000 unit / mL Penicillin, 10,000mg/mL Streptomycin - Life technologies). Digested tissue was cultured under standard conditions (5% carbon dioxide; 37°C) in 75-cm^2 tissue culture flasks (1gr of tissue/flask). Two days after isolation,

non-adherent cells were washed off and CM was added. Subsequently, at 80% confluency, MSCs were trypsinized and subcultured at a density of 5000 cells / cm2 (268).

3.3.3. MSCs characterization

Immunophenotypic characterization of MSCs was performed according to criteria established by the International Society for Cellular Therapy (258) by multiparametric flow cytometry (BD LSRII; Becton Dickinson Co, Mountain View, CA). Passage 2 MSCs were treated with Fc receptor blocking reagent and stained with the following fluorochrome-conjugated monoclonal antibodies (BD Biosciences): fluorescein isothiocyanate (FITC)-conjugated anti-CD90 and anti-CD45; phycoerythrin (PE)-conjugated anti-CD73; allophycocyanin (APC)-conjugated anti-CD34, anti-CD19 and anti-HLA-DR; peridinin chlorophyll (PerCP)-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. 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% (Supplementary Fig. 3.1A).

3.3.4. Multilineage differentiation assays

At passage 3, MSCs were plated in 24-well plates at a density of 5000 cells/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). Cells were then fixed with 4% formaldehyde and stained with alizarin red S (Sigma-Aldrich), oil red O (Sigma-Aldrich) or safranin O (Sigma-

Aldrich) to assess osteogenic, adipogenic and chondrogenic differentiation, respectively (Supplementary Fig. 3.1B).

3.3.5. Peripheral blood mononuclear cell (PBMC) isolation, carboxyfluorescein succinimidyl ester (CFSE) fluorescent dye labeling, and activation

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 10uM carboxyfluorescein succinimidyl ester (CFSE) (Sigma), stimulated with anti-CD3/CD28 beads (1 bead/cell) (Dynabeads® Human T-Activator CD3/CD28, Life Technologies) (269) and cultured for 4 days with MSCs.

3.3.6. Co-cultures

The capacity of MSCs to suppress proliferative responses of activated $CD4^+$ and $CD8^+$ T-cells was assessed in a 4-day allogeneic co-culture system (i.e., MSCs from different ATH donors were cultured with monocyte depleted PBMCs obtained from a single unrelated healthy donor) (270). MSCs were plated at 75×10^3 cells / well in flat-bottom 24-well plates (Corning) 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 co-cultures) or -independent conditions (transwell cultures) (MSCs:PBMCs ratio 1:8). In the later, MSC and T-cells were separated by a 0.4

micrometer pore size membrane (Millipore). At day 4, cells were stained with CD8-PE, CD4-APC and with a cell viability marker: 7-Aminoactinomycin D (7-AAD). 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 *Ni* is the number of events in generation *i* (286).

3.3.7. 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 cat#001-000-162, Sigma cat#D966)] for 60 min 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.

3.3.8. Flow cytometry analysis of reactive oxygen species (ROS)

Intracellular 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 min. Fluorescence intensity was measured by FACS and data was analyzed with FlowJo software v9.7.2.

3.3.9. Cytokine array and enzyme-linked immunosorbent assays

MSCs were plated in 6-well plates at a density of 1 x 10^5 cells/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 and the multispot electrochemiluminescence immunoassay V-Plex Pro inflammatory Panel (MesoScale Discovery: 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 A-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).

3.3.10. 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), anti-IL-8/ CXCL8 (10 µg/mL), anti- MCP-1/CCL2 (Abcam) (45 µg/mL) (287) monoclonal antibodies or a MIF antagonist (S,R)-3-(4-Hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid (ISO-1) (85 nM/mL) (Santa Cruz Biotechnology) (288) at the time the co-cultures were started.

3.3.11. Statistical analysis

All analyses were performed using the GraphPad Prism software (Graph-Pad, San Diego, 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 2-sided and a *P*-value of <0.05 was considered statistically significant.

3.4. Results

3.4.1. 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 (289). Studies directly comparing MSCs from different tissues have consistently shown that adipose derived MSCs have stronger immunosuppressive capabilities than alternative sources. However it is not known whether pericardial and subcutaneous

adipose tissue-derived MSCs (adMSCs) possess similar functional properties (165). 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 mesenchymal stromal cells (i.e. plastic adherence, tri-lineage differentiation and expression of positive and negative surface markers); and express similar levels of reactive oxygen species (ROS, DCFDA) and double-strand DNA breaks (gamma H2AX), two hallmarks of cellular aging (Supplementary Fig 3.2). 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 co-cultures (Figure 3.1). Although we cannot exclude the possibility of other functional differences between these two MSCs sources, our data suggests that the easily accessible subcutaneous adipose derived MSCs 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 adipose tissue-derived MSCs in subjects with acute myocardial infarction (APPOLO Trial; (260)) and chronic ischemic heart disease (PRECISE Trial; (254)) emphasize the relevance of quantifying and potentially optimizing the function of those cells for clinical use.

3.4.2. 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 (290, 291). 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 (Gamma H2AX) in MSCs (Figure 3.2A), and also reduced their efficiency to suppress both $CD4^+$ and $CD8^+$T-cell proliferation (Figure 3.2B). It has been suggested that MSCs can induce apoptosis of T-cells (180), which could account for the impaired immunomodulatory function of irradiated MSCs. However, MSCs irradiation did not impact $CD4^+$ and $CD8^+$ T cell viability in co-culture experiments (Figure 3.2C).

3.4.3. 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 (292, 293). 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-year-old) to those of adult ATH patients (A- MSCs; <65 years-old). E- MSCs not only had a larger cellular size (Supp Fig 3.3A) but also displayed ~2-fold increase in both gamma- H2AX levels (Supp

Fig 3.3B), a marker of DNA double strand breaks (294), and intracellular ROS levels (Supp Fig 3.3C). We next conducted cell-cell contact dependent and independent (transwells) co-cultures 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 (Figure 3.3A and 3.3B). 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 adult and elderly MSCs (Supp Figure 3.4) nor differences in MSCs-induced T-cell apoptosis (Figure 3.3C, 3.3D). As a result, we conclude that i) MSCs- suppression of T-cell proliferation is primarily mediated by secreted soluble factors, and ii) A-MSCs are superior to E-MSCs in inhibiting $CD4^+$ and $CD8^+$ T-cell proliferation.

3.4.4. 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 (255). 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 macrophage migration inhibitory factor (MIF) was elevated in E-MSCs relative to A-MSCs (Supp Figure 3.5). 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 (Figure 3.4A). Key

factors of the 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 (Figure 3.4B, C, D, E). A positive correlation between IL-6 and MCP-1/CCL2 levels assessed by ELISA (Supp Figure 3.6) was observed, which can relate to the fact that IL-6 is a potent inducer of MCP-1/CCL2 (295). 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 co-cultures as a proof-of-concept for the role of these factors in the reduced immunomodulatory function of E-MSCs (Figure 3.5). Indeed, neutralization of IL-6 (Figure 3.5A, D), IL-8/ CXCL8 (Figure 3.5B, E) and MCP-1/CCL2 (Figure 3.5C, F) 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 (Supp Figure 3.7).

3.5. Discussion

An enhanced understanding of the biology of MSCs has led to clinical trials testing their therapeutic effects in various conditions including cardiovascular diseases (254, 260). Overall, these trials have demonstrated that MSCs-based therapies are promising; however, notable intra- and inter-trial 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 pre-activated state of MSCs (188). 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 adipose tissue-derived MSCs (165). 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 (296). Our results show that pericardial and subcutaneous adipose tissue-derived MSCs 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 7-AAD staining, respectively). These data does 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 (190).

Collectively, our data suggests that the easily accessible subcutaneous adipose derived MSCs could be used as a surrogate to estimate the T-cell suppressive effects of epicardial MSCs. Furthermore, results from human trials using subcutaneous adipose tissue-derived MSCs in subjects with acute myocardial infarction (APPOLO Trial; (260)) and chronic ischemic heart disease [PRECISE Trial; (254)] 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 (188). 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.

Atherosclerosis is now considered a chronic inflammatory disease. Vascular inflammation in ATH is initiated in the adventitia and progresses towards to the intima (297). MSCs have been isolated from all layers of the vasculature (298); 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 towards the expression of senescence-associated factors (293). 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 (82). Moreover, MCP-1/CCL2 is linked to an increased risk of myocardial infarction and left ventricular heart failure (299). Evidence from *in vitro* models, animal studies and case-control series suggest a key role of IL-8/CXCL8 in the establishment

and preservation of the inflammatory microenvironment of the insulted vascular wall contributing to atherosclerosis onset and progression [reviewed in (300)]. Furthermore, increased IL-6 levels are also associated with atherosclerotic plaque development, plaque destabilization and increased risk of future cardiovascular events (301). 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.

3.6. Conclusions

Collectively, our study provides novel insights into the characterization of adipose tissue derived MSCs from subjects with atherosclerosis. 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.

3.7. Authors' contributions

OKM: collection, assembly, analysis and interpretation of data, manuscript writing; DST: provision of study material; SN: analysis and interpretation of data FR: data interpretation, manuscript writing; IC: conception and design, analysis and interpretation of data, manuscript writing, final approval of manuscript and financial support.

3.8. Acknowledgments

This work was supported by an operating grant from the Canadian Institutes of Health Research (CIHR, MOP-125857) and the Programme de bourses de Chercheur-boursier clinicien (IC) and Chercheur boursier (FR) from the Fonds De Recherche Sante Quebec (FRSQ). SN was supported by a Canderel student fellowship from the Institut du cancer de Montréal.

3.9. Disclosure of Potential Conflicts of Interest:

None

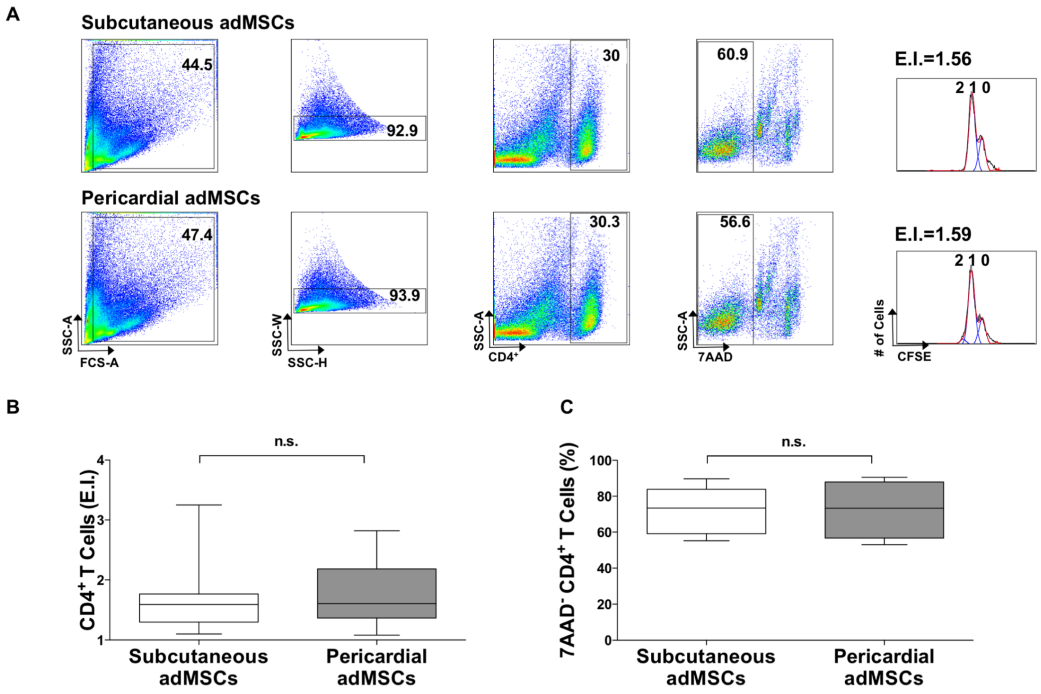


Figure 3.1. MSCs from pericardial and subcutaneous adipose tissue equally suppress T-cell proliferation.

(A) Representative example of a flow cytometry proliferation analysis of monocyte depleted PBMCs in co-culture 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).

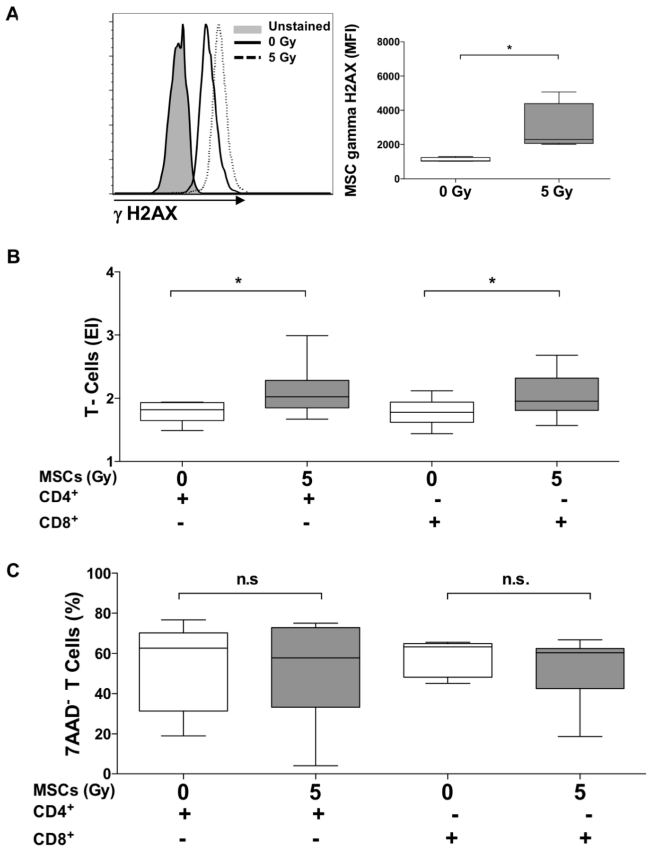


Figure 3.2. DNA damage impairs MSCs immunopotency.

(A) MSCs radiation (day 2 post-5 Gy) induces gamma-H2AX phosphorylation reported as mean fluorescence intensity (MFI) (*p=0.04, n=4). (B) Irradiated MSCs have impaired $CD4^+$ and $CD8^+$T-cell suppressive ability (*p=0.03, n=6). (C) Irradiated MSCs do not affect $CD4^+$ and $CD8^+$ T cell viability (7AAD viability staining-FACS) (n=6). (Gy: gray unit; EI: expansion index)

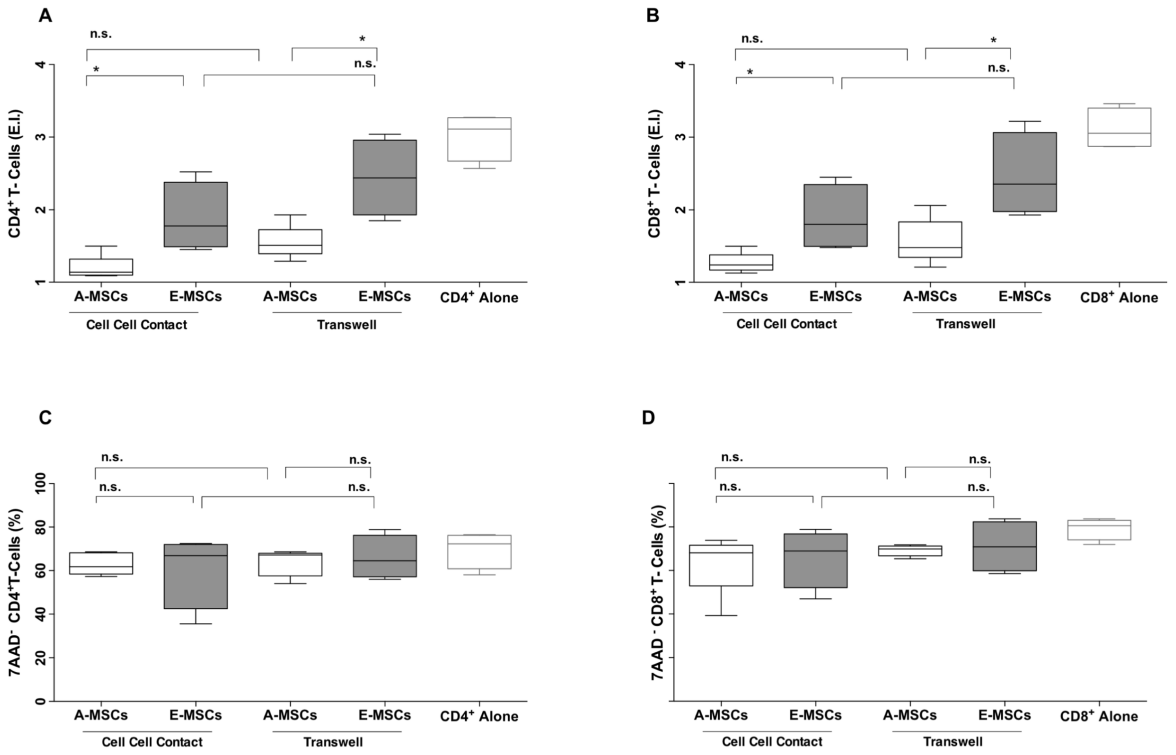


Figure 3.3. Soluble factors mediate the impaired immunopotency of elderly MSCs. MSCs immunopotency was assessed in co-cultures either in direct contact with T lymphocytes (Cell Cell Contact) or in a transwell system. Reduced suppressive effect of elderly MSCs (E-MSCs) compared to adult MSCs (A-MSCs) on (A) $CD4^+$ and (B) $CD8^+$ T-lymphocyte proliferation in either direct contact (*p=0.01, A-MSCs n=5, E-MSCs n=4) or transwell (*p=0.03, *p=0.05, 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.

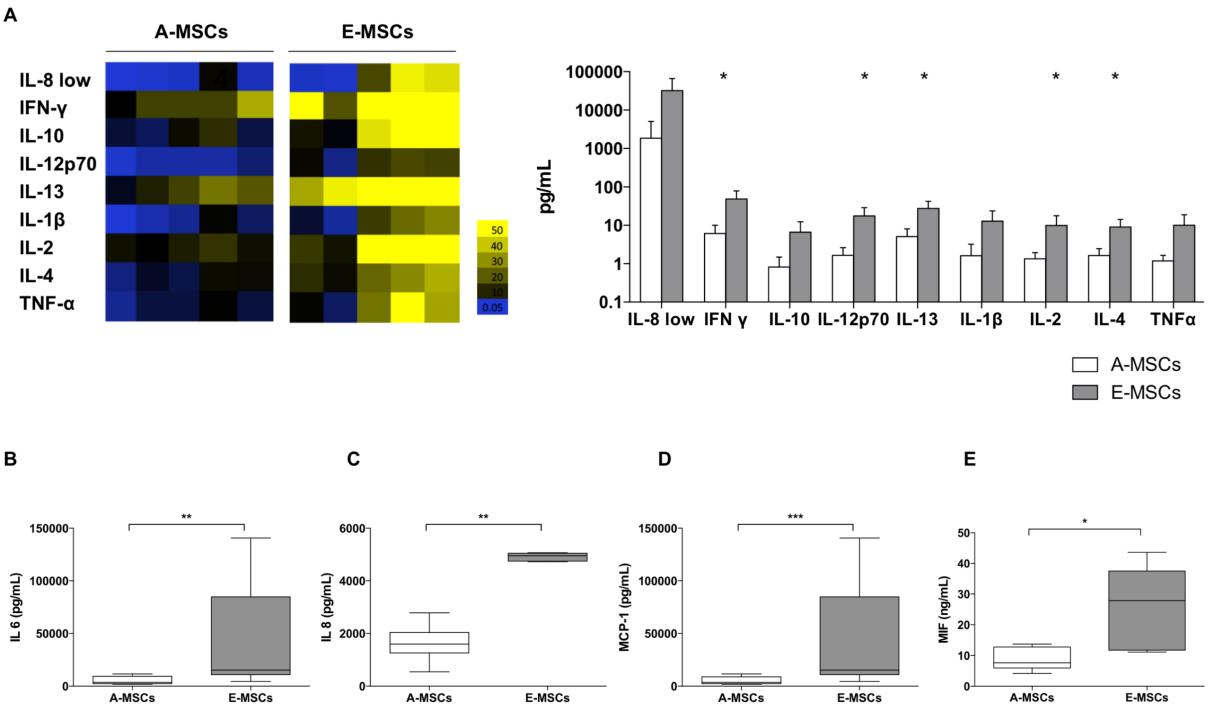


Figure 3.4. Elderly MSCs secrete higher levels of senescence associated cytokines. (A) Baseline production of cytokines and chemokines by MSCs from adult (A-MSCs) and elderly (E-MSCs) individuals assessed by V-Plex assay. Data is reported as a ratio of secretion compared to the average of the A-MSCs groups. The color scale represents fold change (n=5). (B, C, D, E) Senescent associated cytokines and chemokines were confirmed by ELISA. IL-6, IL-8/ CXCL8 (**p<0.01, n=11), MCP-1/CCL2 (***p<0.001, n=11), MIF (*p=0.01 n=6).

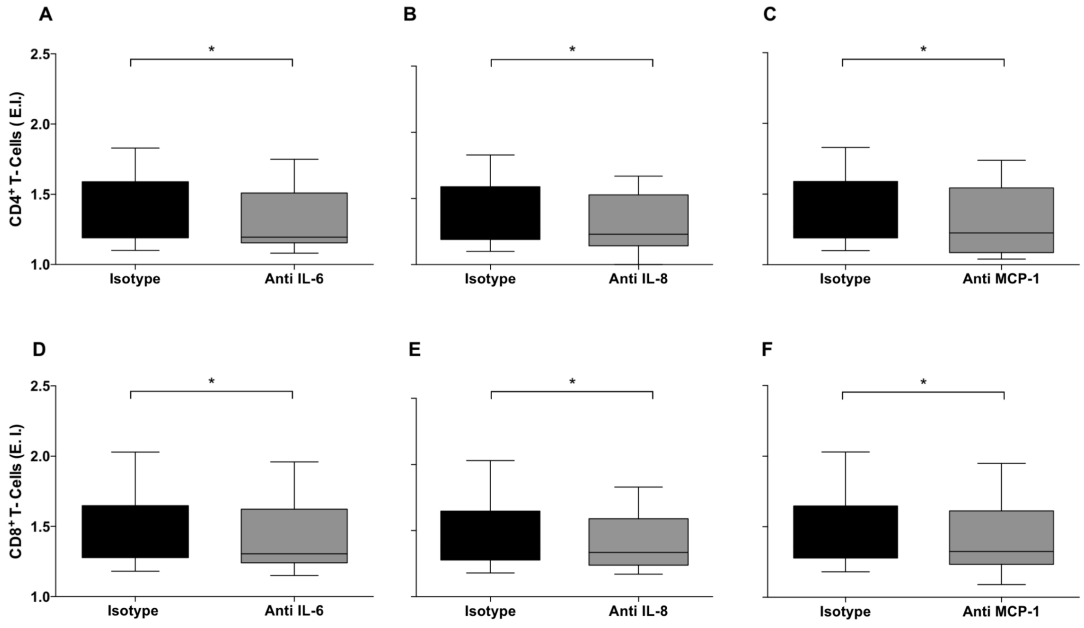
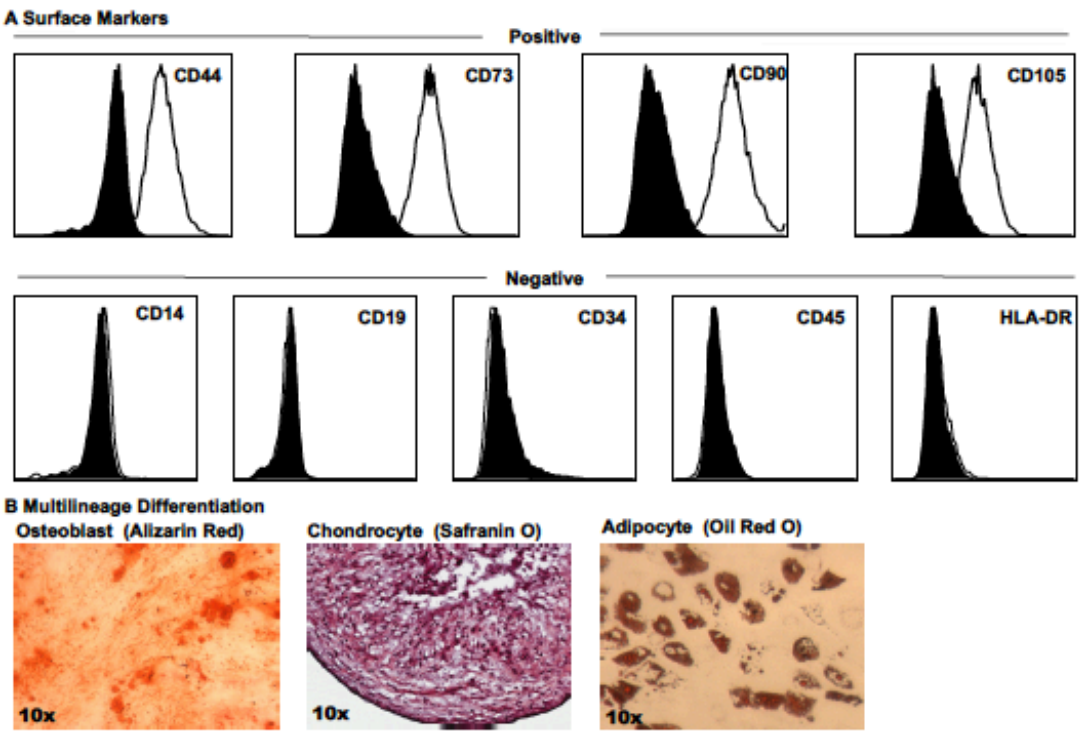


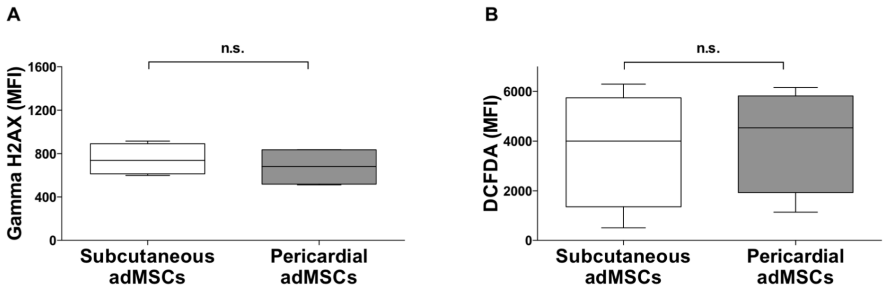
Figure 3.5. Antagonizing components of the senescence-associated secretory phenotype in co-cultures enhances MSCs immunopotency.

(A) IL-6, (B) IL-8/ CXCL8 and (C) MCP-1/CCL2 neutralization in MSCs:$CD4^+$ T-cell co-cultures improves MSCs immunopotency (*p=0.03, n=6). Similarly (D) IL-6, (E) IL-8/ CXCL8 and (F) MCP-1/CCL2 neutralization improves MSCs:$CD8^+$T-cell suppression (*p=0.03, n=6).



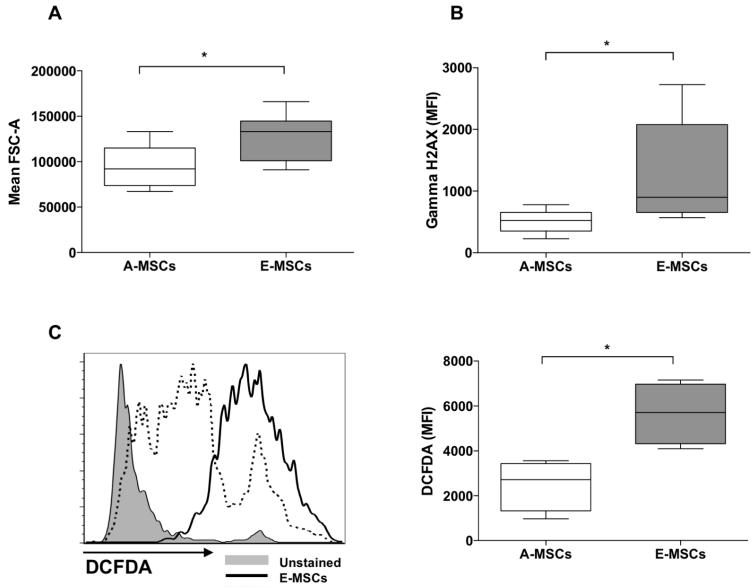
Supplementary Figure 3.1. Characterization of human adipose tissue derived MSCs according to the International Society of Cellular Therapy.

The expression of positive (CD44, CD73, CD90, CD105) and negative (CD14, CD19, CD34, CD45, HLA-DR) surface markers was analyzed by flow cytometry in each MSCs sample at passage 2. All the samples expressed positive markers in more than 95% of the cells and negative markers in less than 5% of them. Multipotency was tested by inducing trilineage differentiation into osteoblasts, chondrocytes and adipocytes.



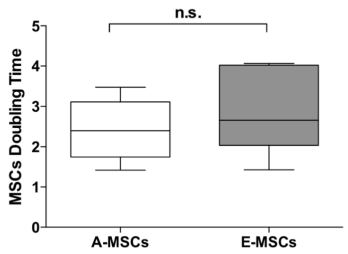
Supplementary Figure 3.2. Similar DNA damage and intracellular ROS levels in pericardial and subcutaneous MSCs

(n=4).



Supplementary Figure 3.3. Hallmarks of senescence in elderly MSCs (E-MSCs)

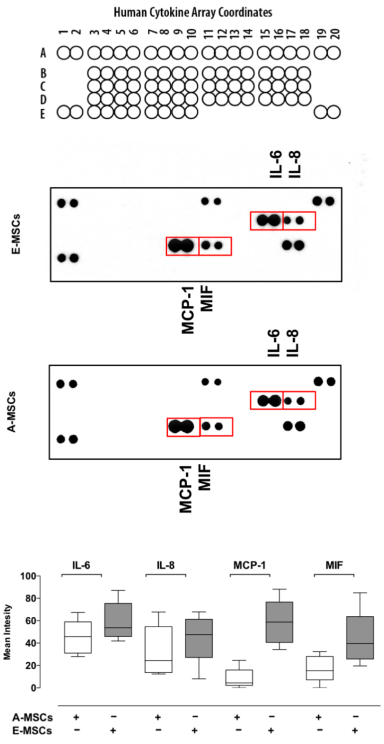
(A) Increased cell size (p=0.02, n=8), (B) DNA damage (p=0.01, n=6) and (C) intracellular ROS levels (p=0.02, n=4) in E-MSCs.



Supplementary Figure 3.4. Similar proliferation of A-MSCs and E-MSCs.

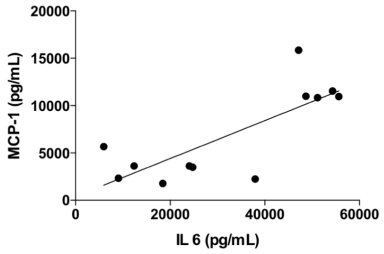
MSCs proliferation rates were measured with the MTT cell proliferation assay, no differences between A-MSCs and E-MSCs doubling time were observed (p=0.6, n=5).

Coordinate	Target/Control
A1, A2	Reference Spot
A3, A4	C5/C5a
A5, A6	CD40 Ligand
A7, A8	G-CSF
A9, A10	GM-CSF
A11, A12	GROα
A13, A14	I-309
A15, A16	sICAM-1
A17, A18	IFN-γ
A19, A20	Reference Spot
B3, B4	IL-1α
B5, B6	IL-1β
B7, B8	IL-1ra
B9, B10	IL-2
B11, B12	IL-4
B13, B14	IL-5
B15, B16	IL-6
B17, B18	IL-8
C3, C4	IL-10
C5, C6	IL-12 p70
C7, C8	IL-13
C9, C10	IL-16
C11, C12	IL-17
C13, C14	IL-17E
C15, C16	IL-23
C17, C18	IL-27
D3, D4	IL-32α
D5, D6	IP-10
D7, D8	I-TAC
D9, D10	MCP-1
D11, D12	MIF
D13, D14	MIP-1α
D15, D16	MIP-1β
D17, D18	Serpin E1
E1, E2	Reference Spot
E3, E4	RANTES
E5, E6	SDF-1
E7, E8	TNF-α
E9, E10	sTREM-1
E19, E20	Negative Control

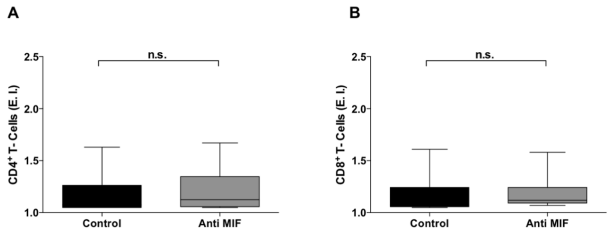


Supplementary Figure 3.5. Baseline production of cytokines and chemokines by MSCs from adult and elderly individuals assessed by proteome profiler.

(A) Representative image of the A-MSCs and E-MSCs proteome profiler where red squares indicate those secreted factors that were different between both groups (B) Summary data of differentially secreted cytokines and chemokines (n=5)



Supplementary Figure 3.6. Correlation between the levels of IL-6 and MCP-1/CCL2. determined by ELISA ($p=0.002$, $R^2 =0.61$)



Supplementary Figure 3.7. Antagonizing MIF does not rescue MSCs immunopotency.

(n=6).

Preface to Chapter 4

After showing that MSCs from elderly atherosclerotic donors have a pro-inflammatory secretome and reduced immunopotency, I assessed a mechanism that could underlie these defects. Supported by previous evidence indicating that redox imbalance, a hallmark of aging, promotes atherosclerosis; we hypothesized that mitochondrial dysfunction in ATH-MSCs lead to an abnormal redox state that ultimately promoted MSC polarization towards a pro-inflammatory phenotype. Further, we tested the impact of modulating the MSC mitochondrial function as a strategy to enhance the immunomodulatory function of ATH-MSCs.

CHAPTER IV: MITOCHONDRIAL OXIDATIVE STRESS REDUCES THE IMMUNOPOTENCY OF MESENCHYMAL STROMAL CELLS IN ADULTS WITH CORONARY ARTERY DISEASE

MITOCHONDRIAL OXIDATIVE STRESS REDUCES THE IMMUNOPOTENCY OF MESENCHYMAL STROMAL CELLS IN ADULTS WITH CORONARY ARTERY DISEASE

Ozge Kizilay Mancini, Maximilien Lora, Alexanne Cuillerier, Dominique Shum-Tim, Reggie Hamdy, Yan Burelle, Marc J. Servant, Ursula Stochaj and Inés Colmegna

4.1. Abstract

Mesenchymal stromal cells (MSCs) are promising therapeutic strategies for coronary artery disease (CAD), however, donor-related variability in cell quality is a main cause of discrepancies in preclinical studies. *In vitro*, MSCs from individuals with CAD have reduced ability to suppress activated T-cells. The mechanisms underlying the altered immunomodulatory capacity of MSCs in the context of atherosclerosis (ATH) remain elusive.

The aim of this study was to assess the role of mitochondrial dysfunction in the impaired immunomodulatory properties of MSCs from ATH patients.

Adipose tissue-derived MSCs were isolated from ATH (n=38) and Non ATH (n=42) donors. MSCs:$CD4^+$T-cell suppression, was assessed in allogeneic co-culture systems. Compared to Non ATH-, ATH-MSCs displayed higher levels of both intracellular ($p=0.006$) and mitochondrial ($p=0.03$) reactive oxygen species (ROS) reflecting altered mitochondrial function. The increased mitochondrial ROS levels of ATH-MSCs promoted a phenotypic switch characterized by enhanced glycolysis and an altered cytokine secretion (interleukin-6 (IL-6) $p<0.0001$, IL-8/CXCL8 $p=0.04$, and monocyte chemoattractant protein-1 (MCP-1/CCL2) $p=0.01$). Furthermore, treatment of ATH-MSCs with the ROS scavenger N-acetyl-L-cysteine (NAC) reduced the levels of IL-6, IL-

8/CXCL8, and MCP-1/CCL2 in the MSC secretome and improved MSCs immunosuppressive capacity (p=0.03).

Conclusion: An impaired mitochondrial function of ATH-MSCs underlies their altered secretome, and reduced immunopotency. Interventions aimed at restoring the mitochondrial function of ATH-MSCs improve their *in vitro* immunosuppressive ability, and may translate into enhanced therapeutic efficiency.

4.2. Introduction

Atherosclerosis (ATH) is a chronic inflammatory syndrome affecting large and medium sized arteries, the prevalence of which has increased steadily as the population ages. Coronary artery disease (CAD) is the underlying condition in most acute coronary events and the leading cause of death in developed countries (271, 302). Previous studies have shown that excessive mitochondrial oxidative stress within the vasculature contribute to the initiation and progression of endothelial dysfunction and atherogenesis (217, 303). Genetic ablation of manganese superoxide dismutase or uncoupling protein-2, accelerate atherogenesis in mouse models of ATH via an increase in mitochondrial oxidative stress (304,305). Conversely, overexpression of thioredoxin-2 decreases the atherosclerotic lesion size by enhancing nitric oxide production and lowering reactive oxygen species (ROS) (218). Furthermore, it has recently been shown that increased mitochondrial oxidative stress in lesional macrophages promotes atherogenesis through Nuclear Factor (NF)-κB-mediated monocyte-migration into the plaque (306). It is unknown whether mitochondrial oxidative stress in other lesional cell types contributes to atherogenesis.

In addition to cells of the innate immune system, the adaptive immune response also partake in orchestrating the progression of ATH. $CD4^+$ T-cells are one of the most abundant adaptive immune cells in human atherosclerotic plaques (307, 109), and are pro-atherogenic by promoting inflammation, lesion growth, and plaque instability (308). Furthermore, depletion of $CD4^+$ T-cells in *Ldlr*$^{-/-}$ and *ApoE*$^{-/-}$ murine models of ATH leads to atheroprotection with a substantial reduction in plaque formation and progression (309, 310). Due to growing evidence supporting the role of inflammation in ATH pathophysiology, ongoing clinical trials evaluate the impact of targeting pro-inflammatory cytokines [e.g., interleukin-1β (CANTOS trial), IL-6 (ASSAIL-MI), and tumor necrosis factor-α (CIRT trial) (128)]. Although, these studies will inform on the effects of targeting key single effectors of the ATH inflammatory process, complex pro- and anti-atherogenic immune networks operate during disease progression. Consequently, there is a need for broader, multi-target approaches to modulate the immune-mediated events involved in ATH.

Mesenchymal stromal cells (MSCs) are multipotent cells with unique immunomodulatory properties (276), that justify their use in clinical trials as cellular therapy for prevalent chronic inflammatory conditions (e.g. CAD) (281,282, 311). A major challenge in this field has been the unpredictable and partially conflicting results from some of those trials (312). Among the factors contributing to such inconsistent outcomes are issues related to donor heterogeneity, *ex vivo* MSCs expansion, and cryopreservation (313). We and others have shown that MSCs from individuals with chronic inflammatory conditions (e.g., type 2 diabetes, ATH) have reduced immunopotency due to a pro-inflammatory shift in their secretome (8, 68). The aim of this study is to assess whether increased

mitochondrial oxidative stress in ATH-MSCs reduces their immunopotency and alters their secretome, thereby promoting atherogenesis.

4.3. Material and Methods

4.3.1. Study subjects

This study was approved by the McGill University-Ethics Board and participants provided written informed consent. Subcutaneous adipose tissue was obtained from 42 healthy adults undergoing orthopedic surgery (Non ATH) and 38 adults undergoing programmed coronary artery bypass surgery (ATH). The demographic information of the study participants is summarized in Table 4.1.

4.3.2. Isolation and characterization of MSCs

MSCs were isolated from adipose tissue (AT) as previously described (8). Briefly, following enzymatic digestion of AT (1-8 g), cells were plated in 75-cm^2 tissue culture flasks (1g of tissue/flask). Two days after isolation, non-adherent cells were washed off and complete medium (CM), DMEM supplemented with 10% MSC qualified fetal bovine serum [FBS] and 1% penicillin / streptomycin) was added. At 80% confluency, MSCs were trypsinized and subcultured at a density of 5,000 cells/cm^2. Immunophenotypic characterization of MSCs was performed according to criteria established by the International Society for Cellular Therapy (ISCT) by multiparametric flow cytometry (BD LSRII; Becton Dickinson Co, Mountain View, CA). Passage 2 MSCs were stained with fluorochrome-conjugated monoclonal antibodies: fluorescein isothiocyanate (FITC)-conjugated anti-CD90 and anti-

CD45; phycoerythrin (PE)-conjugated anti-CD73; allophycocyanin (APC)-conjugated anti-CD34, anti-CD19 and anti-HLA-DR; peridinin chlorophyll (PerCP)-conjugated anti-CD105, anti-CD44 and anti-CD14. Data were analyzed with FlowJo software v9.7.2. In all samples, CD73, CD105, and CD90 surface markers were present in more than 95% of the events, while CD45, CD34, CD19, CD14 and HLADR were detected in less than 5% (Supp Figure 4.1A). At passage 3, MSCs were plated in 24-well plates at a density of 5,000 cells/cm^2 (Supp Figure 4.1B). Cells were incubated in CM for 3 days which was then replaced with one of the three differentiation media and cultured for 3 weeks as per the manufacture's protocol (StemPro® Adipo-, Osteo-, Chondrogenesis Differentiation Kit). MSCs were then fixed with 4% formaldehyde and stained with Alizarin-Red S, Oil red O, or safranin O; to assess osteogenic, adipogenic and chondrogenic differentiation, respectively (Supp Figure 4.1C).

4.3.3. Peripheral blood mononuclear cell (PBMCs) isolation, carboxyfluorescein succinimidyl ester (CFSE) fluorescent dye labeling, and PBMC activation

PBMCs were separated using lymphocyte separation medium (Corning) and density-gradient centrifugation. Then, they were cultured in 10% FBS RPMI medium (Wisent Biotechnologies) overnight for monocyte depletion. Monocyte-depleted PBMCs were stained with carboxy-fluorescein succinimidyl ester (CFSE; Sigma) and activated with anti-CD3/CD28 beads (Dynabeads®, Life Technologies).

4.3.4. Immunopotency assay

The capacity of MSCs to suppress proliferating $CD4^+$ T- cells was assessed in a 4-day allogeneic co-culture system (i.e., MSCs from different donors exposed to the same unrelated primary $CD4^+$ T-cells). MSCs were plated at 25×10^3 cells/well in flat-bottom 96-well plates (Corning) and cultured overnight. Activated monocyte-depleted CFSE-stained PBMCs (2×10^5 cells) were then cultured with MSCs in cell-cell contact conditions (MSCs:PBMCs ratio 1:8). At day 4, cells were stained with 7-Aminoactinomycin D (7-AAD), and APC-conjugated anti-CD4. The Expansion Index (E.I.) of $7AAD^-$ $CD4^+$ cells was determined with FlowJo software v9.7.2.

4.3.5. MSC priming ('licensing) by PBMCs

MSCs were plated at 75×10^3 cells/well in 24-well plates (Sarstedt) and cultured overnight. Activated monocyte-depleted PBMCs (6×10^5 cells) were then cultured in 0.4 µm PET transwell inserts (Millipore) added to the MSC plate (MSCs:PBMCs ratio 1:8). At day 4, the inserts were removed, the MSCs trypsinized, and assigned to the corresponding experiments.

4.3.6. Reactive oxygen species (ROS) and mitochondrial mass measurement

ROS was determined by the oxidant-sensing probe 2',7'-dichlorodihydrofluorescein diacetate (DCFDA 10 µM; Sigma, Schnelldorf, Germany) in $7\text{-}AAD^-$ MSCs (viable MSCs). Mitochondrial mass was measured by staining live MSCs with MitoTracker Green FM (400 nmol/L) for 30 minutes at 37°C. MSCs were washed with PBS and analyzed in a LSR II flow cytometer. Data were analyzed with FlowJo software.

4.3.7. Mitochondrial superoxide ($O_2^{\cdot -}$) production

MSCs (1×10^4) were seeded in 96-well, black flat-bottom microplates. After 4 days, cells were incubated in HBSS buffer containing 5 µM MitoSOX Red for 30 min. Fluorescence was measured using a SpectraMax® M2 Microplate reader at 0, 10, and 15 min (314).

4.3.8. Mitochondrial membrane potential

MSCs mitochondrial membrane potential was measured as previously described (315). Briefly, MSCs were cultured overnight on poly-L-lysine coated glass cover slips. The following day the medium was changed, supplemented with MitoTracker Red and MSCs were incubated for 30 minutes at 37˚C. MSCs were fixed with 3.7% formaldehyde/ PBS, permeabilized with 0.1% Triton X-100, and blocked with 0.05% Tween 20, 5% FBS, 1 mM NaN_3. Next, MSCs were incubated with primary antibodies against mitochondrial import receptor subunit Tom70 overnight. The following day, AlexaFluor®488-conjugated secondary antibodies were added and MSCs were stained with DAPI. Images were acquired with a Zeiss LSM510 confocal microscope. The Multi Wavelength Cell Scoring Module of MetaXpress® software was used for analysis.

4.3.9. Electron microscopy

MSCs were prepared for electron microscopy as previously described (314). MSCs were plated in Nunc® Lab-Tek® Chamber Slide™ 8-well slides coated with Permanox® at a seeding density of 1×10^4 cells per well. The following day, cells were fixed in 0.1 M phosphate buffer with 2.5% glutaraldehyde and stored at 4°C overnight. Cells were rinsed in 0.1 M phosphate buffer, incubated in fixation buffer supplemented with 1%

osmium tetroxide for 1 h, dehydrated through a graded ethanol series, and embedded in epoxy resin. Ultra-thin sections from the selected areas were contrasted with uranyl acetate and lead citrate, and viewed using an FEI Tecnai™ 12 transmission electron microscope at 120 kV with an XR-80C AMT, 8 megapixel CCD camera. Micrographs of randomly selected mitochondria were obtained at a final magnification of 1300X. Each mitochondrion was identified manually; the length of mitochondria was measured with an optical pen using ImageJ software.

4.3.10. Metabolic assays

Respirometry (oxygen consumption rate, OCR) and the extracellular acidification rate (ECAR) of MSCs were measured with an XF96 Extracellular Flux Analyzer (Seahorse Bioscience) as described previously (316, 317). Briefly, MSCs were plated at 1×10^4 cells/well in 200 µl non-buffered Dulbecco's modified Eagle's medium containing 25 mM glucose and 2 mM glutamine. Cells were incubated in a CO_2-free incubator at 37°C for 1 h to allow for temperature and pH equilibration prior to loading into the XF96 apparatus. XF assays consisted of a sequential mix (2 min), pause (2 min), and measurement (4 min) cycles, allowing for determination of OCR/ECAR every 7 min. All measurements were normalized to cell number, determined with the crystal violet assay.

4.3.11. ATP concentration

The ATPlite luminescence assay system (PerkinElmer) was used to determine ATP levels. Resting or primed (after co-culturing with PBMCs) MSCs were plated in 96-well

plates at a density of 10,000 cells per well. After 24 h, the ATP assay was conducted according to the manufacturer's specifications.

4.3.12. Enzyme-linked immunosorbent assays

MSCs were plated in 96-well plates at a density of 1 x 10^4 cells/well in 200 µL CM, and cultured overnight. Following a 24-hour treatment with either NAC (2 mM, Sigma) or oligomycin (1µM) the MSCs were washed extensively with PBS. CM was added for 24 hours and the supernatants were collected and frozen at −80°C. Quantification of IL-6, IL-8/CXCL8, and MCP-1/CCL2 was done by ELISA (BD Biosciences).

4.3.13. Determination of histone H2AX phosphorylation

MSCs were fixed in cytofix solution and permeabilized in 0.5% Triton X-100 (Sigma). Next, cells were incubated in blocking solution, and then with a mouse anti-γH2AX (pS139) Alexa Fluor 488 antibody overnight at 4°C, and analyzed by flow cytometry (FACS). Background was determined using unstained MSCs. Data were analyzed with FlowJo software.

4.3.14. Western blotting

MSCs were cultured either in resting conditions with CM or primed with interferon-gamma (10 ng/ml, R&D systems) and TNF-alpha (15 ng/ml, R&D systems) for 72 h. Total protein extracts (10-20 µg) were separated on 10% acrylamide–sodium dodecyl sulfate gels and transferred to polyvinylidene fluoride membranes (Millipore). Specific proteins were detected with anti- Mitofusin (Mfn)-2, anti-OPA1, anti-Dynamin-1-like protein (DRP-

1), anti-β-tubulin, anti- Hypoxia-inducible factor 1-*alpha* (HIF1-α), Complex I subunit NDUFB8, Complex II subunit 30kDa, Complex III subunit Core 2, or Complex IV subunit II antibodies. Beta-actin, GAPDH or alpha-tubulin were used as loading controls. Anti-mouse or anti-rabbit secondary antibodies were used, and signals were detected using a chemiluminescence system (ECL Plus, Amersham). Image lab software (BioRad) was used for analysis. Specific characteristics of all antibodies are provided in Online Table2

4.3.15. Statistical analysis

All analyses were performed with GraphPad Prism software (Graph-Pad, San Diego, USA) and graphs are presented as box plots. Wilcoxon signed-rank test was used to assess differences following NAC, MitoTempo, MitoCP and Trolox treatments. Mann-Whitney U test was used to compare Non ATH- and ATH-MSCs. Mean ± standard deviation (SD) from all comparisons are reported. All hypotheses tests were 2-sided; a *p*-value of <0.05 was considered statistically significant and is indicated by an asterisk in the figures.

4.4. Results

4.4.1. Increased intracellular ROS reduce the immunopotency of MSCs

At physiologic concentrations, ROS act as signaling molecules and play an important role in the regulation of various cellular functions including cell growth, proliferation, apoptosis, and inflammatory responses (318). However, excessive ROS levels cause oxidative stress, which is implicated in the pathogenesis of various cardiovascular diseases including ATH (83, 319). To determine whether oxidative stress alters

MSC immunopotency, we first treated MSCs with H_2O_2 to increase intracellular ROS levels (Supp Figure 4.2A). The immunopotency of H_2O_2-treated MSCs was assessed in $CD4^+$ T-cell suppression assays. H_2O_2 treatment increased MSCs intracellular ROS levels (Supp Figure 4.2B) reducing their efficiency to suppress $CD4^+$ T-cell proliferation (Figure 4.1A, B). This was not associated with changes in T-cell viability (Figure 4.1C).

4.4.2. MSCs from ATH subjects have increased levels of mitochondrial ROS

While our group previously reported that ATH impairs the immunomodulatory function of MSCs (8), the mechanisms underlying this defect remain elusive. Therefore, we assessed whether MSCs from ATH patients (ATH-MSCs) recapitulate the oxidative stress-induced reduction in MSC immunopotency. Specifically, we compared the redox state of ATH-MSCs to that in MSCs from individuals without ATH (Non ATH-MSCs). Our results indicate that ATH-MSCs have higher intracellular ROS levels (Figure 4.2A). As mitochondria are the major source of ROS, we next assessed mitochondrial oxidative stress by quantifying mitochondrial ROS levels. Indeed, ATH-MSCs have increased mitochondrial oxidative stress than Non ATH-MSCs (Figure 4.2B). Moreover, ATH-MSCs are less potent suppressors of $CD4^+$ T-cell proliferation (Figure 4.2C), and the increase in mitochondria-specific ROS correlated with the reduced immunopotency of MSCs (Figure 4.2E). $CD4^+$ T-cell viability was similar in Non ATH- and ATH-MSCs co-cultures (Figure 4.2D). A perturbation in the balance between oxidants and antioxidants in favor of oxidants, potentially promoting cellular damage, is termed 'oxidative stress'. Therefore, we further investigated if the increased intracellular ROS levels in ATH-MSCs resulted from a reduction in the antioxidant defense. Non ATH and ATH-MSCs have equal

abundance of two major antioxidant enzymes: catalase and superoxide dismutase (SOD). However, we cannot exclude differences in the enzymatic activity of these scavenger systems (Supp Figure 4.3).

4.4.3. Mitochondrial morphology is altered in ATH-MSCs

Morphological and structural alterations of mitochondria can lead to mitochondrial dysfunction and excessive intracellular ROS levels (320). Since ATH-MSCs display elevated mitochondrial oxidative stress, we next assessed the content (i.e. mitochondrial mass), structure, and morphology of their mitochondria. Our results revealed that MSCs from adults with ATH have increased mitochondrial mass (Figure 4.3A) and elongated mitochondria (Figure 4.3B); two features that may reflect an increase in fusion activity. Mitochondrial fusion maximizes oxidative capacity in response to stress by mixing the contents of partially damaged mitochondria by means of complementation (321). Ultimately, this protective mechanism ensures the maintenance of a functional mitochondrial population. To test whether in ATH-MSCs the structural mitochondrial changes were associated with defects in fusion or fission, we assessed the levels of two members of the dynamin GTPase superfamily that regulate mitochondrial fusion/fission dynamics (i.e., OPA-1 and DRP-1). Our data revealed that ATH-MSCs have increased total OPA-1 levels and reduced p-DRP-1 (Ser637), thus providing a potential mechanism for the differences in ATH-MSCs mitochondrial morphology (Supp Figure 4.4).

Mitochondrial morphology is inherently related to function. This is supported by the dynamic re-configurations of mitochondria that occur in some normal cells and the

morphological changes that are associated with various human diseases (e.g., diabetes mellitus, neurodegeneration, aging and cancer) (322, 323). Consequently, we investigated whether the altered morphology of ATH-MSCs was accompanied by changes in mitochondrial membrane potential. Compared to Non ATH-MSCs, ATH-MSCs display a reduced mitochondrial membrane potential (Figure 4.3C). In summary, ATH-MSCs display profound changes in mitochondrial morphology and function relative to Non ATH-MSCs.

4.4.4. Glycolytic metabolic shift of ATH-MSCs

Next, we assessed the impact of the increased mitochondrial oxidative stress on the bioenergetic function of ATH-MSCs by metabolic profiling. Under resting and primed (i.e., following MSC activation by PBMCs) conditions we measured the metabolic activity of Non ATH- and ATH-MSCs by simultaneously monitoring mitochondrial respiration and glycolysis.

The primary metabolic function of mitochondria is oxidative phosphorylation (OXPHOS), an energy-generating process that couples the oxidation of respiratory substrates with ATP production. Resting and primed ATH-MSCs have reduced mitochondrial respiration (OCR: key metric of OXPHOS), and sensitivity to electron transport chain inhibitors than Non ATH-MSCs (Figure 4.4A, B, C). Furthermore, upon priming, ATH-MSCs have similar mitochondrial oxygen consumption but an increased rate of extracellular acidification (ECAR: rate of conversion of pyruvate to lactic acid, measure of glycolytic activity) compared to Non ATH-MSCs. Interestingly, intracellular ATP levels are not different in resting and primed ATH-MSCs (Figure 4.4D), while primed Non ATH-

MSCs increase their ATP levels through both OXPHOS and glycolysis (Figure 4.4B, C, D). These results suggest that mitochondrial dysfunction in ATH-MSCs leads to a metabolic switch from OXPHOS to glycolytic energy production.

To assess whether the enhanced glycolytic flux of ATH-MSCs was associated with a low efficiency and/or impairment of OXPHOS, we investigated the abundance of OXPHOS protein complexes under resting and primed conditions. The OXPHOS system consists of five multimeric protein complexes (I, II, III, IV, and V). ATH-MSCs have decreased levels of Complex I subunit NDUFB8, Complex II subunit (SDHB), Complex III subunit Core 2 and Complex IV subunit COX II (Supp Figure 4.5). These results suggest a potential link between the enhanced glycolysis of ATH-MSCs and their low mitochondrial respiratory chain activity.

In aged tissues, the accumulation of HIF-1α under normoxic conditions causes a metabolic shift towards glycolysis (324). Since ATH is an age-related syndrome, we addressed this in ATH-MSCs. Despite being cultured under normoxic conditions, ATH-MSCs have increased HIF-1α protein levels (Supp Figure 4.6). This could be an additional explanation for the ATH-MSCs metabolic shift (Supp Figure 4.4).

Spare respiratory capacity (SRC) is the difference between the maximum mitochondrial OCR and the basal mitochondrial OCR. Cells can use SRC to adapt to increased energy demands. The mitochondrial SRC is critical for cellular survival and function(325, 326); and higher SRC has been associated with lower ROS generation (326). Compared with Non ATH-MSCs, ATH-MSCs have a lower SRC both under resting and primed conditions (Figure 4.4E).

Collectively, our data suggest that ATH-MSCs not only shift their metabolism toward glycolysis and produce less ATP, but they are also more susceptible to stress and possibly to cell death.

4.4.5. ATH-MSCs have high levels of HIF-1α and an altered secretome

ROS may act as secondary signaling messengers and activate NF-κB. In turn, NF-κB enhances the transcription and accumulation of HIF-1α, which then drives the expression of pro-inflammatory cytokine/chemokine genes (327). Given the higher abundance of HIF-1α in ATH-MSCs (Supp Figure 4.6), we evaluated key cytokines/chemokines in the MSC secretome. ATH-MSCs secrete higher levels of IL-6, IL-8/CXCL8 and MCP-1/CCL2 than Non ATH-MSCs in both resting (Figure 4.5A, B, C) and primed conditions (Supp Figure 4.7). Moreover, oligomycin-induced mitochondrial dysfunction of Non ATH-MSCs leads to an increase in ROS levels, and to a shift in the cytokine/chemokine secretome similar to ATH-MSCs (Figure 4.5D, E, F). Of relevance, oligomycin treatment had no impact on the ATH-MSC secretome (Supp Figure 4.8).

4.4.6. Increased apoptosis in ATH-MSCs

Excessive oxidative stress is cytotoxic due to increased oxidative damage to DNA, proteins, and lipids (328). Since ATH-MSCs have higher intracellular ROS levels than Non ATH-MSCs, we assessed the impact of increased oxidative stress on MSC survival (Figure 4.6A). After priming MSCs, we evaluated mitochondrial membrane potential, DNA damage, autophagic vesicles and apoptosis. ATH-MSCs have lower membrane potential (Figure 4.6B), higher DNA damage (Figure 4.6C), increased number

of autophagic vesicles (Figure 4.6D, E) and are more prone to apoptosis (Figure 4.6F, G).

4.4.7. ROS reduction shifts the ATH MSC secretome and rescues their immunopotency

The MSC secretome plays a key role mediating MSC immunopotency. Modulation of the MSC secretome through pharmacological preconditioning, or genetic manipulation prior to transplantation improves the outcome of MSC-based therapy (329, 330). We showed that ATH-MSCs have reduced immunopotency due to a shift in their secretome; and that neutralizing inflammatory secreted factors enhances MSCs immunomodulatory function (68). Therefore, we assessed the effects of reducing ROS on the MSC secretome profile. We treated both Non ATH- and ATH-MSCs with the ROS scavenger N-acetyl-L-cysteine (NAC), and measured key cytokines and chemokines of the MSC secretome. ROS reduction in ATH-MSCs but not in Non ATH-MSCs (Supp Figure 4.9) diminishes the levels of IL-6, IL-8/CXCL8 and MCP-1/CCL2 (Figure 4.7A, B, C) in the secretome, and enhances ATH-MSCs survival and immunopotency (Figure 4.7D, E, F, G). Furthermore, the mitochondria targeted ROS scavengers MitoCP and MitoTempo similarly improve the ATH-MSCs immunomodulatory capacity (Figure 4.7G).

4.5. Discussion

Clinical trials demonstrated the feasibility, safety and potential therapeutic benefits of autologous adipose-derived MSCs transplantation in various inflammatory conditions, including cardiovascular diseases. The main barrier limiting the translation of these results to clinical practice is inter-trial inconsistency (331, 332). A major contributor to

these discrepancies is the variability in the MSCs' immunopotency due to donor-related differences (333). Previously, our group showed that ATH impairs MSCs' immunomodulatory function, and this could impact their therapeutic efficiency (8). Defining the mechanisms underlying the reduced immunopotency of ATH-MSCs is relevant for MSCs optimization to ultimately enhance their therapeutic efficiency. In the present study, we demonstrate that mitochondria in ATH-MSCs are dysfunctional leading to an overproduction of ROS. In turn, oxidative stress negatively affects the MSCs immunomodulatory function and promotes a phenotypic switch characterized by enhanced glycolytic metabolism and an altered secretome. Furthermore, we provide evidence that the modulation of ROS levels in ATH-MSCs enhance their immune-modulatory function.

Excessive ROS levels reduce the osteogenic differentiation and proliferation of MSCs (334). Here we demonstrate that H_2O_2 also reduces the ability of MSCs to suppress T-cell proliferation, a surrogate marker of the overall immunosuppressive capacity of MSCs (190). Further we show that ATH-MSCs have higher levels of total intracellular ROS [a negative predictor of the MSCs expansion potential (335)] and, in particular, elevated mitochondrial ROS levels that correlate with their reduced *in vitro* immunopotency. Considering that MSCs may play a role in inhibiting inflammation and stabilizing vulnerable ATH plaques (183), and the implications of mitochondria to ATH development (336), our findings provide the rationale for testing whether restoring mitochondrial dysfunction in ATH-MSCs could impact ATH progression reducing the risk of acute events.

The abundance of HIF-1α in ATH-MSCs is a relevant finding linking their high ROS levels and their metabolic shift. ROS activate the HIF-1α promoter via a functional NF-κB site (327), and the HIF1α protein is stabilized by high mitochondrial ROS concentrations (337). HIF1α regulates the production of numerous glycolytic enzymes (338), and can underlie the glycolytic metabolic shift seen in ATH-MSCs. In certain cancer cells, HIF1α mediates a metabolic reprogramming for which those cells predominantly produce energy by a high rate of aerobic glycolysis followed by lactic acid fermentation in the cytosol (Warburg effect) (339, 340) . The generation and modulation of ROS is one of the benefits of the Warburg effect to cancer cells (341). Similarly, this metabolic reprogramming may also serve as a protective antioxidant mechanism in ATH-MSCs. Indeed, these cells are exposed to a pro-oxidative (inflammatory) milieu *in vivo*, and they display elevated basal (resting) ROS levels. Consequently, ATH-MSCs may shift their metabolism towards glycolysis to avoid excessive and cytotoxic levels of ROS accumulation thus ensuring their survival.

Increased mitochondrial ROS and downstream HIF1α expression in ATH-MSCs, may also account for the altered secretome of these cells. Indeed, elevated mitochondrial ROS are implicated in the *de novo* synthesis of NLPR3 inflammasome protein components (342). Further, mitochondrial ROS also promote sustained activation of pro-inflammatory Mitogen-activated protein kinases (MAPK) signaling through inhibition of MAPK phosphatases (343). Finally, ROS-induced HIF1α directly regulates inflammatory gene expression and processes in various myeloid and cancer cells (344, 345). The phenotypic shift of immunomodulatory Non ATH-MSCs towards pro-inflammatory ATH-MSCs resembles the changes seen during macrophage polarization

(159). Specifically, polarization towards the pro-inflammatory M1 phenotype is also characterized by a shift from OXPHOS towards glycolytic metabolism via HIF1α-dependent mechanisms (346).

The survival rate of transplanted MSCs is a determining factor in the success of cellular therapies (347). Considering the harsh micro-environment of the ischemic myocardium similar to that of other inflammatory conditions for which MSCs-based therapies are applied, the quality of MSCs pre-transplantation is crucial to ensure their viability and post-transplantation effects. Here, we show that PBMCs' primed ATH-MSCs, which partially mimics their transplanted micro-environment, are more prone to apoptosis than Non ATH-MSCs. These data suggest that in ATH-MSCs the elevated basal ROS levels, when combined with ROS generated upon PBMC priming, become cytotoxic.

Globally, our observations could be explained by a concentration-centered model of ROS as proposed by Finkel (348). In this model, low levels of mitochondrial ROS are associated with hypoxic responses mediated (at least in part) by HIF1α involving metabolic adaptations. Moderate levels of mitochondrial ROS are linked to the regulation of inflammatory responses; while high levels are involved in activating apoptosis/autophagy pathways, capable of inducing cell death. Supporting this model, our data shows that most of the resting (non-primed) ATH-MSCs have moderate levels of ROS. However, PBMC priming of ATH-MSCs increases their intracellular ROS levels triggering MSCs apoptosis.

Our data point to mitochondria as a suitable pharmacological target to improve the therapeutic potential of MSCs from individuals with ATH. Due to the study design it is not possible to discriminate between the effects of age and disease on mitochondrial dysfunction. To assess this, we have compared Non ATH-MSCs with Non ATH-*adult* MSCs (patients undergoing orthopedic surgery, age-matched to ATH-MSCs donors) for the main study outcomes (i.e. ROS levels, MSC secretome and apoptosis rates). The lack of differences in these readouts between both groups suggests that ATH is the main culprit of the reported differences between ATH- and Non ATH-MSCs (Supp Table 4.1, Figure 4.10). Furthermore, previously we showed that the negative effect of ATH on MSC immunosupressive ability is more pronounced than that of age (8). In addition, a recent comparative analysis of a large number of human adipose derived MSCs showed that the *in vitro* and *in vivo* properties were similar and maintained during aging (349). Although we can't rule out the confounder effect of medications used by the ATH-MSCs donors, our previous data indicate that neither statins, ACE inhibitors/ARB, or Beta blockers predicted differences in the MSCs immunomodulatory function (8). All together, these data suggest the role of disease as the main culprit of the mitochondrial dysfunction in adult ATH-MSCs. *In vitro* pre-treatment of ATH-MSCs with either a free radical scavenger or with mitochondria targeted redox-sensitive agents, reduced their pro-inflammatory secretome and increased their survival upon priming. This is relevant as co-treatment with MSCs and NAC, in an acute myocardial infarction model, improved MSCs engraftment and survival, thus enhancing the overall therapeutic benefit (350). Both *in vitro* and *in vivo* studies documented the protective effects of mitochondria-targeted

antioxidants for various conditions (351, 352). It remains to be tested whether *ex vivo* treatment of ATH-MSCs with such compounds could enhance their function.

In conclusion, our work shows that MSCs from subjects with ATH have higher levels of intracellular ROS due to impaired mitochondrial function. Excessive ROS levels compromise the MSC immunopotency, and increase their susceptibility to cell death. Overall these data suggest that the therapeutic effectiveness of ATH-MSCs could be impaired compared to those of Non ATH subjects. This study uncovered cellular mechanisms implicated in the potency of MSC-based therapies that likely contribute to the inter- and intra-trial variability of therapeutic outcomes. Finally, our results suggest strategies to enhance the therapeutic efficacy of autologous ATH-MSCs.

4.6. Acknowledgements

We thank Dr Russel Jones laboratory for their technical assistance with the metabolic assays. This work was supported by operating grants from the Canadian Institutes of Health Research (CIHR) to IC (MOP-125857) and MJS (MOP-123482). IC is a Chercheur-boursier clinician – Junior 2 from the Fonds de recherche du Québec- Santé.

4.7. Disclosures

None

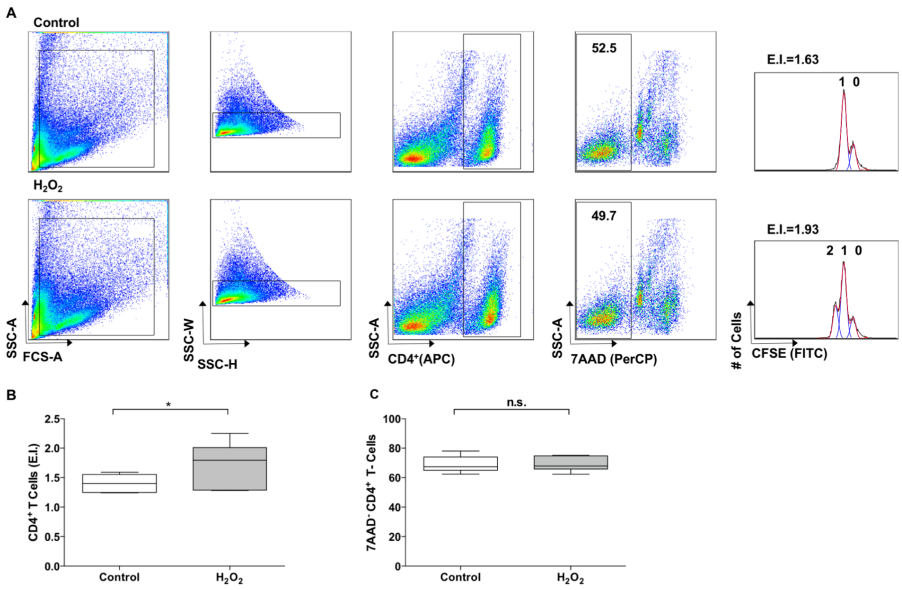


Figure 4.1. Increased intracellular ROS levels reduce MSC immunopotency.

(A) Representative example of a flow cytometry proliferation analysis of $CD4^+$ T-lymphocytes in co-culture with MSCs pre-treated with 40μM H_2O_2 or not pre-treated (Control). (B) Reduced immunosuppressive function of MSCs following H_2O_2 treatment (n=6, mean±SD Control: 1.4±0.16, H_2O_2: 1.72±0.37; *p=0.03). (C) H_2O_2 pre-treatment of MSCs does not affect $CD4^+$ T cell viability ($7\text{-}AAD^-$). Abbreviations: H_2O_2: Hydrogen peroxide; FSC-A: forward scatter area; SSC-A: side scatter area; SSC-H: side scatter height; SSC-W: side scatter width; EI: expansion index; 7-AAD: 7-aminoactinomycin D; MSCs: human adipose derived mesenchymal stromal cells.

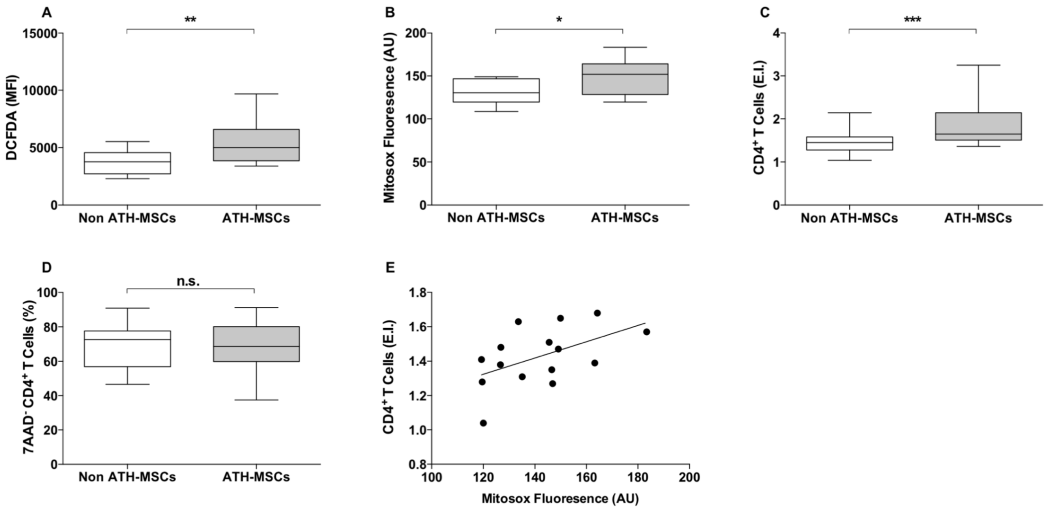


Figure 4.2. MSCs from ATH individuals produce higher levels of ROS and have reduced immunopotency.

(A) Increased intracellular (n=16, mean±SD Non ATH: 3730±974.3, ATH: 5341±1738; **p=0.006), and (B) mitochondrial (n=12, mean±SD Non ATH: 130.6±13.2, ATH: 148.8±19.5; *p=0.03) ROS levels in ATH-MSCs. (C) Reduced suppressive effect of ATH-MSCs on $CD4^+$ T-lymphocyte proliferation compared to Non ATH-MSCs (n=23, mean±SD Non ATH: 1.45±0.21, ATH: 1.86±0.5; ***p=0.0003). (D) Similar $CD4^+$ T cell viability ($7\text{-}AAD^-$) in ATH and Non ATH-MSCs immunopotency assays. (E) Increased mitochondrial ROS levels correlate with an increased E.I. of $CD4^+$ T-cells (i.e reduction in the suppressive effect of MSCs) (R^2=0.26, p=0.04). Abbreviations: ATH: Atherosclerotic; DCFDA: 2',7' –dichlorofluorescein diacetate; MFI: mean fluorescence intensity; AU: arbitrary unit; EI: expansion index; 7-AAD: 7-aminoactinomycin D; MSCs: human adipose derived mesenchymal stromal cells

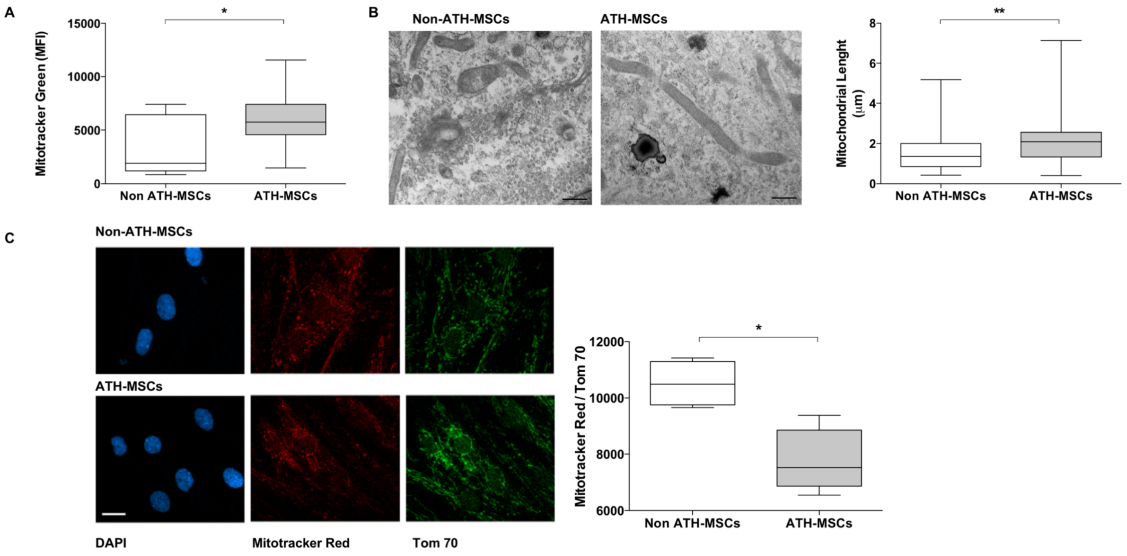


Figure 4.3. MSCs from ATH subjects have elongated mitochondria and lower membrane potential.

Mitochondrial mass was measured by Mitotracker green. (A) Pooled data of the Mitotracker green flow cytometric analysis in Non ATH- and ATH-MSCs (n=10, Non ATH: 3237±2617, ATH:6028 ±2655; *p=0.04). Transmission electron microscopy was performed for mitochondrial ultrastructure analysis. (B) Representative images of mitochondria are shown for each group. On these images, mitochondria were identified manually and the length was measured with an optical pen using ImageJ software. Mitochondrial length comparison between Non ATH- and ATH-MSCs (n=3, 15 mitochondria per cell, Non ATH: 1.54±0.86, ATH: 2.14±1.27; **p=0.006). (C) Representative images of mitochondrial membrane potential are shown for both groups. The multi wavelength cell scoring, module of MetaXpress® software was used to analyze mitochondrial membrane potential. Scale bar is

20 µm. Comparison between Non ATH- and ATH-MSCs. (n=4, Non ATH: 10516±803, ATH: 7792±1095; *p=0.02). All values between brackets are mean±SD. Abbreviations; ATH: Atherosclerotic; MFI: mean fluorescence intensity; MSCs: human adipose derived mesenchymal stromal cells.

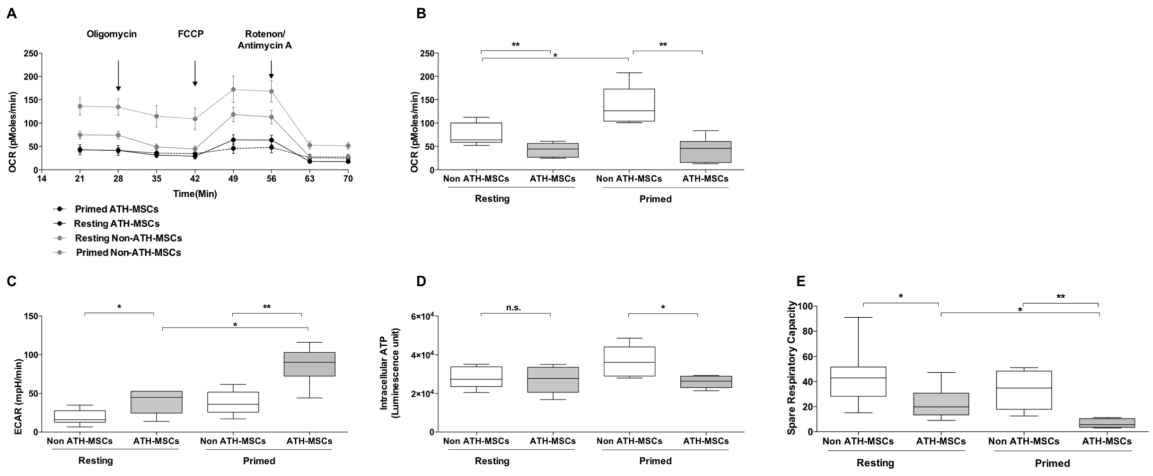


Figure 4.4. Mitochondrial dysfunction leads to a metabolic shift from OXPHOS to glycolysis in ATH-MSCs.

In vitro metabolic analysis of MSCs was assessed with the XF96 Extracellular Flux Analyzer. (A) Mitochondrial bioenergetics profiles of Non ATH- and ATH-MSCs resting and after priming with $CD4^+$ T-lymphocytes. (B-C) Basal oxygen consumption rate (OCR, pM O_2/min) and extracellular acidification rate (ECAR, mpH/min) in Non ATH- and ATH-MSCs for resting (OCR n=6, Non ATH: 74.68±22.9, ATH: 42.78±14.8; **p=0.008. ECAR Non ATH: 19.28±9.53, ATH: 39.57±15.47; **p=0.006) and primed (OCR n=5, Non ATH: 135.8±42.7, ATH: 42.8±26.1; **p=0.004. ECAR n=5, Non ATH: 38.1 ± 16, ATH: 86.9 ± 24.6; *p=0.02) conditions. Priming increases OCR in Non ATH-, and ECAR in ATH-MSCs compared to resting conditions (*p=0.01, *p=0.03). (D) ATP levels of resting and primed MSCs (n=5, Non ATH: 36445±8206, ATH: 26080±3188; *p=0.03). (E) Spare respiratory capacity in MSCs resting (n=6, Non ATH: 44.1 ± 22.5,

ATH: 22.7 ± 13.3; *p=0.04), and primed (n=5, ATH: 33.5 ± 15.8, ATH: 6.5 ± 3.5; **p=0.004). Metabolic analyses were performed in quadruplicates. All values between brackets are mean±SD. Abbreviations: ATH: Atherosclerotic; ATP: Adenosine triphosphate; MSCs: human adipose derived mesenchymal stromal cells.

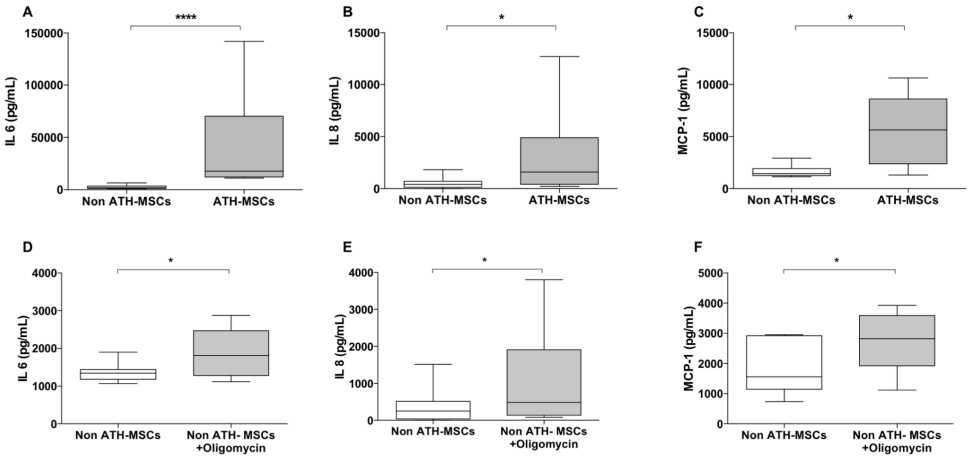


Figure 4.5. Induction of mitochondrial dysfunction in Non ATH-MSCs 'recapitulates' features of ATH-MSCs.

Baseline production of cytokines and chemokines by MSCs from Non ATH and ATH individuals were assessed by enzyme-linked immunosorbent assays. (A) IL-6, (n=7, Non ATH: 2434 ± 1557, ATH: 46137± 48504; ****<0.0001). (B) IL-8/ CXCL8 (n=7, Non ATH: 538.5 ± 529.2, ATH: 3185 ± 4502; *p=0.04). (C) MCP-1/CCL2 (n=7, Non ATH: 1615 ± 655.6, ATH: 5779 ± 3265; *p=0.01). Oligomycin treatment of Non ATH-MSCs increased their secretion of (D) IL-6 (n=6, Non ATH: 1350 ± 246.4, Non ATH + Oligomycin: 1903 ± 644; *p=0.01), (E) IL-8/ CXCL8 (n=8, Non ATH: 385 ± 501, Non ATH + Oligomycin: 1019 ± 1340; *p=0.04), and (F) MCP-1/CCL2 (n=6, Non ATH: 1833 ± 900, Non ATH + Oligomycin: 2726 ± 999; *p=0.03). All values between brackets are mean±SD. Abbreviations: ATH: Atherosclerotic; IL-6: interleukin-6; IL-8: interleukin-8; MCP-1: monocyte chemoattractant protein-1; MSCs: human adipose derived mesenchymal stromal cells.

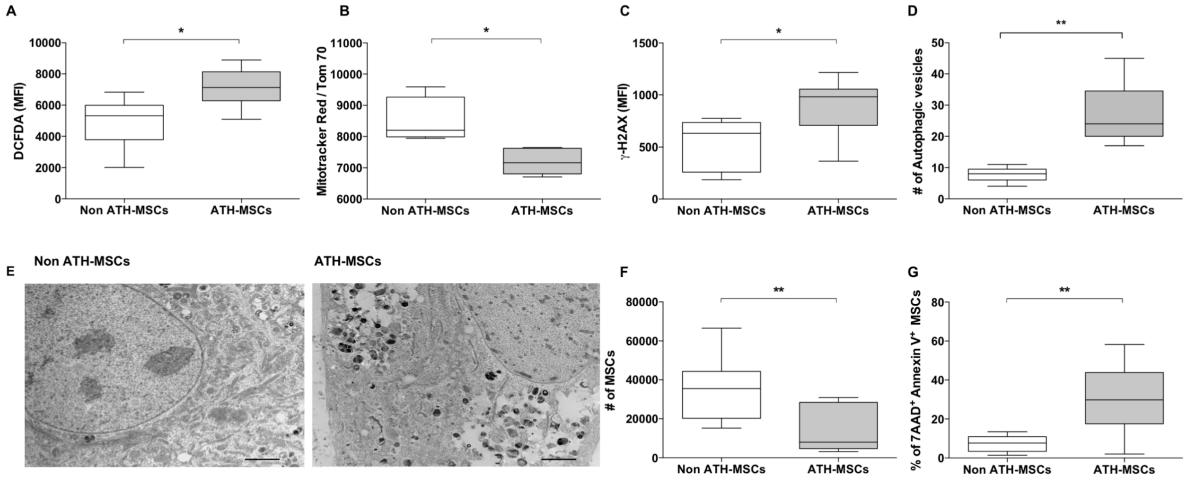


Figure 4.6. Priming leads to excessive ROS levels in ATH-MSCs and increased apoptosis

(A) Comparison of intracellular ROS levels in primed (i.e. co-culture with T-cells) Non ATH and ATH-MSCs (n=6, Non ATH: 4931±1643, ATH: 7135±1267; *p=0.02). (B) Comparison of mitochondrial membrane potential in Non ATH- and ATH-MSCs (n=5, Non ATH: 8485±749.6, ATH: 7206±416.9; *p=0.02). (C) Increased DNA damage in primed ATH-MSCs (n=6, Non ATH: 538.3±244.9, ATH: 888±279.1; *p=0.03). (D-E) Transmission electron microscopy of autophagic vesicles in primed MSCs. The number of autophagic vesicles/cell was determined in each sample (n=5, Non ATH: 7.8±2.4, ATH: 27±9.9; **p=0.004). Scale bar is 2 μm. (F) Reduced cell count (n=10, Non ATH: 35982±16846, ATH: 14603±12002; **p=0.003), and (G) higher apoptosis (n=11, Non ATH: 6.9±4.3, ATH: 29.4±17.9; **p=0.001) in ATH-MSCs. All values between brackets are mean±SD. Abbreviations: ATH: Atherosclerotic; DCFDA: 2',7' –dichlorofluorescin diacetate; MFI: mean fluorescence intensity; g-H2AX: phosphorylated H2A histone family member X; 7-AAD: 7-aminoactinomycin D; MSCs: human adipose derived mesenchymal stromal cells.

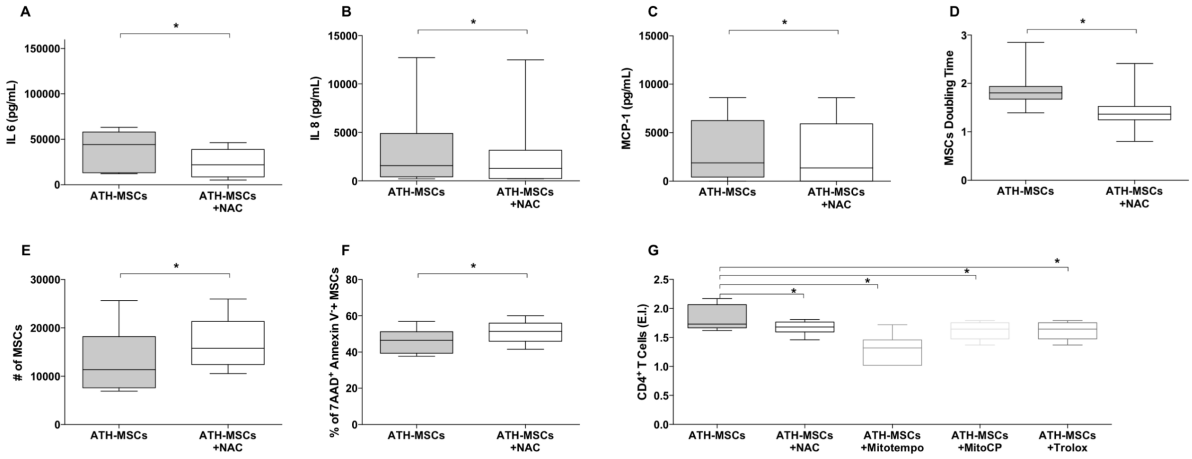


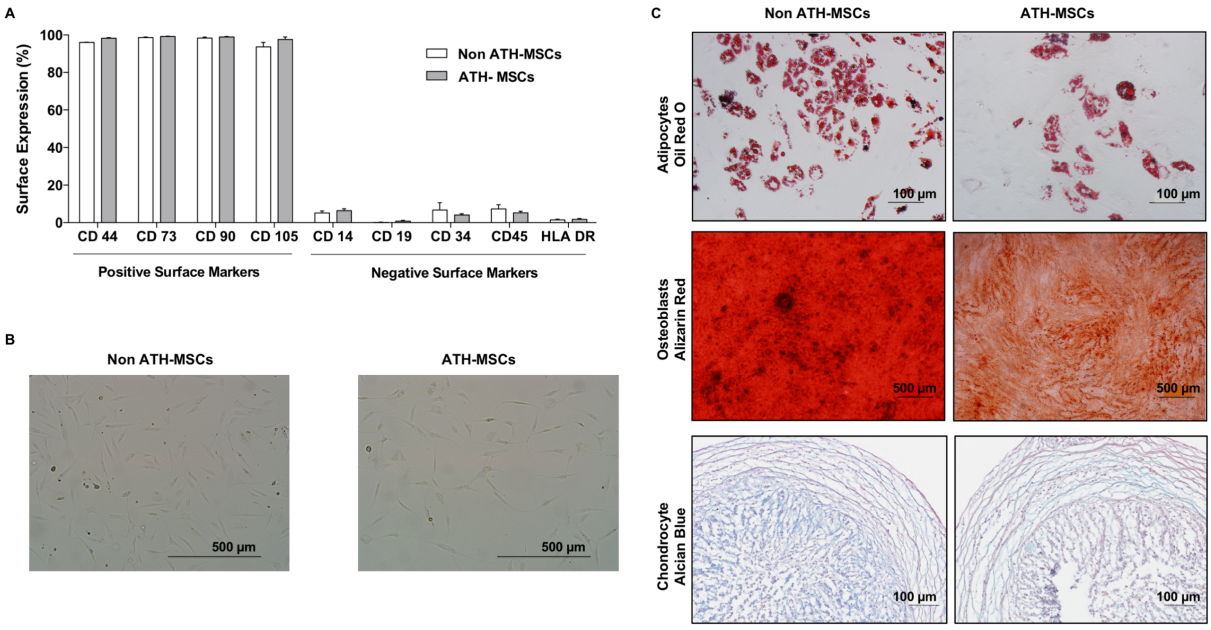
Figure 4.7. Reduction of intracellular ROS levels in ATH-MSCs alters their secretome profile and improves immunopotency

Changes in the secretion of the following cytokines and chemokines (A) IL-6 (n=6, ATH: 38966±21921, ATH+NAC: 23676±16593; *p=0.03), (B) IL-8/CXCL8 (n=6, ATH: 3185±4504, ATH+NAC: 2771±4405; *p=0.03), and (C) MCP-1/CCL2 (n=6, ATH: 3080±3475, ATH+NAC: 2728±13475; *p=0.03) by ATH-MSCs after NAC treatment. NAC treatment of ATH-MSCs (D) increased their doubling capacity (n=7, ATH: 1.44±0.48, ATH+NAC: 1.89±0.45; *p=0.01), (E) increased MSCs frequencies (n=6, ATH: 13137±6892, ATH+NAC: 16816±5651; *p=0.03), and (F) reduced apoptosis (n=6, ATH: 46±6.9, ATH+NAC: 51±6.5; *p=0.03). (G) The intracellular ROS scavenger NAC and the mitochondria-targeted antioxidants MitoTempo, MitoCP, or Trolox improvement MSC:$CD4^+$T-cell suppressive capacity (n=6, ATH: 1.82±0.22, ATH+NAC: 1.6±0.1, ATH+MitoTempo: 1.2±0.2, ATH+MitoCP: 1.6±0.2, ATH+Trolox: 1.6±0.2; *p=0.03). All values between brackets are mean±SD. Abbreviations: ATH: Atherosclerotic; NAC: N-acetylcysteine; IL-6: interleukin-6; IL-8:

interleukin-8, MCP-1: monocyte chemoattractant protein-1; 7-AAD: 7-aminoactinomycin D; EI: expansion index; MSCs: human adipose derived mesenchymal stromal cells.

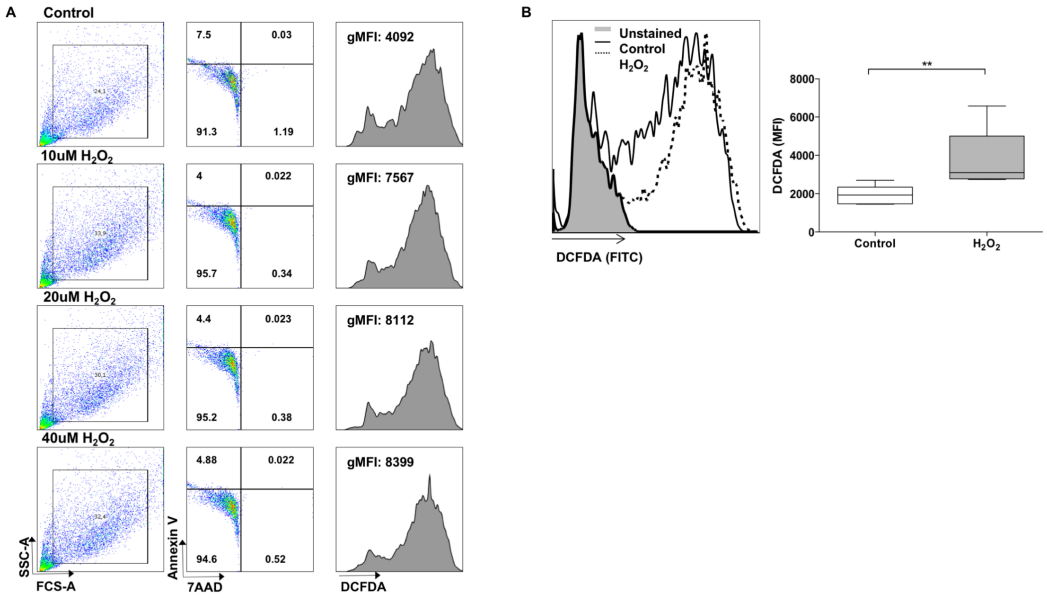
Table 4.1. Demographic Characteristics of the Study Subjects

	Surgical Procedure	
	Cardiovascular (ATH) n =38	**Orthopedic (Non ATH) n =42**
Gender (F:M)	**10:28**	**18:24**
Age (mean ± SD)	**59.9±11**	**22.6±20.1**
Cardiovascular Risk Factors (%)		
Tobacco	**29**	**6**
Hypertension	**79**	**14.2**
Hypercholesterolemia	**71**	**0**
Type II Diabetes	**39**	**0**
Medications (%)		
Aspirin	**74**	**2.3**
Statin	**66**	**0**
Metformin	**26**	**0**

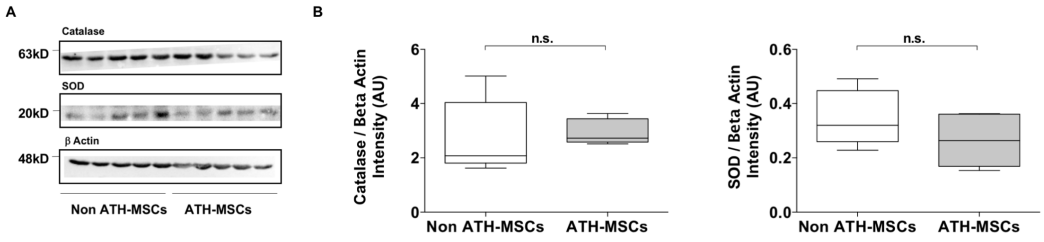


Supplementary Figure 4.1. Characterization of human adipose tissue derived MSCs.

(A) Similar frequency of positive (CD44, CD73, CD90, CD105) and negative (CD14, CD19, CD34, CD45, HLA-DR) surface markers. (B) Plastic adherence and (C) Trilineage differentiation of Non-ATH and ATH MSCs into adipocytes, osteoblasts and chondrocytes.

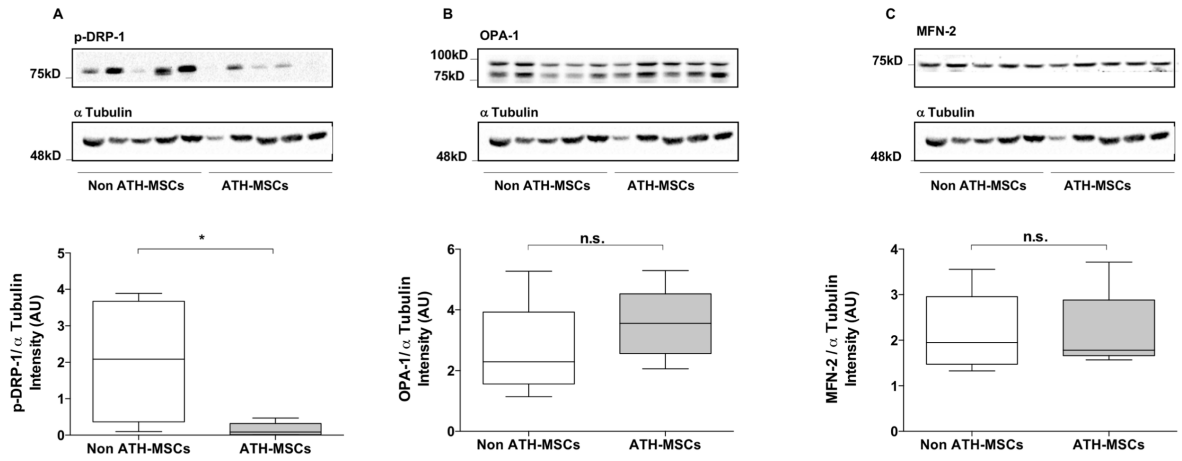


Supplementary Figure 4.2. Increased intracellular ROS levels after H_2O_2 treatment. (A) Dose response of H_2O_2 MSCs treatment and intracellular ROS levels. (B) H_2O_2 (40uM) treatment increases intracellular ROS levels in MSCs (n=5, mean±SD, Control: 1912±503.1, H_2O_2: 3730±1616; **p=0.008). Abbreviations: MFI: geometric Mean Fluorescence Intensity; H_2O_2: Hydrogen peroxide; DCFDA: 2',7' – dichlorofluorescein diacetate.



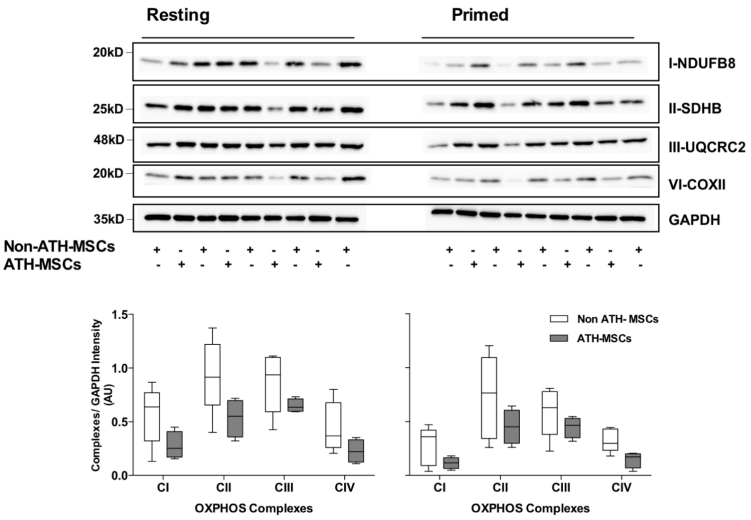
Supplementary Figure 4.3. Similar antioxidant enzyme abundance in both ATH- and Non-ATH-MSCs.

(A) Representative Western blots for catalase and superoxide dismutase (SOD). beta actin was used as loading control (B) No significant changes in the abundance of either catalase or SOD in both groups. Abbreviations: ATH: Atherosclerotic; MSCs: Human adipose derived mesenchymal stromal cells.



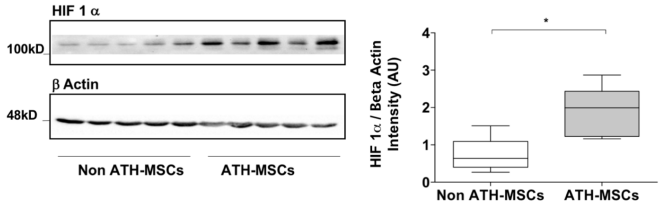
Supplementary Figure 4.4. Similar mitochondrial fusion protein abundance in ATH- and Non ATH-MSCs.

Representative Western blots for p-DRP-1, MFN2 and OPA-1. Comparison of p-DRP-1, OPA-1, and MFN2 protein abundance (n=5). a tubulin was used as loading control. Abbreviations: ATH: Atherosclerotic; p-DRP-1: dynamin-related protein; OPA-1: mitochondrial dynamin like GTPase; MFN-2: Mitofusin-2; AU: arbitrary unit; MSCs: Human adipose derived mesenchymal stromal cells.



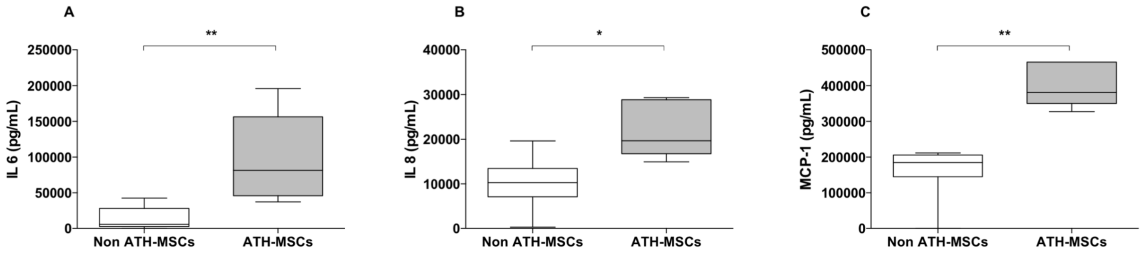
Supplementary Figure 4.5. Reduced OXPHOS complexes in ATH-MSCs.

Representative Western blots for OXPHOS complexes under resting and primed conditions. Comparison of the abundance of each protein (Complex I subunit NDUFB8, Complex II subunit SDHB, Complex III subunit Core 2, Complex IV subunit II). GAPDH was used as loading control. Abbreviations: ATH: Atherosclerotic; MSCs: Human adipose derived mesenchymal stromal cells.



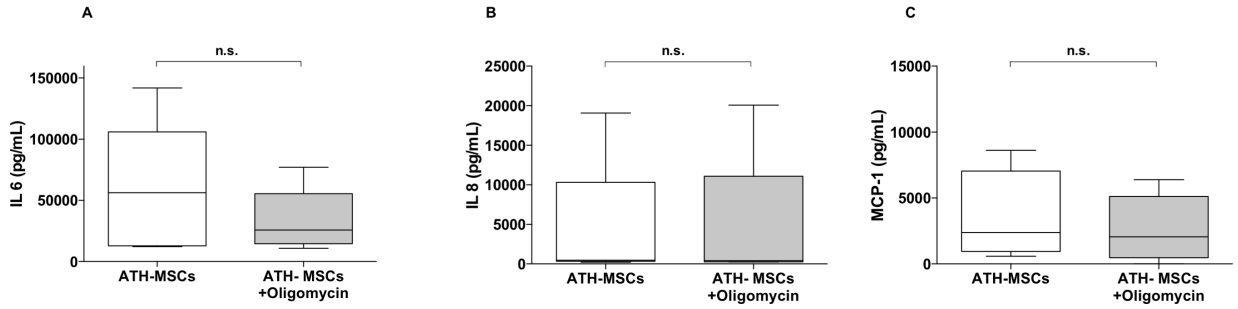
Supplementary Figure 4.6. Increased protein abundance of HIF1α in ATH-MSCs.

Representative Western blots for HIF1α. Comparison of HIF1α protein abundance. β actin was used as loading control. Abbreviations; ATH: Atherosclerotic; HIF: Hypoxia induced factor; AU: arbitrary unit; MSCs: Human adipose derived mesenchymal stromal cells.



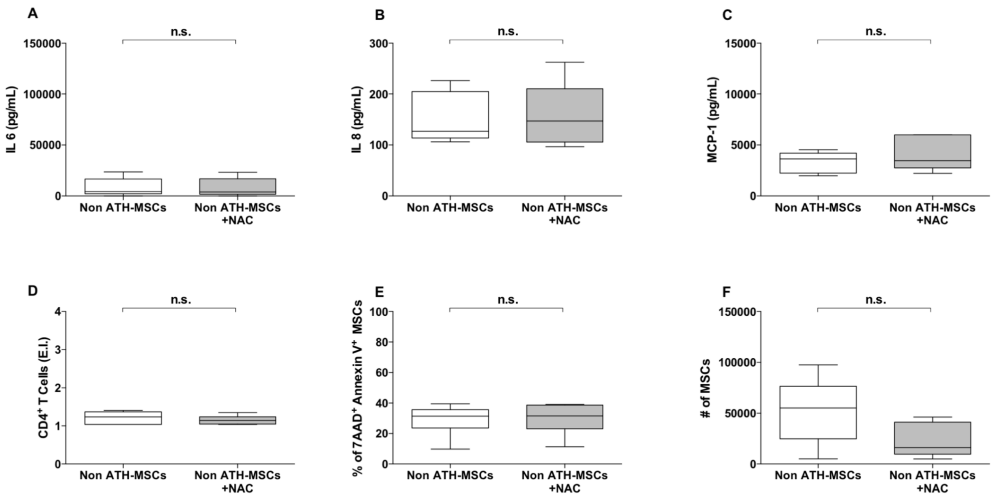
Supplementary Figure 4. 7. Higher cytokine and chemokine production by ATH-MSCs upon priming.

MSCs from Non-ATH and ATH individuals were primed with interferon-gamma (10 ng/ml) and TNF-alpha (15 ng/ml), and key components of their secretome were assessed by enzyme-linked immunosorbent assays. (A) IL-6, (n=7, Non ATH: 13725± 15736, ATH:97203± 62848; **p=0.005), (B) IL-8/ CXCL8 (n=6, Non ATH: 10216 ± 6179, ATH: 22204 ± 6339; *p=0.01), and (C) MCP-1/CCL2 (n=7, Non ATH: 154862 ± 73021, ATH: 402573 ± 61305; **p=0.002). All values between brackets are mean±SD. Abbreviations: ATH: Atherosclerotic; IL-6: interleukin-6; IL-8: interleukin-8, MCP-1: monocyte chemoattractant protein-1; MSCs: Human adipose derived mesenchymal stromal cells.



Supplementary Figure 4.8. Oligomycin treatment does not alter the secretome of ATH-MSCs.

levels of (A) IL-6, (B)) IL-8/ CXCL8, and (C)) MCP-1/CCL2 in ATH-MSCs ± oligomycin treatment (n=5). Abbreviations: ATH: Atherosclerotic; IL-6: interleukin-6; IL-8: interleukin-8; MCP-1: monocyte chemoattractant protein-1; MSCs: Human adipose derived mesenchymal stromal cells.

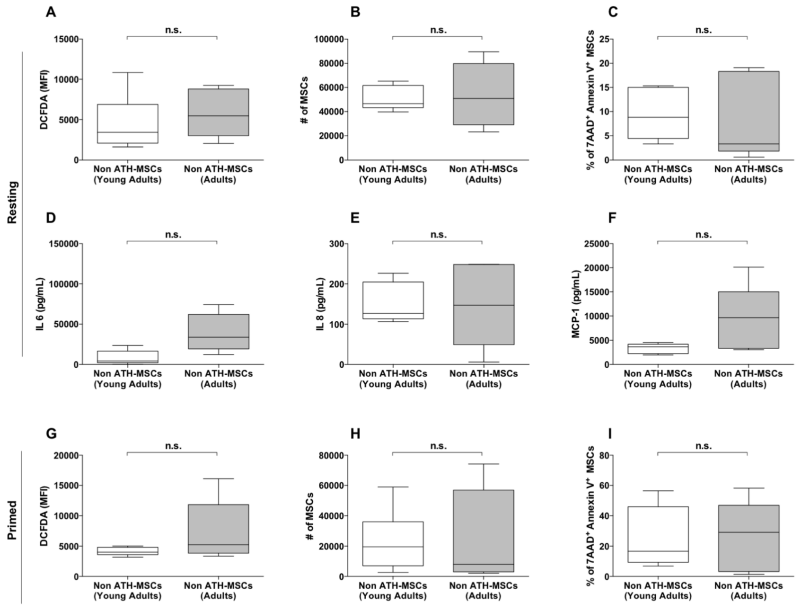


Supplementary Figure 4.9. NAC pretreatment does not affect the function of Non-ATH MSCs.

The content of (A) IL-6, (B) IL-8/ CXCL8, and (C) MCP-1/CCL2 (n=6) in the secretome of Non ATH-MSCs is unchanged by NAC pre-treatment. There are no differences in the (D) immunopotency (n=6), (E) apoptosis (n=6), and (F) frequency of Non ATH-MSC ± NAC. Abbreviations: ATH: Atherosclerotic; NAC: N-acetylcysteine; IL-6: interleukin-6; IL-8: interleukin-8; MCP-1: monocyte chemoattractant protein-1; 7-AAD: 7-aminoactinomycin D; EI: expansion index; MSCs: Human adipose derived mesenchymal stromal cells.

Supplementary Table 4.1. Demographic Characteristics of Young and Adult Non ATH-MSCs' donors.

Orthopedic Surgery Donors (Non-ATH)	Adults n =6	Young Adults n =7
Gender (F:M)	6:0	4:3
Age (mean ± SD)	69.8±10.3	15.1±1.1
Cardiovascular Risk Factors (%)		
Tobacco	0	0
Hypertension	100	0
Hypercholesterolemia	0	0
Type II Diabetes	0	0
Medications (%)	16	0
Aspirin	0	0
Statin	0	0
Metformin		



Supplementary Figure 4.10. Young adult and Adult Non ATH-MSCs have a similar phenotype.

There are no differences in (A) intracellular ROS levels, (B) cell count, (C) apoptosis, and secreted levels of (D) IL-6, (E) IL-8, and (F) MCP-1 (n=6) in both Young adults and Adults Non ATH-MSCs under resting conditions. Post-priming (G) intracellular ROS levels, (H) cell count, and (I) apoptosis (n=6) remain similar in both groups. Abbreviations: ATH: Atherosclerotic; DCFDA: 2',7' –dichlorofluorescin diacetate; MFI: mean fluorescence intensity; 7-AAD: 7-aminoactinomycin D; IL-6: interleukin-6; IL-8: interleukin-8; MCP-1: monocyte chemoattractant protein-1; MSCs: Human adipose derived mesenchymal stromal cells.

Supplementary Table 4.2. Specific Characteristics of antibodies.

	Product	Source	Host /Reactivity	Ig fragment	Titer	Catalog number /Company
Flow Cytometry Experiments	CD44	Clone G44-26	Mouse anti-Human	IgG_{2b},κ	1:10	560531 /BD Pharmingen
	CD73	Clone AD2	Mouse anti-Human	IgG_1, κ	1:10	550257 /BD Pharmingen
	CD90	Clone 5E10	Mouse anti-Human	IgG_1, κ	1:10	555595 /BD Pharmingen
	CD105	Clone 266	Mouse anti-Human	IgG_1, κ	1:10	560819 /BD Pharmingen
	CD14	Clone MφP9	Mouse anti-Human	IgG_{2b}, κ	1:10	562692 /BD Pharmingen
	CD19	Clone HIB19	Mouse anti-Human	IgG_1, κ	1:10	555415 /BD Pharmingen
	CD34	Clone 581	Mouse anti-Human	IgG_1, κ	1:10	555824 /BD Pharmingen
	CD45	Clone HI30	Mouse anti-Human	IgG_1,κ	1:10	555482 /BD Pharmingen
	HLA-DR	Clone G46-6	Mouse anti-Human	IgG_{2a},κ	1:10	560896 /BD Pharmingen
	CD4	Clone RPA-T4	Mouse anti-Human	IgG_1, κ	1:10	555349 /BD Pharmingen
	γ-H2AX	Clone N1-431	Mouse anti-Human	IgG_1	1:10	560445 /BD Pharmingen

	Product	Source	Host /Reactivity	Ig fragment	Titer	Catalog number /Company
Western Blot Experiments	Catalase	n/a	Rabbit anti-Human	IgG	1:1000	ab52477 /abcam
	Superoxide dismutase	Clone 2A1	Mouse, anti-human	$IgG_{1, \kappa}$	1:1000	ab16956 /abcam
	p-DRP-1	n/a	Rabbit anti-Human	n/a	1:1000	6319s /Cell signaling
	OPA-1	Clone 18/OPA1	Mouse anti-Human	IgG_1	1:1000	612606/BD Biosciences
	MFN2	n/a	Rabbit anti-Human	n/a	1:1000	M6319 /Sigma
	Complex I subunit NDUFB8,	-	-	-	1:1000	ab110411/abcam
	Complex II subunit SDHB,	-	-	-	1:1000	ab110411 /abcam
	Complex III subunit Core 2	-	-	-	1:1000	ab110411 /abcam
	Complex IV subunit II	-	-	-	1:1000	ab110411 /abcam
	HIF1α	Clone 54/HIF-1α	Mouse anti-Human	$IgG_{1, \kappa}$	1:1000	610958/BD Biosciences

	Product	Source	Host /Reactivity	Ig fragment	Titer	Catalog number /Company
Immunofluorescence	Mitotracker Red	-	-	-		M7512/ThermoFisher Scientific
	Tom 70		Rabbit anti-Human	His-tagged cytosolic fragment of human Tom70	1:500	
Viability	Annexin V	-	-	-	1:10	556421 /BD Pharmingen
	7 AAD	-	-	-	1:10	559925 /BD Pharmingen
Loading controls	GAPDH	n/a	Rabbit anti-Human	IgG	1:10000	ab9485 /abcam
	α tubulin	Clone DM1A	Mouse anti-Human	IgG_1	1:5000	T9026 /Sigma-Aldrich
	β actin	Clone AC-15	Mouse anti-Human	IgG_1	1:10000	A5441/Sigma-Aldrich
Secondary Antibodies	Peroxidase-labeled antibody to Mouse IgG(H+L)		Goat	IgG	1:2500	074-1806/KPL
	Peroxidase-labeled antibody to Rabbit IgG(H+L)		Goat	IgG	1:2500	074-1506/KPL
	Secondary antibody, Alexa488-anti-rabbit		Donkey	IgG	5ug/ml	711-545-152(/Jackson Immuno Research Inc)

CHAPTER V: CONCLUSION AND SUMMARY

5.1. Conclusion and summary

Due to its prevalence and high associated mortality; new and effective therapeutic interventions are needed for ATH. MSCs are attractive, as therapeutic agents due to their immunodulatory and tissue regenerative capacities. A growing body of evidence suggests the protective role of MSCs in ATH. However, it was not known whether MSCs from ATH individuals are functionally equivalent to those of healthy individuals.

In my thesis, I proposed that MSCs from atherosclerotic patients are functionally defective and less efficient in immune modulation and in restoring tissue homeostasis. To address this hypothesis, I first characterized the MSCs function from patients with ATH and Non ATH controls. Among these patients, we assessed the effect of ATH, chronological age, and T2DM on the MSCs immunopotency. We reported that independently, each of these conditions contribute to a reduction of the MSCs immunosuppressive capacity (Chapter 2). Previous studies demonstrated that both replicative senescence and cell freezing/thawing process lead to a functional decline in MSCs (353, 354). In order to prevent any culture induced effects on MSCs, we used freshly isolated and early passage (P4) cells. As a readout we used an immunopotency assay, which could predict the MSC *in vivo* therapeutic function. Our data is consistent with animal studies showing that ATH and T2DM reduce MSCs effectiveness in experimental models of MI (266, 355). To the best of our knowledge, this was the first study that explored the effects of ATH and two of its major risk factors, on the MSC immunomodulatory potential in a large cohort of ATH subjects. The relevance of our findings is that if autologous MSCs progress as a therapeutic option for ATH, optimization of donor selection or the graft should be

considered to enhance the MSCs functionality and thus increase the potential clinical effect.

Considering that the MSCs secretome is a key effector of MSCs function, next I hypothesized that age-associated changes in the secretome underlie the impaired function of MSCs from elderly atherosclerotic patients. ATH is not only considered as a disease of aging but also cellular senescence is a key pathogenic mechanism in ATH. First, I assessed senescence markers in MSCs from adult and elderly atherosclerotic patients. MSCs from elderly atherosclerotic patients displayed an aged phenotype, with increased cell size, DNA damage and intracellular ROS levels. This is consistent with other studies showing that VSMCs, ECs and macrophages from atherosclerotic plaques show a senescent phenotype with decreased cell proliferation, increased DNA damage, shorter telomere lengths and irreversible growth arrest [reviewed in (247)]. We then assessed another senescence marker: the SASP. We screened the supernatants of MSCs from both groups and found that aged MSCs from atherosclerotic patients secrete higher levels of pro-inflammatory cytokines specifically MCP-1/CCL2, IL-8/CXCL8 and IL-6. Further the neutralization of these cytokines with specific antibodies resulted in the improved MSCs ability to suppress activated T-cells. The relevance of these findings include that 1- the pro-inflammatory phenotype of elderly ATH-MSCs can promote inflammation in the ATH plaque either by directly secreting proinflammatory cytokines/chemokines or by influencing local immune responses; 2- modulating senescent cells (e.g., navitoclax) regress plaque growth via decreased secretion of senescence associated secretory phenotype (356); 3- antagonizing IL-6 (i.e. tocilizumab)

systemically could theoretically impact ATH progression through the modulation of MSCs function.

Cytokine/chemokines (i.e., IL-6, IL-8 and MCP-1) that we found to be secreted by ATH-MSCs in high levels have been also implicated in ATH progression and ongoing clinical trials are testing the impact of using monoclonal antibodies targeting those cytokines in ATH. Among those, high levels of serum IL-6 have been shown to correlate with increased endothelial dysfunction and contribute to atherosclerotic plaque development and destabilization. Moreover, every SD increased in log IL-6 indicates 25% higher risk of having future vascular events (357). In addition, IL-6 is a potent promoter of CRP. This serum inflammatory marker is currently included in the AHA risk assessment guidelines and allows for the sub-classification of individuals previously assigned an intermediate CVD risk (according to traditional risk factors) into higher or lower risk categories (358). Altogether, these observations have led to clinical trials of IL-6 inhibition (359) . The fact that tocilizumab upregulates apolipoprotein B and LDL cholesterol in rheumatoid arthritis patients in a dose-dependent and inflammatory status-independent manner is a concern when this mAb is used in ATH. However, tocilizumab-induced increases in LDL cholesterol could be curtailed via high-dose statin co-therapy [reviewed in (357)].

MCP-1/CCL2 is a key chemokine that regulates the trans-endothelial trafficking of monocytes, memory T lymphocytes, and NK cells in atherosclerotic lesions. It is produced locally by various immune and non-immune cell types (e.g., fibroblasts, ECs, VSMCs and monocytes themselves). MCP-1's overexpression in patients with ATH, especially in lesioned areas, makes this chemokine and its receptor (CCR2) of therapeutic relevance in ATH. Indeed, MCP-1 or CCR2 knockout mice display a significant reduction in arterial

lipid deposition that translates into a reduction in the formation of atherosclerotic lesions (360). Monocyte/macrophage MCP-1 secretion was shown to be induced by oxLDL in a TLR4-dependent and ERK-mediated fashion. Pre-treatment of oxLDL-stimulated primary monocyte/macrophages with an anti-TLR4 antibody or with a specific ERK inhibitor abrogated oxLDL-induced MCP-1 secretion (361) .

IL-8 (CXCL8) is another cytokine that is highly expressed in atherosclerotic lesions. In ATH, the chemokine is secreted by ECs, T cells and VSMCs but is especially prominent in macrophage-rich areas of atherosclerotic lesions [reviewed in (32)]. IL-8 serves as a monocyte and neutrophil chemoattractant and activator and promotes monocyte adhesion to arterial endothelial cells, a critical step in the initiation of ATH. Further, IL-8 favors angiogenesis in the late stages of plaque formation and may also participate in atherosclerotic plaque destabilization and thrombosis (32, 362). Importantly, like MCP-1, IL-8 production in macrophages was also shown to be induced by oxLDL via activation of TLR4 (363). Activation of monocyte/macrophage TLR4 by oxLDL thus appears to be a central mechanism underlying oxLDL's pro-inflammatory actions in ATH.

Similarly, to monocyte/macrophages, which can assume a pro-inflammatory "M1-like" and anti-inflammatory "M2-like" phenotype, MSCs can also adopt both pro- and anti-inflammatory characteristics. Interestingly, such MSC polarization is believed to occur via TLR-dependent mechanisms, with TLR4-primed MSCs developing pro-inflammatory characteristics (159). Thus, it is conceivable that in the context of ATH, prolonged oxLDL stimulation of TLR4 in elderly MSCs drives these cells towards the pro-inflammatory (MCP-1/IL-6/IL-8-secreting) phenotype observed in our work. Moreover, it has been shown that TLR4 activation in MSCs reduced their reparative capacity (364). Therefore,

targeting TLR4 or TLR4 signaling prior to MSC transplantation should be tested as a way of improving their therapeutic potential.

Knowing that MSCs from elderly ATH patients have a pro-inflammatory secretome and reduced immunopotency, I tested the contribution of mitochondrial dysfunction and ROS-stimulated production of inflammatory factors (Chapter 4). By comparing MSCs from ATH patients to healthy counterparts, I demonstrated that MSCs from atherosclerotic individuals have mitochondrial dysfunction defined by reduced OXPHOS potential and ATP production which in turn leads to excessive intracellular ROS production. In order to prevent overproduction of ROS and ultimately apoptosis, these cells switch their metabolism from OXPHOS to glycolysis. Although using glycolysis as the main energy production pathway protects ATH MSCs from apoptosis, it also results in a pro-inflammatory secretome and impaired immunomodulatory function. Moreover, reducing intracellular ROS levels in these cells by using mitochondrial ROS-specific antioxidants improved their immunomodulatory function.

Mitochondrial dysfunction is implicated in both circulating cells (e.g. leukocytes) and atherosclerotic plaque cells (e.g. VSMCs and ECs). Moreover, increased oxidative stress due to impaired mitochondrial function has been shown to induce double-stranded DNA breaks promoting plaque progression and instability due to cell senescence, apoptosis, and inflammation in VSMC. Further support for the role of ROS in ATH comes from experimental models of ATH.

Deficiency of antioxidant enzymes glutathione peroxidase and manganese superoxide dismutase (SOD2) have been shown to promote progression of atherosclerotic plaque development by increasing mitochondrial oxidative stress and DNA damage on the cells

in atherosclerotic plaques. In addition, excessive oxidative stress has been found to promote calcification in atherosclerotic plaques [reviewed in (365)] .

A metabolic shift toward aerobic glycolysis has been correlated with a pro-inflammatory phenotype in immune system cells, including macrophages, dendritic cells and T cells (207). Moreover, manipulating this switch resulted in controlling cellular inflammatory responses. HIF1α is a key transcription factor orchestrating this metabolic switch via the expression of glycolytic enzymes. Under hypoxic conditions, HIF1α is known to be induced through a posttranslational-dependent mechanism. However, under normoxic conditions, NF-κB signaling is known to upregulate HIF1α in activated innate immune cells (366). Through hypoxic pre-conditioning, the role of HIF1α was extensively studied in MSCs. It has been implicated in increased migration, proliferation and reduced apoptosis. In addition, a hypoxic microenvironment has been shown to reduce adipogenic differentiation and promotes osteogenic lineage commitment via HIF1α-dependent mechanisms [reviewed in (367)] . However, all these studies evaluated the HIF1α effect under hypoxic conditions. In our study, MSCs from atherosclerotic patients expressed higher levels of HIF1α under normoxic conditions. Furthermore, these MSCs shift their metabolism towards glycolysis in a HIF1α-dependent manner. In line with our findings, a recent study by Killer *et al.* showed a positive correlation between metabolic activity and immunosuppressive ability and suggests using metabolic profiles to determine the 'functional fitness of MSCs' (368). Our work adds to these findings and further suggests that the baseline metabolic activity of MSCs could be a surrogate of their clinical efficacy. While OXPHOS-dependent ATP production predicts a better immunosuppressor MSCs, a glycolytic switch under normoxic conditions predicts a pro-inflammatory phenotype.

Recent studies showed a link between micro RNAs and HIF1α stabilization. miR-21 levels have been shown to be upregulated after ROS exposure (i.e. H_2O_2) in a dose dependent manner, leading ultimately to downregulated HIF1α expression. Moreover, miR-21 been found to be the most abundant miRNA in atherosclerotic plaques and absence of miR-21 in hematopoietic cells has been shown to promote ATH progression through promoting foam cell formation, inducing pro-inflammatory phenotype in plaque macrophages and their apoptosis (369). The effect of miR-21 in MSCs remains elusive. However, studies demonstrated a possibility of modifying MSC activity through modulation of other miRs in MSCs prior to their transplantation. Thus, it remains to be tested whether the overexpression of miR-21 in ATH-MSCs could enhance the therapeutic potential of MSCs from individuals with ATH or other chronic inflammatory conditions.

In summary, my work lead to novel contributions in the field of MSCs of relevance to human ATH; provided insights on the possible mechanistic defects of the MSCs immunomodulatory function in the context of chronic inflammation; uncovered the link between mitochondrial dysfunction and the pro-inflammatory signature of atherosclerotic MSCs; and provided the foundation to consider targeting the functional impairments of ATH-MSCs as a strategy to improve the outcome of autologous MSCs transplantation.

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