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EFFECTS OF OBESITY ON ADIPOSE STROMAL/STEM CELL IMMUNOMODULATION AND MITOCHONDRIAL RESPIRATION CAPACITY

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ABSTRACT

Amna Adnan: Effects of obesity on adipose stromal/stem cell immunomodulation and mitochondrial respiration capacity
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Background and aims: Adipose tissue (AT) derived adipose stem/stromal cells (ASC) are multipotent cells. They not only possess excellent proliferation and differentiation capacity but are also known for their low immunogenicity and the ability for immunomodulation. Obesity is becoming increasingly a worldwide health concern causing low-grade chronic inflammation in AT. Macrophages are known to keep the tissue homeostasis. Obesity-induced inflammation not only disturbs the tissue-resident macrophage ratio but also affects the ASC functions. Metabolic dysfunction of AT has been previously studied but the effect of obesity-related inflammation on ASC functions is still not clearly known. This study aimed to investigate the effect of obesity on ASC immunomodulatory capacity, especially macrophage polarization and cytokine secretion. Also, the aim was to study the effect of donor weight and *in-vitro* inflammatory environment on the mitochondrial respiratory capacity of ASC.
Materials and methods: ASC were isolated from human AT samples that were obtained from two donors undergoing bariatric surgery, before and after weight loss. Monocyte derived macrophages were polarized from frozen PBMCs (peripheral blood mono nuclear cells) into pro-inflammatory (M1), anti-inflammatory (M2), and regulatory macrophages (Mreg). These three types of macrophages were cultured in mono and co-culture with ASC collected from two different donors before and after weight loss. Macrophage polarization was studied with light and fluorescent microscopy, immunophenotype was analyzed with flow cytometry analysis and the amount of cytokine secreted in mono and co-cultures with ASC. Metabolic capacity of ASC collected from lean and obese donors was measured by quantifying the mitochondrial respiration capacity of these cells at two different time points.
Results: M1, M2 and Mreg macrophages with characteristic morphology, phenotype, and cytokine secretion were polarized from frozen PBMCs. M1 macrophages showed high expression of CD86, CD11c and HLA-DR, M2 showed high expression of CD206, CD163 and CD86 while Mreg macrophages showed decreased expression of above-mentioned markers. The mono and co-cultures of M1 and Mreg macrophages secreted pro-inflammatory cytokines. The co-cultures with obese derived ASC secreted more pro-inflammatory cytokines, particularly IL-6, IL-12p70 and MCP-1. Moreover, the cellular respiratory capacity of ASC increased after the weight loss.
Conclusion: Our study showed that different macrophage types can be polarized from frozen PBMCs *in vitro.* It was studied that Mreg macrophages have both regulatory and pro-inflammatory nature. Also, it was observed that secretion of proinflammatory cytokines particularly IL-6, IL-12p70 and MCP-1 was significantly high in ASC obtained from obese donors compared with M1 monocultures and the immunosuppressive capacity of ASC was independent of donor weight. Moreover, it was observed that ASC after weight loss have increased cellular respiration capacity and inflammatory environment rapidly increases the cellular respiration capacity of ASC *in vitro*. Although this study showed some insight of ASC immunomodulation, additional co-cultures of macrophages with higher number of ASC obtained from obese and lean donors should be carried out with optimized study design that will provide further insight of obesity induced inflammation and its effects on immunomodulatory functions of ASC.

Keywords: adipose stem cells, macrophages, adipose tissue, inflammation, cytokines

PREFACE

Resuming studies after over a decade gap was not the easiest undertaking. However, reflecting on this immensely rewarding experience makes me happy to have made this choice which has truly helped me to develop as a person, both professionally and personally. However, it was not possible without the inspiration and motivation of several people who supported me along this journey to whom I feel deeply indebted.

This master thesis was conducted in Adult Stem Cell group, Faculty of Medicine and Health Technology, Tampere university.

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Furthermore, I want to thank my parents and my friends. Thank you for all the encouragement and prayers provided. A special thanks to my husband Adnan Zia for continuously inspiring and supporting me during the master studies. Most importantly, big thanks to my kids for providing endless energy and cheerful moments to keep me going on in this journey until it ends.

Tampere, 23 April 2021

Amna Adnan

CONTENTS

LIST OF SYMBOLS AND ABBREVIATIONS

ASC	adipose derived stem cells
AT	adipose tissue
BM	basic medium
BMI	body mass index
BM-MSCs	bone marrow derived mesenchymal stem cells
BSA	bovine serum albumin
CCR2	C-C chemokine receptor 2
CD	cluster of differentiation
CFU-F	colony forming units
DAPI	4',6-diamidino-2-phenylindole dihydrochloride
DPBS	Dulbecco's phosphate buffered saline
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
GM-CSF	granulocyte/macrophage colony stimulating factor
HIF-1	hypoxia inducible factor 1
IFN-ɣ	interferon gamma
IL-12	interleukin-12
IL-13	interleukin-13
IL-18	interleukin-18
IL-1ß	interleukin-1 beta
IL-4	interleukin- 4
IL-6	interleukin-6
ISCT	international Society for Cellular Therapy
lnASC	lean adipose stem cells
LPS	lipopolysaccharides
M1	proinflammatory macrophages
M2	anti-inflammatory macrophages
MCP-1	monocyte chemoattractant protein-1
Mreg	regulatory macrophages
MSCs	mesenchymal stromal/stem cells
obASC	obese adipose stem cells
OCR	oxygen consumption rate
P/S	penicillin/streptomycin
PBMCs	peripheral blood mononuclear cells
PFA	paraformaldehyde
Phalloidin-TRITC	phalloidin-tetramethyl rhodamine B isothiocyanate
rhu M-CSF	recombinant human macrophage colony stimulating factor
ROS	reactive oxygen species
SVF	stromal vascular fraction
T2DM	type 2 diabetes
TARC	tthymus- and activation-regulated chemokine
TNF-α	tumor necrosis factor- alpha
WAT	white adipose tissue
WHO	world health organization
αMEM	alpha Minimum essential medium

1. INTRODUCTION

Adipose stromal/ stem cells (ASC) derived from adipose tissue (AT) are markedly promising candidates for various clinical applications, not only for their remarkable proliferation and differentiation capacity but also for their low immunogenicity and the ability for immunomodulation (Patrikoski et al. 2019). By contributing to balancing the immune system, they secrete anti-inflammatory cytokines which suppress the pro-inflammatory milieu (Chu et al. 2019).

Major worldwide health concern is an increasing frequency of obesity. Excessive weight gain among individuals causes a rise in related health complications e.g., cardiovascular diseases, cancers, and diabetes (Wang et al. 2011). Obesity is defined based on body mass index (BMI), which is the weight (kg) divided by height squared (m^2). According to the world health organization (WHO), a BMI 25 -29.9 kg/m2 is considered overweight in adults, while BMI equal to 30 or higher is defined as obese (www.who.int, page visited: 15.04.20). In the United States, the percentage of obesity was 42.4% among adults independent of gender in 2017-2018 (Hales et al. 2020). Correspondingly, in Finland, 28% of women and 26% of men are obese in age over 30 (www.thl.fi, page visited: 03.12.20). The complications caused by obesity are becoming a major public health burden that will exacerbate in the coming years (Saltiel & Olefsky 2017).

Metabolic homeostasis in the human body is maintained by the metabolic organs e.g., liver, pancreas, and AT comprising parenchymal and stromal cells. Macrophages are the key players that control tissue homeostasis. In lean individuals AT homeostasis is maintained by anti-inflammatory M2 like macrophages, which prevents inflammation and promotes insulin sensitivity (Sharma et al. 2018). During inflammation, the fate of an organ is governed by the balance between pro-inflammatory M1 and M2 macrophage polarization (Moghaddam et al. 2018). A chronic low amplitude inflammation linked to the metabolic disorder in the AT during obesity is called *meta-inflammation* (Lumeng 2013). Metabolic dysfunction of adipocytes is the primary event leading to chronic inflammation in AT (Meijer et al. 2011). Chronic nutrient excess causes the expansion of AT and dysfunction of adipocytes (Spalding et al. 2008).

During obesity, there is a rapid and transient induction of adipocyte proliferation, particularly in the visceral AT. When the AT expands, there is a marked change in its microenvironment causing increased mechanical and oxidative stress-inducing hypoxia. This altered microenvironment of AT causes adipocyte death. The dysregulated death of adipocytes induces macrophage infiltration to the AT and the direct interaction of dead cells with the macrophages further induces them towards the pro-inflammatory phenotype (Kuroda & Sakaue 2017). Furthermore, hypoxia upregulates the secretion of inflammatory adipokines which inhibits the differentiation of preadipocytes into adipocytes and transforms them into leptin-secreting cells (Trayhurn 2013).

Similarly, mitochondria are multi-functional organelles responsible to conduct a wide range of cellular responses. Mitochondria is the powerhouse of the cell. Its essential role is oxidative phosphorylation in cellular energy production and generation of reactive oxygen species (ROS) (Wallace 1999). They can also modulate innate immune responses in several physiological disorders e.g., autoimmune diseases, metabolic syndrome, neurodegeneration, and cancer. They engage immune cells to keep normal tissue homeostasis. (West 2017). Any mitochondrial damage can trigger inflammation and promotes pathology. Also, mitochondria are important players in keeping the metabolic homeostasis of the white adipocytes in the AT. They are important factors in the synthesis of adiponectin from the adipocytes (Woo et al. 2019). Any imbalance of the mitochondrial function in adipocytes highly affects the metabolic system and causes AT inflammation. Many studies have been done on the metabolic dysfunctions in relation to obesity. However, questions related to AT inflammation and its effects on ASC function especially concerning human ASC are still unknown.

In this thesis, the effects of obesity have been studied on the immunomodulatory and mitochondrial respiratory capacity of ASC from two different donors before and after weight loss. Peripheral blood mononuclear cells (PBMCs) were polarized towards three different types of macrophages i.e., proinflammatory M1, anti-inflammatory M2, and regulatory macrophages Mreg and co-cultured with ASC from two different donors before and after weight loss. Moreover, the cellular respiration capacity of lean and obese derived ASC was studied under inflammatory and standard culture conditions. Based on the previous research work, we hypothesize in this study that obesity-induced inflammation will influence different functions of ASC. It will not only influence the immunomodulatory capacity of these highly regenerative cells but will also reduce their cellular respiration capacity. We expect that ASC obtained from the obese donors before weight loss will possess less immunosuppressive properties and low mitochondrial respiration compared with ASC obtained from the lean donors after the weight loss.

2. LITERATURE REVIEW

2.1 Obesity

2.1.1 Definition, statistics, and related disorders

One of the most serious global health concerns is the prolific occurrence of obesity. Excessive weight gain can cause several major disorders, referred to as metabolic diseases, e.g., type II diabetes mellitus (T2DM), cancer, cardiovascular diseases, and musculoskeletal disorders (Engin 2017). Obesity is defined based on body mass index (BMI), which is the weight (kg) divided by height squared (m^2). According to the world health organization (WHO), a BMI 25 - 29.9 kg/m2 is considered overweight in adults, while BMI equal to 30 or higher is defined as obese (https://www.who.int, Page visited: 150420). Children and adolescent's obesity are defined considering their age and sex (Apovian 2016). In 2020, the WHO reported that the percentage of obesity had increased threefold since 1975. In 2016, 13% of world's adult population was obese and the trend is increasing. Moreover, Hales et al. have shown that in the United States, the percentage of obesity was 42.4% among the adults independent of gender in 2017-2018 (Hales et al. 2020). If this increase continues at the same rate, the percentage of the obese individuals will constitute 20% world's population by 2030 (Singh et al. 2016).

Furthermore, common comorbidities of obesity are type 2 diabetes, coronary heart disease, stroke, dyslipidemia, osteoarthritis, hypertension, and some cancers. Patients with class III obesity, i.e., BMI≥40kg/m2, have increased risks of diabetes, hypertension, asthma, hyperlipidemia and arthritis (Apovian 2016).

2.1.2 Adipose tissue, link between obesity and inflammation

Obesity-linked inflammation is mainly localized in the adipose tissue (AT), which in turns promotes chronic low amplitude inflammation linked to metabolic disorder also known as *meta-inflammation* (Lumeng 2013). Metabolic inflammation is described as chronic low-grade inflammatory events engineered by metabolic cells in response to excess nutrients and energy (Grego r et al.2011). Metabolic inflammation is a characteristic of low-level local or systemic inflammatory responses occurred during metabolic disorders (Tilg et al. 2019).

The AT is a highly heterogenous organ. In addition to its heterogeneity, it consists of different tissue types i.e., brown AT, white AT, and brown in white AT. It is present at

multiple places throughout the human body e.g., subcutaneous, visceral, epicardial (Lina & Judit 2017). AT is considered as mini organ having autonomous characteristics and functionality (Tchkonia et al. 2007). The plasticity of AT i.e., the structural, cellular, and molecular characteristics can be directly affected by physiological and pathological conditions. For example, obesity induces impaired adipogenesis, disrupted ECM homeostasis, and limits the AT plasticity (Pellegrinelli et al. 2016). Obesity is one of the most determining condition that can lead to dysfunctional AT. Moreover, resident immune cells in the AT play a critical role in keeping the normal tissue homeostasis, removes detritus and apoptotic cells under normal physiological conditions. However, the excess accumulation of fat tissue increases the number and performance of immune cells, e.g., macrophages, neutrophils, B and T lymphocytes (Figure 1) (Cildir et al. 2013). This inflammation in AT is considered as a core mechanism in connecting obesity with various vascular and metabolic complications.

Also, the milieu of AT during obesity becomes highly inflammatory due to the active releases of inflammatory cytokines by the pro-inflammatory macrophages, which promotes insulin resistance and further polarizes the macrophages towards the pro-inflammatory type (Sharma et al. 2018).

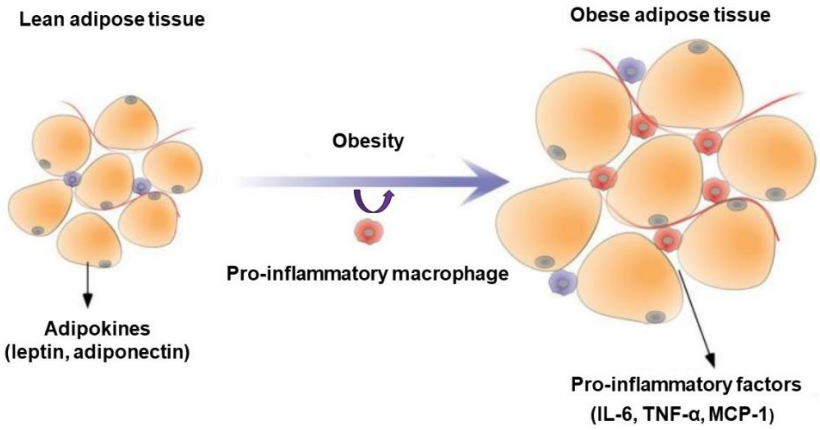

Figure 1. *AT modulation with the inflammation. From Odegaard & Chawla 2011 (Figure modified)*

2.2 Adipose stem/stromal cells

Adult stem cells are the undifferentiated cells located in a differentiated organ in a specialized microenvironment called a niche which helps their growth and regulation. These cells are clonogenic and multipotent in nature (Gurusamy et al. 2018). They have been identified in different tissues e.g., blood, skin, muscle, and heart, etc. The structural and functional regenerative capacity of adult stem cells such as bone marrow-derived mononuclear cells, hematopoietic stem cells, mesenchymal stromal/stem cells (MSCs), and

endothelial precursors have been illustrated with several clinical and preclinical studies. Further, MSCs are multipotent cells exhibiting self-renewal and differentiation capacity (Figure 2). They possess an ability to migrate towards the sites of injury, inflammation, and tumor (Mishra & Banerjee 2017). In addition to multipotent, they also acquire immunomodulatory functions that have been utilized in the treatment of various immune disorders (Li & Hua 2017). They have the capacity to interact vigorously with cells of adaptive and innate immune system either through direct cell-cell contact or through their paracrine activity.

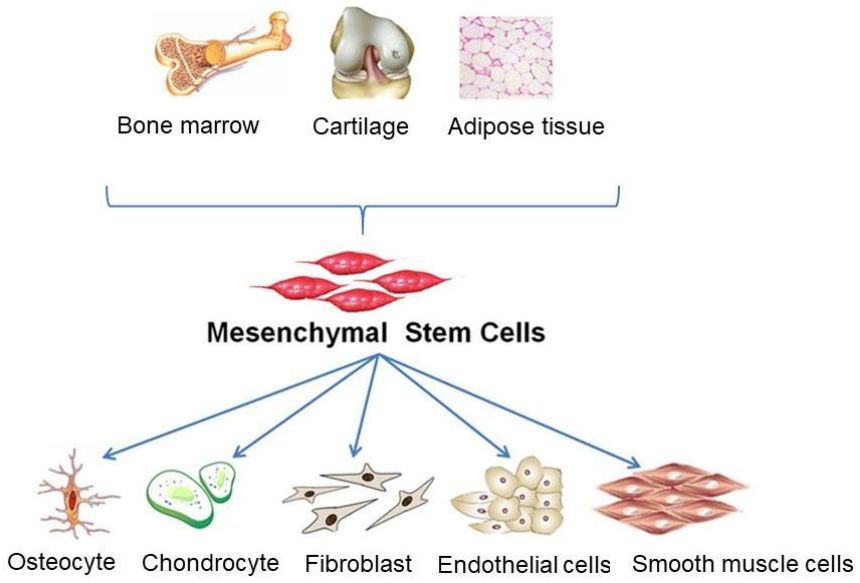

Figure 2. *A multilineage differentiation potential of MSCs from different sources. From Xie et al. 2016 (Figure modified)*

Adipose stromal/ stem cells (ASC) derived from AT are markedly promising candidates for various clinical applications, not only for their remarkable proliferation and differentiation capacity but also for their low immunogenicity and the ability for immunomodulation (Patrikoski et al. 2019). ASC have the potential to differentiate into various mesodermal lineages of myoblast, chondroblast, osteoblast, and adipocytes. These multi-lineage stem cells can be utilized in several tissue engineering and regenerative medicinal applications (Huang et al. 2013). The ASC can be isolated from AT by washing extensively and digesting with collagenase (Figure 3) (Zuk et al. 2001). The digested tissue is then centrifuged to obtain stromal vascular fraction (SVF). After centrifugation, the supernatant is discarded, and the SVF pellet is incubated with red blood cell lysis buffer and centrifuged to remove cellular debris. Plastic adherent ASC are obtained by culturing the SVF.

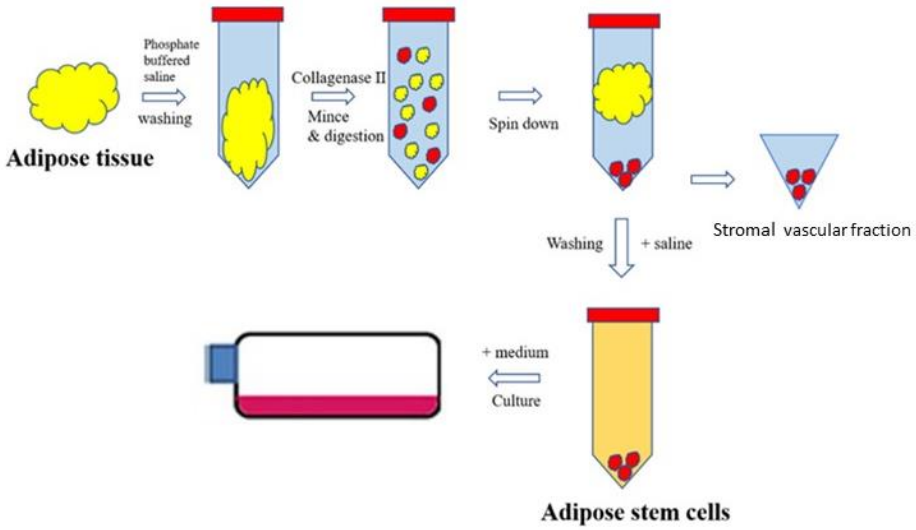

Figure 3. *Enzymatic isolation of ASC from AT. From Chu et al. 2019 (Figure modified)*

Moreover, AT is a greater reservoir of MSCs than any other organ. Generally, 1 × 10^7 ASC can be isolated from 300 ml of lipoaspirate with a purity greater than 95% (Mizuno et al. 2012). In other words, the average yield of ASC from lipoaspirate is 5000 colony forming units (CFU-F) per gram of AT compared to 100-1000 CFU-F per milliliter of bone marrow. Also, ASC are highly heterogeneous population of cells, features include differentiation potential, immunophenotype, proliferation capacity, and the secretome. There are several factors that affect their heterogeneous behavior e.g., donor sex, BMI, age, and clinical conditions; site and process of sampling; culture surface and the type of medium (González 2019). Additionally, Choudhery and fellows found that ASC obtained from younger donors (less than 40 years) have great viability, differentiation, and proliferation capacity (Choudhery et al. 2014). Furthermore, according to the International Society for Cellular Therapy (ISCT), ASC are defined based on their plasticity, adherence, undifferentiated state maintenance, self-renewal ability, multipotency, and multi-lineal differentiation (Sovrea et al. 2019). Also, there are no definitive markers for ASC, but they express differentiation antigens (CD): CD10, CD13, CD29, CD34, CD44, CD49d, CD73, CD90, CD105 and CD166 while the cells do not express CD11b, CD14, CD19, CD31, CD40, CD40L, CD45, CD79a, CD80, CD86 and CD106 (Bourin et al. 2013 & Dominici et al. 2006).

2.2.1 Adipose stem/stromal cell immunomodulation

The anti-inflammatory potential of ASC helps to regulate the immune system. For this reason, they are used as immunomodulators in regenerative medicine. They contribute to keep the balance of the immune system by regulating T cells as well as secretion and

activity of interleukin - 10 (IL-10) (Chu et al. 2019). ASC possess immense immunomodulatory, predominately anti-inflammatory capacity when comes in direct contact with immune cells. They can skew the inflammatory macrophages (M1) towards anti-inflammatory macrophages (M2) (Stojanovic & Najman 2019). ASC normally secrete different cytokines, chemokines, and growth factors regardless of the need and provided an inflammatory signal. The reverse of macrophage phenotype depends greatly upon the inflammatory microenvironment in which ASC polarizes the phenotypes of macrophages from pro to anti-inflammatory. The capacity to modulate immune responses is one reason for the success of using ASC in the treatment of different inflammatory diseases and autoimmune disorders.

2.2.2 Effects of inflammation on plasticity and ASC function

Inflammation induces drastic changes in the ASC. It is considered that ASC differentiation and plasticity are highly affected by inflammation. Metabolic disturbances activate adipocyte hypertrophy and hyperplasia through the elevation of pro-inflammatory cytokines. The direct influence of inflammation on ASC plasticity affects their proliferative and differentiation potential (Badimon & Cubedo 2017). The changes in differentiation and proliferation capacity have an obvious impact on ASC functionality altering their regenerative capacity. Additionally, obesity-induced inflammation limits the stemness of ASC, making their regenerative capacity impaired (Onate et al 2013). ASC in a niche of subcutaneous white adipose tissue (WAT) of obese individuals commit themselves to adipocyte differentiation showing upregulation of inflammatory gene expression related to the loss of their stemcellness. Also, different studies have shown contradictory results regarding the adipogenic capacity of ASC in the inflammatory environment.

Furthermore, from previous studies it has been clear that inflammation in AT during obesity contributes to the development of insulin resistance and T2DM. In the patients with T2DM, preadipocytes reduced the expression of genes involved in adipogenic differentiation (Van Tienen et al. 2011). The decreased expression of adipogenic and lipogenic genes was linked with the decreased insulin sensitivity in the subcutaneous AT from obese women (Goedecke et al. 2011). These findings markedly show that metabolic dysfunction and inflammatory environment affect the differentiation potential of ASC and their ability in maintaining the tissue repair and homeostasis turning them into proinflammatory stimulators (Louwen et al. 2018). Furthermore, increased senescence and increased adipogenic and osteogenic differentiation of ASC was observed in an obese animal model (Badimon & Cubedo 2017). On the other hand, lower adipogenic and osteogenic proliferation was studied in obese individuals. Inflammation also decreases the

ASC stemness in obese individuals with a long term pro-inflammatory environment (Figure 4).

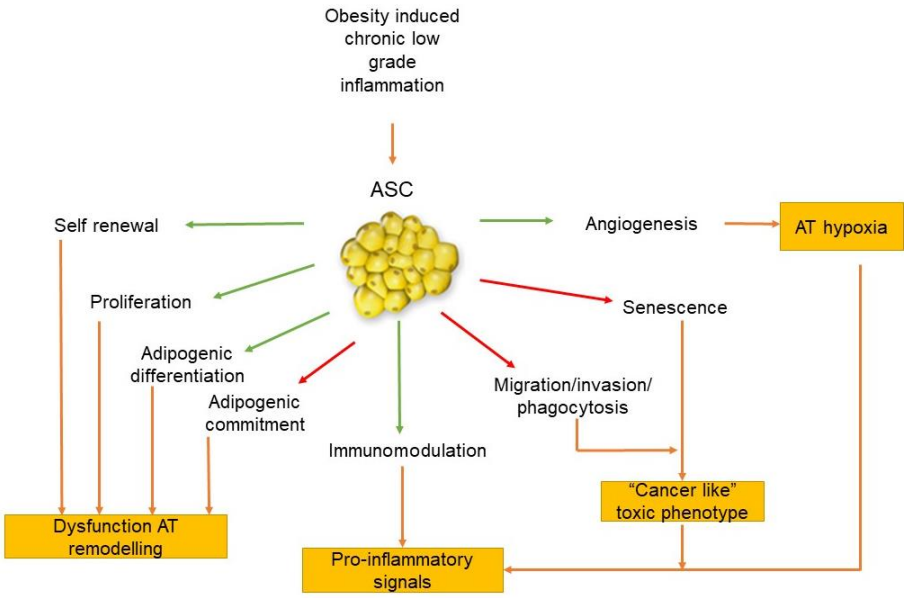

Figure 4. *Impact of the proinflammatory state on ASC properties. Red arrows indicate enhanced and green arrows indicate weakened properties. From Badimon & Cubedo 2017 (Figure modified)*

2.2.3 Utilization of ASC in clinical cell therapy

Stem cell therapy has shown immense advantages in the treatment of a variety of disorders, including diseases related to the immune system (Bateman et al. 2018). In the past decade, researchers have developed several applications of ASC for clinical use. Earlier in 2009, cosmetic surgery was the major treated application. Later in 2010, the use of ASC and SVF increased tremendously. The therapeutic potential of ASC has been experimentally observed for many disorders skeletal regeneration, facial reconstruction, neurological, autoimmune, urological, cardiovascular, and immunological diseases. Several ASC-based clinical trials are undergoing worldwide e.g., in Europe, Mexico, Korea, and the United States (Sovera et al. 2019).

In May 2020, a total of 367 clinical trials evaluating the potential of ASC for treating different types of diseases were found at http://www.clinicaltrials.gov (Table 1). The most common stem cell therapies were the treatment of joint disorders e.g., osteoarthritis, also the treatment of gastrointestinal disorders, such as Crohn's disease, as well as facial injuries and wound scars.

Table 1. *Examples of multiple Adipose stem cell clinical trials treating different diseases in different phases. From clinicaltrials.gov (Page visited on 25th April 2020)*

Title	Status	Conditions	Interventions	Location	Phase
Autologous AT Derived MSCs Therapy for Patients with Knee Osteoarthritis	Completed	Knee osteoarthritis	Biological: Mesenchymal stem cells low-dose group	China	1
Allogeneic Stem Cell Therapy in Heart Failure	Active, not recruiting	Heart Failure	Biological: Cardiology Stem Cell Centre Adipose Stem Cell (CSCC_ASC)	Denmark	2
Adipose Derived Stem Cells Transplantation for Chronic Obstructive Pulmonary Disease (COPD)	Unknown	COPD	Biological: Adipose derived stem cells	Vietnam	1 & 2
Expandability of Aging AT	Completed	Aging, Obesity	Other: Overfeeding	US	NA
Treatment of Complex Anal Fistulas Using Centrifuged AT Containing Progenitor Cells (CAT)	Completed	Anal Fistula	Drug: ASC (Cx401, company code) Drug: Fibrin adhesive	Italy	NA
A Clinical Trial to Determine the Safety and Efficacy of Hope Biosciences Autologous MSCs Therapy (HB-adMSCs)	Active, not recruiting	Alzheimer Disease	Drug: HB-adMSCs	US	1 & 2

for the Treatment of Alzheimer's Disease					
Accelerated Aging of the Cells of Visceral AT in Morbid Obese Subjects	Unknown	Morbid Obesity		France	NA
ASC for Traumatic Spinal Cord Injury (CELLTOP)	Active, not recruiting	Spinal Cord Injuries Paralysis	Biological: Autologous, Adipose derived Mesenchymal Stem Cells	US	1
The Effects of Capsinoids on Brown AT Activation in Obesity	Recruiting	Weight loss	Dietary Supplement: CapsinoidDietary Supplement: Placebo	US	NA
Effectiveness of Sitagliptin for HIV Insulin Resistance and Inflammation	Completed	Inflammation Macrophage Infiltration Cardiovascular Disease	Drug: Sitagliptin Drug: Placebo	US	3
Reparative Therapy in Acute Ischemic Stroke with Allogenic MSCs from AT, Safety Assessment, a Randomized, Double Blind Placebo Controlled Single Centre Pilot Clinical Trial (AMASCIS-01)	Completed	Ischemic Stroke	Drug: Allogenic mesenchymal stem cells from adipose tissue Drug: Placebo	Spain	2

AT Derived Stem Cell Based Hair Restoration Therapy for Androgenetic Alopecia	Completed	Combination of SVF and PRP for Androgenetic Alopecia	Drug: stem cells Drug: platelet rich plasma	Pakistan	2
A Clinical Trial to Determine the Safety and Efficacy of Hope Biosciences Autologous MSCs Therapy (HB-adMSCs) to Provide Protection Against COVID-19	Enrolling by invitation	COVID-19	Drug: HB-adMSCs	US	2
Myeloid to Adipocyte Trans differentiation in Human Cells	Suspended	Obesity	Adipose Tissue Biopsy	US	
Application of Cell Regeneration Therapy with MSCs from AT in Critical Chronic Ischemic Syndrome of Lower Limbs (CLI) in Nondiabetic Patients.	Completed	Critical Limb Ischemia	Other: Mesenchymal stem cells from adipose tissue	Spain	2
Development of Bone Grafts Using Adipose Derived Stem Cells and Different Scaffolds	Completed	Overweight	Procedure: Cell Culture	Switzerland	Not found

Comparison of Hematopoietic Stem Cell Activity in AT From T2DM Patients and Healthy Volunteers	Completed	T2DM	Procedure: Abdominal subcutaneous biopsies and blood test	France	NA

2.3 Adipose tissue inflammation

2.3.1 Macrophages during inflammation

Inflammation is a complex phenomenon consisting of series of events as reviewed by Fujiwara and Kobayashi (Fujiwara & Kobayashi 2005). These cellular responses are tightly regulated which involves both the initiation and maintenance of its downregulation. Macrophages are the key cells participating in these processes. The three main functions of macrophages are antigen presentation, phagocytosis, and immunomodulation. Macrophages not only play an active role in the host defense mechanism, but they also produce biologically active molecules which serve many active roles in the innate and adaptive immune responses. At the initial stimulus, the resting macrophages convert into activated ones with the help of activation signals which include T lymphocyte-derived cytokines, microbial products, fibronectin, and chemical mediators. In acute inflammation, after performing their tasks macrophages disappear but during chronic inflammation, their accumulation persists for a long time. Macrophages are also involved in the downregulation of inflammation. The components of resolution produced by them include soluble mediators such as anti-inflammatory cytokines, antioxidants, protease inhibitors, and cellular responses. Any unchecked inflammation due to imbalance between these two signals can cause major cellular or tissue damage.

2.3.2 Macrophages in obese and lean AT

Metabolic homeostasis in the human body is maintained by the metabolic organs e.g., liver, pancreas, and AT consist of parenchymal and stromal cells such as macrophages. In lean individuals AT homeostasis is maintained by M2 like macrophages, which prevents inflammation and promotes insulin sensitivity (Sharma et al. 2018).

The fate of an organ during inflammation is governed by the balance between M1 and M2 macrophage polarization (Moghaddam et al. 2018). During infections in lean or normal-weight individuals, activated macrophages release proinflammatory cytokines TNF-α (Tumor necrosis factor-alpha), IL-1ß (interleukin-1 beta), and IL-6 (interleukin-6) which

collectively trigger peripheral insulin resistance to decrease nutrient storage (Wynn et al. 2013). The activated immune cells utilize the glycolysis pathway providing energy sources for the function of host response. Tissue-resident macrophages play important role in such responses. On the other hand, malfunction of these metabolic responses occurs during meta-inflammation. During obesity-induced inflammation, TNF-α, IL-1ß, and IL-6 secreted by AT macrophages act against the action of adiponectin and leptin leading to insulin resistance (Castoldi et al. 2015 & Kalupahana et al. 2012). In obesity, the inflamed milieu disturbs tissue homeostasis. This not only promotes insulin resistance but also increases the macrophage polarization towards M1 phenotype.

2.3.3 Adipocytes and macrophages crosstalk

Activation of meta-inflammation is a major characteristic of obesity. One primary and major component in this respect is the close interaction of adipocytes with macrophages. Here, the metabolic status of adipocytes determines the inflammatory yield of macrophages (Engin 2017). Several factors induce the immense infiltration and accumulation of macrophages in the obese AT. The necrosis of hypertrophic adipocytes regulates the macrophage infiltration in the AT. Galectin-3 a human protein involved in free lipid clearance induces the influx of macrophages at the site of necrotic adipocyte inflammation (Cinti et al. 2005). Its anti-apoptotic behavior also promotes the survival of macrophages during inflammation (Hsu & Liu. 2004). Also, the hypertrophic adipocyte-derived chemotactic monocyte chemoattractant protein-1 (MCP-1) CC chemokine receptor 2 (CCR2) pathway promotes macrophage infiltration in obese AT (Figure 5) (Kanda et al. 2006). These macrophages not only secrete pro-inflammatory cytokines but also affect the gene expression of adipocytes resulting in the insulin resistance. The activation of caspase-1 in the AT of obese animals induces the activation of IL -1 ß leading to insulin resistance. This indicates crosstalk between adipocytes specific caspase-1 and IL-1 ß produced by macrophages in obese AT (Stienstra et al. 2010).

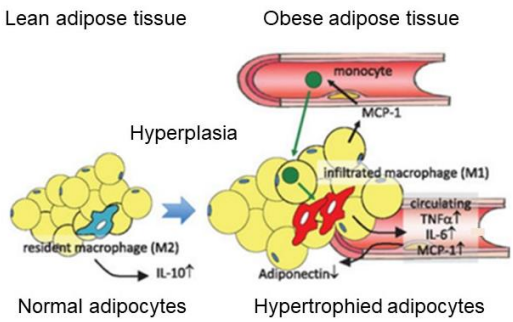

Figure 5. *Macrophages in lean and obese AT. From Tateya et al. 2013 (Figure modified)*

2.3.4 Adipokines and inflammation

AT cytokines, growth factors, and extracellular matrix proteins collectively termed adipokines which keep the AT homeostasis (Unamuno et al. 2018). The inflammation caused by obesity disturbs the adipokine levels in the AT e.g., adiponectin and leptin. Adiponectin is the anti-inflammatory adipokine that in lean individuals promotes the polarization of macrophages towards M2 phenotype. During AT dysfunction, an increase in TNF-α, IL-6, and interleukin-18 (IL-18 secretion) of the pro-inflammatory environment downregulates the adiponectin production from the adipocytes (Engin 2017).

Further, leptin in lean individuals maintains energy homeostasis by inhibiting hunger and diminishing the fat storage in the adipocytes. During the metabolic imbalance of the AT, the size and the number of adipocytes rise, which in turn increases the amount of leptin production. In the immune system leptin affects the shift in AT macrophage immunometabolic profile and mediates the production of pro-inflammatory cytokines TNF-α, interleukin-12 (IL-12), IL-6, and IL-1ß (Monteiro et al. 2019). The pro-inflammatory signals also regulate the leptin receptors. In the microenvironment of obese AT, the increased macrophage polarization towards M1 occurs due to the constant influence of excessive leptin amounts.

2.4 Obesity-related adipose tissue dysfunction

2.4.1 Hypoxia in adipocytes

In the state of obesity, there is a decreased AT oxygen consumption due to the poor blood flow in the AT. Hence in obese subjects, there is 58% lower vascular endothelial growth factor amount and 44% lower capillary density as compared to lean subjects (Engin 2017). These decreased levels cause hypoxia in the tissue affecting many biological functions e.g., angiogenesis, apoptosis, cell proliferation, and inflammation.

During the AT inflammation in the obesity, the size of adipocytes increases inducing hypoxia in white tissue depots. The large size of adipocytes limits the oxygen perfusion especially for those fat cells distant from the capillary network. The hypoxia inhibits the differentiation of preadipocytes to adipocytes and transforms them into leptin-secreting cells (Trayhurn 2013). Hypoxia in AT stimulates the secretion of cytokines e.g., TNF- α, IL-6, interleukin-10 (IL-10), and CCL-2 (MCP-1) causing the infiltration of M1 macrophages. Hypoxia inducible factor 1 (HIF-1) induces upregulation of toll-like receptor 4 (TLR-4) expression in adipocytes which in turn stimulates the inflammatory mediators by

enhancing the response to lipopolysaccharides (LPS). This shows how hypoxia and inflammatory responses are interconnected in the state of obesity.

Also, during obesity, there is a rapid and transient induction of adipocyte proliferation, particularly in the visceral AT. When the AT expands, there is a marked change in its microenvironment including increased mechanical and oxidative stress also causing hypoxia. This altered microenvironment causes adipocyte death during obesity. This dysregulated death of adipocytes induces macrophage infiltration to the AT and the direct interaction of dead cells with the macrophages further aggravates the M1 macrophages (Kuroda & Sakaue 2017). Increased hypertrophy and hyperplasia in the visceral AT eventually led to the expansion of macrophage infiltration to the tissue. Furthermore, Goossens et al also confirm that a state of hyperoxia is also found in the AT during obesity which affects its normal function and leads to systemic inflammation. In humans AT blood flow plays an important role in regulating the AT partial oxygen pressure. In obese individuals having insulin resistance, low AT blood flow exhibits AT hyperoxia due to impaired AT capillarization and reduced oxygen consumption (Goossens et al. 2011).

2.4.2 Inflammation and mitochondrial dysfunction

Mitochondria are multi-functional organelles responsible to conduct a wide range of cellular responses. They modulate innate immune responses in several physiological disorders e.g., autoimmune diseases, metabolic syndrome, neurodegeneration, and cancer. They engage immune cells to keep the normal tissue homeostasis (West 2017). Any mitochondrial damage triggers inflammation and promotes pathology.

Also, mitochondria are important players in keeping the metabolic homeostasis of the white adipocytes in the AT. They are important factors in the synthesis of adiponectin from the adipocytes (Woo et al. 2019). Any imbalance of the mitochondrial function in adipocytes highly affects the metabolic system and causes AT inflammation. Defects in mitochondrial function decrease the fatty acid oxidation in the adipocytes leads to AT hypoxia which in turn triggers inflammation (Heinonen et al. 2015).

In obesity, the inflammatory processes cause the production of ROS causing oxidative stress upon the adipocytes (de Mello et al. 2018). The excessive nutrient uptake leads the mitochondria to release more ATP which at the same time generates excessive ROS. The metabolic imbalance affects the capacity of mitochondria to generate and sustain enough ATP levels which cause the abnormal function of mitochondria. The oxidative stress caused by mitochondrial dysfunction onset many inflammatory responses. This

ultimately affects the normal apoptotic function of adipocytes and aggravates the AT inflammation. There are different pathways initiated by mitochondrial dysfunction that led towards the inflammatory responses (Figure 6).

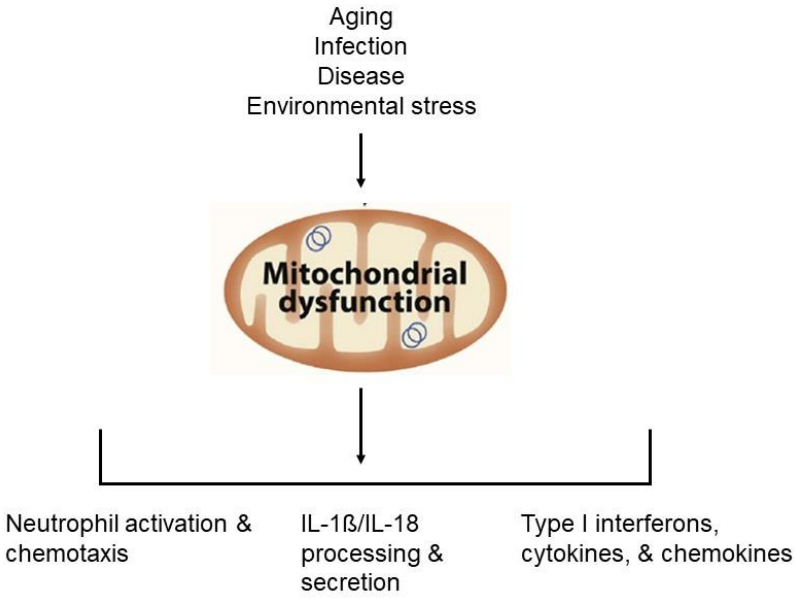

Figure 6. *Mitochondrial dysfunction and onset of inflammatory responses. From West 2017 (Figure modified)*

3. AIMS

The objective of this research was to study the effect of obesity on ASC immunomodulatory and mitochondrial respiratory capacity.

The specific aims of this thesis were to:

Compare the effect of ASC derived from lean and obese conditions from the same donor on macrophage polarization and cytokine secretion.

Study the effect of donor weight and *in-vitro* inflammatory environment on mitochondrial respiratory capacity of ASC.

4. MATERIAL AND METHODS

4.1 Ethical consideration

The collection of AT and peripheral blood was approved by the ethics committee of the Pirkanmaa Hospital District in Tampere (ethical approval R16036). ASC were isolated from AT samples that were obtained with written informed consent from two donors. The first, “obese” ASC were collected from bariatric surgical operations and the second, “lean” ASC after weight loss from AT biopsies in Tampere University Hospital, Finland. Peripheral blood mononuclear cells (PBMCs) were obtained from buffy coat samples obtained from Finnish Red Cross blood service Helsinki. All analyses described below were performed separately with four ASC donor cell lines.

4.1.1 Donor’s characteristics

Below is the data of two different donors, before and after weight loss (Table 2). The data regarding age and height was collected to calculate BMI but is not shown here to protect the privacy of two donors since the donor number is so small.

Table 2. *Characteristical data of two donors at lean and obese state*

No	Gender	Tissue source	Weight (kg)	BMI kg/m2	∆Weight (kg)	∆BMI kg/m2
1.	Female	Subcutaneous	149.8	55.02	77.9	28.61
		Subcutaneous	71.9	26.41		
2.	Male	Subcutaneous	141	39.89	52	14.71
		Subcutaneous	89	25.18		

4.2 Isolation and culture of peripheral blood mononuclear cells (PBMCs)

Before this study, allogenic human PBMCs cell line 2/17 was isolated from buffy coat sample by density gradient centrifugation using Ficoll-Paque PLUS (density 1.077 g/mL:

GE Healthcare, U.K) according to the manufacturer's instruction. After isolation, the cells were aliquoted and cryopreserved in the nitrogen gas phase.

4.3 Adipose stem cell culture

Cell lines of ASC from both donors were isolated and cryopreserved earlier the study. This was performed by mechanical and enzymatic procedure described previously by Zuk and co-workers (Zuk et al. 2001). Lean (ln) and obese (ob) derived ASC cell lines isolated and cryopreserved earlier were cultured and expanded in T175 polystyrene flask (Nunc, Denmark) in Alpha Minimum Essential Medium (αMEM) (Life Technologies™, Thermo Fisher Scientific, USA) supplemented with 5 % human serum (human serum type AB male; Biowest, France), 1 % L-Glutamine (GlutaMAX I; Life Technologies™, Thermo Fisher Scientific, USA), 1 % antibiotics (p/s; 100 U/mL penicillin, 100 U/mL streptomycin, Lonza, Switzerland). The medium was changed two times a week until the cells reached confluency or until required cell number was reached. The experiments with ASC from donor 1 were made at passage 4 and ASC from donor 2 were made at passage 2.

4.4 Flow cytometric surface marker expression analysis for ASC

Flow cytometry is a laser-based technique which provides quick and multi parametric study of isolated cells in solution (McKinnon 2018). It is used to measure the physical and chemical characteristics of cells and their organelles labelled with variety of fluorescent dyes and monoclonal antibodies. Lasers are used as light sources by flow cytometers, producing both scattered and fluorescent light signals which are then read by detectors. As the cell population flows in front of the light source it can be studied based on its fluorescent and light scattering properties (Givan 2011).

Flow cytometry of lnASC and obASC from donor 1 was performed at passage 3 while that of donor 2 at passage 5-6. ASC were harvested and analysed by flow cytometry (fluorescence-activated cell sorting; FACS) (FACS Canto®; BD Biosciences, Belgium). Below are the utilized monoclonal antibodies (Table 3). Analysis was performed on 10, 000 cells per sample and the level of fluorescence greater than 99% of the corresponding unstained cell sample was defined as positive expression.

Table 3. *Surface markers for ASC*

Surface Marker	Manufacture information	Antigen
CD14	BD Bioscience USA	Serum lipopolysaccharide binding protein
CD19	BD Bioscience USA	B lymphocyte-lineage differentiation antigen
CD34	ImmunoTools GmbH Germany	Sialomucin-like adhesion molecule
CD36	BD Bioscience USA	Scavenger receptor class B member 3 (SCARB3)
CD45RO	BD Bioscience USA	Leukocyte common antigen
CD54	BD Bioscience USA	Intercellular adhesion molecule
CD73	BD Bioscience USA	Ecto-5' nucleotidase
CD90	BD Bioscience USA	Thy-1 (T cell surface glycoprotein)
CD105	R&D Systems USA	SH-2 endoglin
HLA-DR	ImmunoTools GmbH Germany	Major histocompatibility class II protein
CD106	BD Pharmingen USA	Vascular cell adhesion protein 1 (VCAM1)
CD142	BD Bioscience USA	Platelet tissue factor, factor III
CD146	BD Bioscience USA	Melanoma cell adhesion molecule (MCAM)

4.4.1 Addition of interferon gamma to lnASC and obASC

lnASC and obASC were cultured in basal RPMI-1640(SIGMA®, Life Science) medium supplemented with 10ng/ml interferon gamma (IFN-ɣ) (SIGMA®, Germany), 10% Fetal Bovine Serum (FBS) (SIGMA®, Germany) and 1% Penicillin/Streptomycin (P/S) for 48hrs prior to co-culture with macrophages. T-175 polystyrene flask (Nunc™, Thermo Fisher Scientific, USA) was used for expansion of ASC prior to co-culture.

4.5 Macrophage polarization assay

Three different types of macrophages were polarized from PBMC 2/17. A total of 2 million cells in basal RPMI-1640 medium were seeded in each well of 12 well plate (Nunc™, Thermo Fisher Scientific, USA). Cells were incubated for 2hrs at 37°C, 5% CO_2. Cells were washed with warm Dulbecco's Phosphate Buffered Saline (DPBS) (Lonza™ Bio-Whittaker™, USA) and polarization media was added to each well according to the plate layout (Table 4). Plates were incubated for 6 days at +37 °C, 5% CO_2. On 6th day the polarization media was replaced with warm activation media (Table 4).

Plates were incubated for 24hrs at +37 °C, 5% CO_2. Next day 80,000/well (22,900/cm^2) lnASC and obASC in each activation media, were added to each condition separately and incubated +37°C, 5% CO_2. Cells were incubated further for 3 days. Activation media from each condition was also utilized as a control condition to study the cytokines release. Mono and co-cultures of macrophages were detached with TrypLE™ express enzyme (1X) (Gibco™) with the help of cell scrapper (USA Scientific Inc).

Table 4. *Composition of polarization and activation media for macrophage polarization assay*

Cell type	Polarization media	Activation media
Mreg	5ng/ml recombinant human macrophage colony stimulating factor (rhu M-CSF) (PromoCell®), 10% FBS in RPMI + GlutaMAX (GlutaMAX™, Gibco by Life Technologies™)	25ng/ml INFγ, 10 ng/ml lipopolysaccharide from E. coli (LPS) (SIGMA®) and 5 ng/ml M-CSF/10 % FBS in RPMI + GlutaMAX
M1	50ng/ml recombinant human granulocyte/macrophage colony stimulating factor (GM-CSF) (PromoCell®), 10% FBS in RPMI + GlutaMAX	50ng/ml INFγ, 10 ng/ml LPS and 50 ng/ml GM-CSF, 10 % FBS in RPMI + GlutaMAX
M2	50ng/ml rhu M-CSF, 10% FBS in RPMI + GlutaMAX	50ng/ml rhu M-CSF, 10% FBS in RPMI + GlutaMAX

4.6 Flow cytometric surface marker expression analysis of macrophages

The immunophenotype of M1, M2 and Mreg macrophages in monoculture and their co-culture with lnASC and obASC was studied with multicolor flow cytometric analysis (fluorescence-activated cell sorting; FACS) (FACSAria®; BD Biosciences, Belgium). Utilized monoclonal antibodies were against CD86-PE (R&D System Inc), HLA-DR-FITC, CD163-PE-CF594, CD90-APC (BD Bioscience, USA); CD206-BV421, CD11C-PECy (BD Bioscience, Belgium). The compensation was performed with BD™ CompBead Plus beads (BD Biosciences) and FVS compensation with Arc™ Amine Reactive Kit (Invitrogen™). The raw data was analyzed using FlowJo™ software (BD Biosciences).

4.7 Cytochemical staining

The cell morphology of ASC was examined by light microscopy imaging with Nikon TS100 light microscopy unit and Nikon DS-5M-L1 camera with 10x air objective (Nikon, Japan). Morphological characteristics of macrophages in mono and co-cultures with ASC were observed with Olympus IX51 (Olympus Corporation of the Americas, PA, USA). First, the culture medium was removed from the wells and samples were rinsed once with 1 × PBS, fixed (4% PFA for 15–30 min, RT, dark), and rinsed three times with 1 × PBS. The permeabilization was performed (0.1% triton-x-100, Sigma-Aldrich for 15 mins, RT) followed by three times 1 × PBS rinse. The samples were then blocked (1% BSA, Sigma-Aldrich for 1h, + 4 °C) and rinsed three times with 1 × PBS. For the staining of the actin cytoskeleton, the samples were stained with phalloidin (1/800 Phalloidin-Tetramethyl rhodamine B isothiocyanate (Phalloidin-TRITC, Sigma-Aldrich) in 1% BSA). The samples were then rinsed five times with 1 × PBS. For the staining of nuclei, samples were stained with DAPI (1/2000 4',6-diamidino-2-phenylindole dihydrochloride (Sigma-Aldrich) in 1 × PBS for 5 mins, RT, dark) followed by a single 1 × PBS rinse and twice rinses with milliQ H_2O. Images of the cells were imaged with manually adjusted light exposure times for phalloidin-TRITC (510–550 nm) and DAPI (420 nm). Images were processed with image processing software ImageJ.

4.8 Cytokine analysis

The cytokines produced by lnASC and obASC monocultures and M1, M2 and Mreg macrophages in mono and co-cultures with lnASC and obASC during the macrophage polarization assay were also studied. The medium from each condition of activation media

was collected, centrifuged at 1000g, 10 mins, + 4 °C. Supernatants were collected for cytokine analysis.

V-PLEX assay MSD (Meso scale diagnostics, LLC) are ELISA based assay kits which includes multi-spot plates to measure different analytes (Figure.7), calibrators, individual labeled detection antibodies and diluents (www.mesoscale.com).

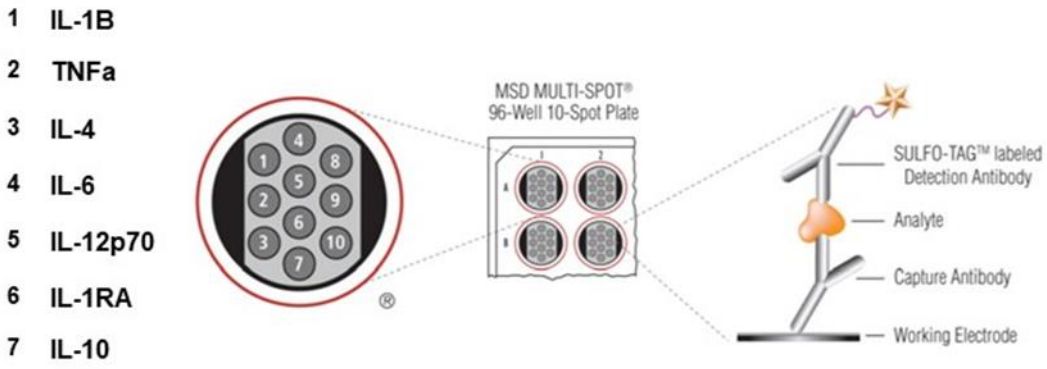

Figure 7. *A picture showing the multiplex plate spot and the analytes placement in pro-inflammatory panel 1 human kit (From www.mesosclae.com)*

Dilutions were prepared from all samples of both ASC donors (Table 5). Calibrants were also prepared following the assay manual. Plates were washed 3 times with wash buffer (PBS + 0.05% Tween + 0.1% PBA), samples and calibrants were added. Plates were sealed tightly with adhesive plate seals and incubated (2hrs, RT with shaking). Plates were washed 3 times with wash buffer and detection antibodies were added to each well. Plates were sealed properly and incubated (2hrs, RT with shaking). Plates were washed 3 times with wash buffer, 2x read buffer (4x read buffer + milliQ H_2O) was added to each well and plates were analyzed with MSD instrument (MESO™ QuickPlex SQ 120, Meso Scale Diagnostics, LLC). The results were analyzed with MSD Discovery Workbench software (Meso Scale Diagnostics, LLC).

Table 5. *Dilutions prepared for cytokine, chemokine, and pro-inflammatory assay kits*

Cytokine assay kit 250-fold		**Chemokine assay kit 100-fold**		**Pro-inflammatory assay kit 10-fold**
IL-1RA		TARC, MIP-1a, MDC, MCP-1		IL-1B, IL-4, IL-6, IL-10, IL-12p70, TNFα
Cytokine 1 1:50	Cytokine 2 1:5	Chemokine 1 1:10	Chemokine 2 1:10	Pro-inflammatory 1:10

4.9 Metabolic analysis

The cellular respiratory capacity of lnASC and obASC from both donors was analysed with Agilent Seahorse XFe24 Cell Mito Stress Test Kit (Agilent Technologies Inc, www.agilent.com). Cell Mito Stress Test measures key parameters of mitochondrial function by directly measuring the oxygen consumption rate (OCR) of cells (Figure 8). The sequential compound injections during the analysis measure basal respiration, ATP production, proton leak, maximal respiration, spare respiratory capacity, and non-mitochondrial respiration. Kits comprised of seahorse 24-well plates, XF24 flux assay kit (sensor cartridge + utility plate), components for assay medium (pyruvate: 100mM stock; Sigma Aldrich, L-glutamine: 200mM stock; Gibco, glucose: 1M stock; Sigma Aldrich and seahorse medium: minimal DMEM, without Phenol Red; Seahorse Bioscience), inhibitors (Oligomycin, FCCP, Rotenone, Antimycin A); Sigma Aldrich, calibrant; Seahorse Bioscience.

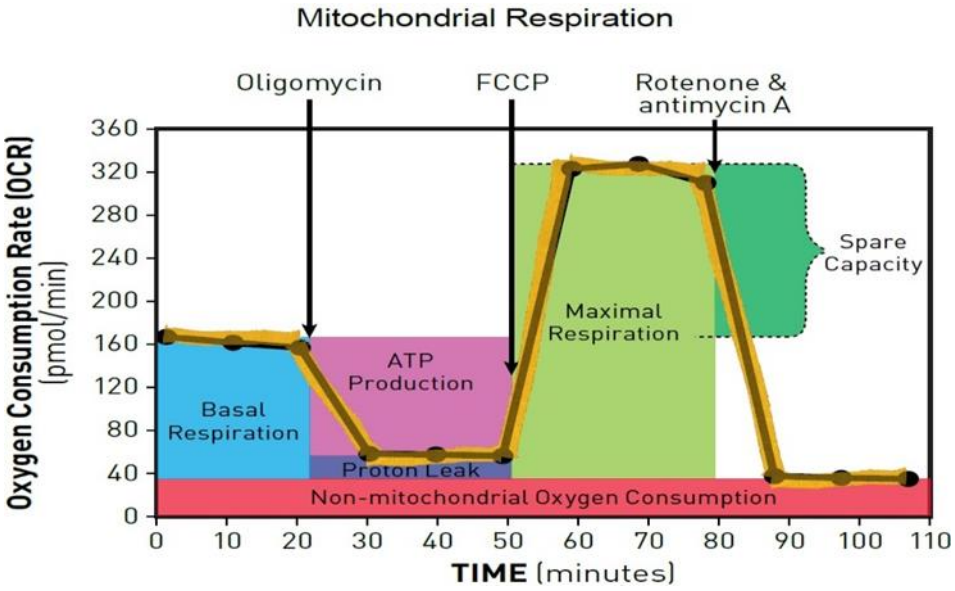

Figure 8. *Seahorse XF Cell Mito Stress Test Profile. (From www.agilent.com)*

Conditioned medium from M1 polarized macrophages was obtained by polarizing the PBMCs towards M1 macrophages by culturing 6 days following the previously described macrophage polarization protocol. Medium was obtained from each well, centrifuged (2500g, 10 mins, + 4 °C), supernatants were filtered and stored for metabolic analysis.

Plates were prepared for two different time points 24hrs and 48hrs. Wells were coated with gelatin (0.1% gelatin; Sigma Aldrich) and incubated (1hr, RT). Gelatin was removed and 25,000 cells/well with 10% FBS in RPMI medium were added. Cells were seeded by two steps seeding at RT to get even distribution (Figure 9). Plates were incubated at 37°C, 5% CO_2 for 24hrs.

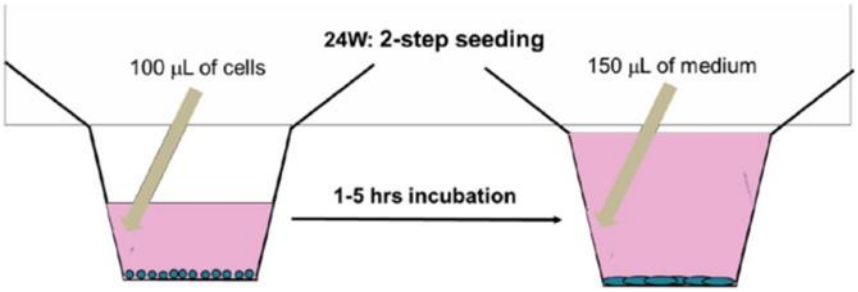

Figure 9. *Diagrammatic representation of 2-step seeding of cells in 24 well seahorse plate (From www.agilent.com)*

M1 conditioned medium was mixed with activation medium with a 60:40 ratio. Previous medium was replaced with M1 conditioned medium and M1 activation medium. Plates were incubated at 37°C, 5% CO_2 for 24hrs. On the same day cartridge plate was hydrated by adding calibrant and hydrated overnight (recommended 16h, minimum 4h) at

37°C in CO_2 – free incubator. Assay medium was prepared according to assay manual, and cells were washed with it. Inhibitors were prepared according to assay manual and loaded to the 3 ports of the hydrated sensor cartridge by following the manual instructions. Plates were loaded and red by Agilent Seahorse XFe analyzer (Seahorse Bioscience Inc). After the run, the raw data was normalized by measuring DNA concentration in each well (CyQUANT® Cell Proliferation Assay). The data was analyzed with Seahorse Wave Controller Software (Seahorse Bioscience Inc).

4.10 Statistical analysis

Statistical analysis was performed with IBM® SPSS® 27.0.1.0 (USA). The significance among the studied cytokines was measured with non-parametric Kruskal-Wallis test to compare the different macrophage monocultures and monocultures with their parallel co-cultures with lnASC and obASC. A Bonferroni correction was performed when multiple comparisons were carried out. Results were considered significantly different when p value was below 0.05 and highly significant when below 0.01.

5. RESULTS

5.1 Macrophage Polarization

5.1.1 Macrophages

The cell morphology of macrophages was examined by light microscope imaging after 6 days of culture (Figure 10). Mixed population of cells was observed. Cells were observed both attached and in suspension. On day 6 differentiated morphologies of M1, M2 and Mreg were observed. M1 appeared round, M2 appeared as spindle and Mreg appeared as in between of M1 and M2 meaning the population of cells was both in round and spindle shape.

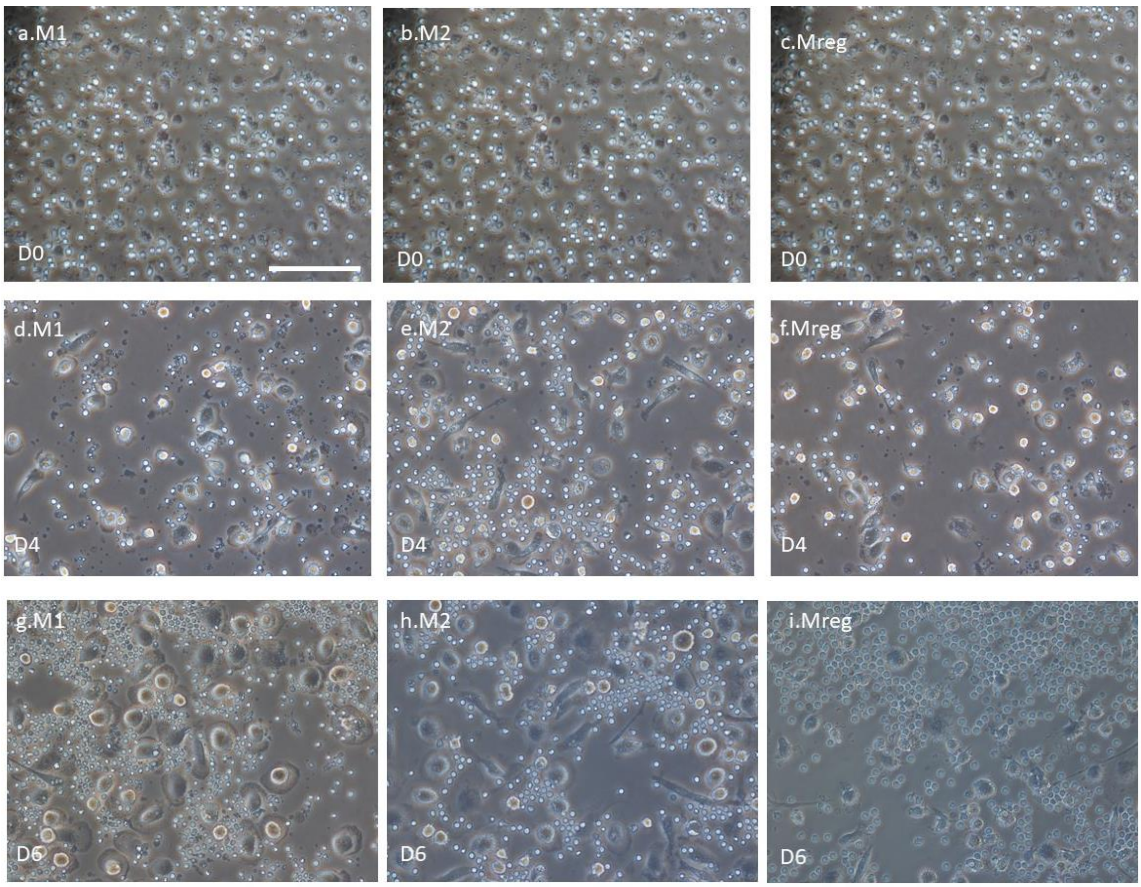

Figure 10. *Morphology of different macrophages after 6 days culture in 10% FBS medium. a) M1 b) M2 c) Mreg at day 0. d) M1 e) M2 and f) Mreg at day 4. g) M1 h) M2 i) Mreg j) at day 6. Scale bar: 100µm*

The cell morphology of macrophages was also observed with Olympus IX51 Fluorescence microscope imaging after 6 days of culture. The cell cytoskeleton was stained with

phalloidin and nucleus with DAPI (Figure 11). The intensity of cytoskeleton staining varied in different macrophages. In M2 macrophages the intensity of phalloidin staining was low as compared to M1 macrophages especially around the nucleus.

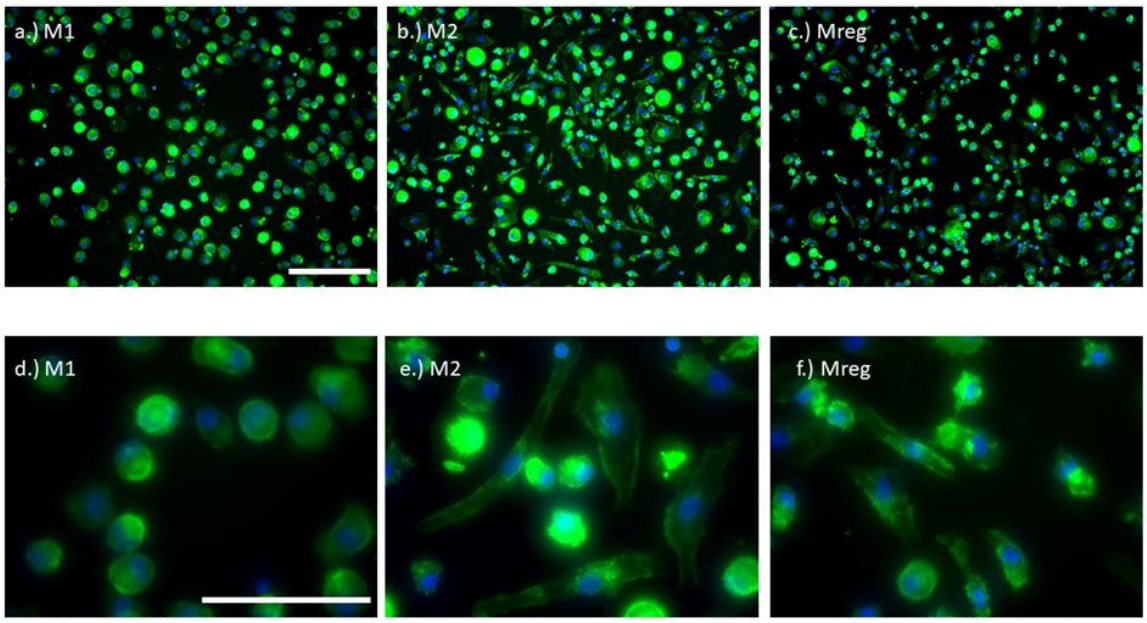

Figure 11. *Morphology of different macrophages after 6 days. a) M1 b) M2 c) Mreg. Scale bar: 200µm. d) M1 e) M2 f) Mreg. Scale bar: 100 µm*

5.1.2 Adipose stem cells

Previously isolated ASC were thawed and cell morphology of lnASC and obASC was examined after 4 days of culture. Cell lines for donor 1 were coded as, lean state: ASC-L3S, obese state: ASC-O3S and donor 2 coded as, lean state: ASC-L6S, obese state: ASC-O6S. ASC from donor 1 were imaged at passage 3 and lean derived ASC of donor 2 at passage 5 while obese derived ASC of donor 2 at passage 6. Cells had typical MSCs like morphology of spindle shaped, adherent cells and no morphological difference was observed between lnASC and obASC (Figure 12).

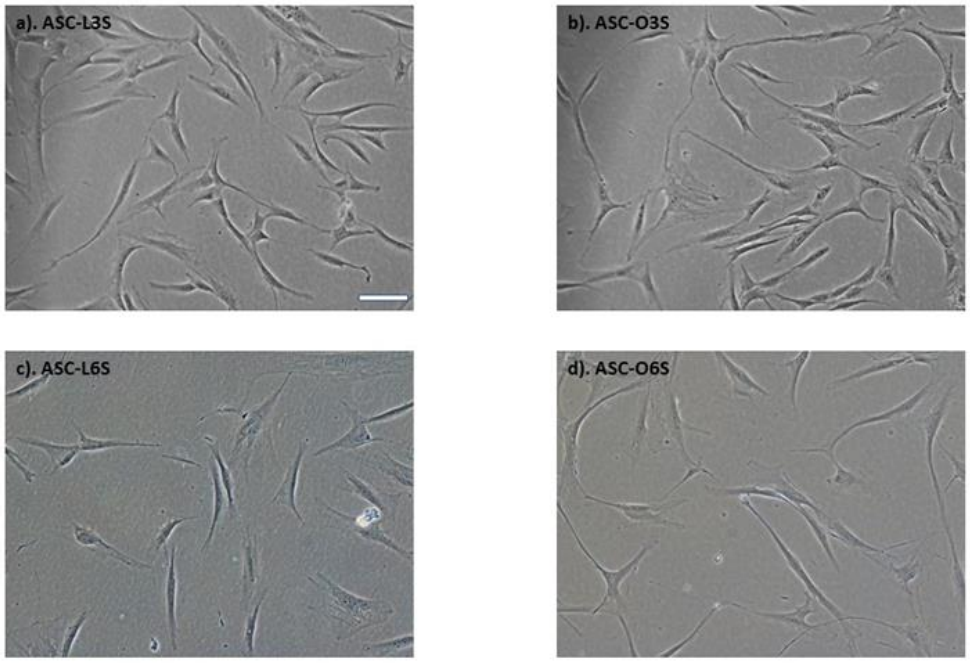

Figure 12. *Morphology of lnASC and obASC from two donors cultured in 5% HS medium. a) ASC-L3S b) ASC-O3S c) ASC-L6S d) ASC-O6S. Scale bar: 100 µm*

5.1.3 Co-cultures of macrophages with ASC

Co-cultures of three different types of macrophages with ASC were observed with Olympus IX51 Fluorescence microscope imaging after 6 days of culture. ASC exhibited a typical spindle or stellate shaped morphology (Figure 13).

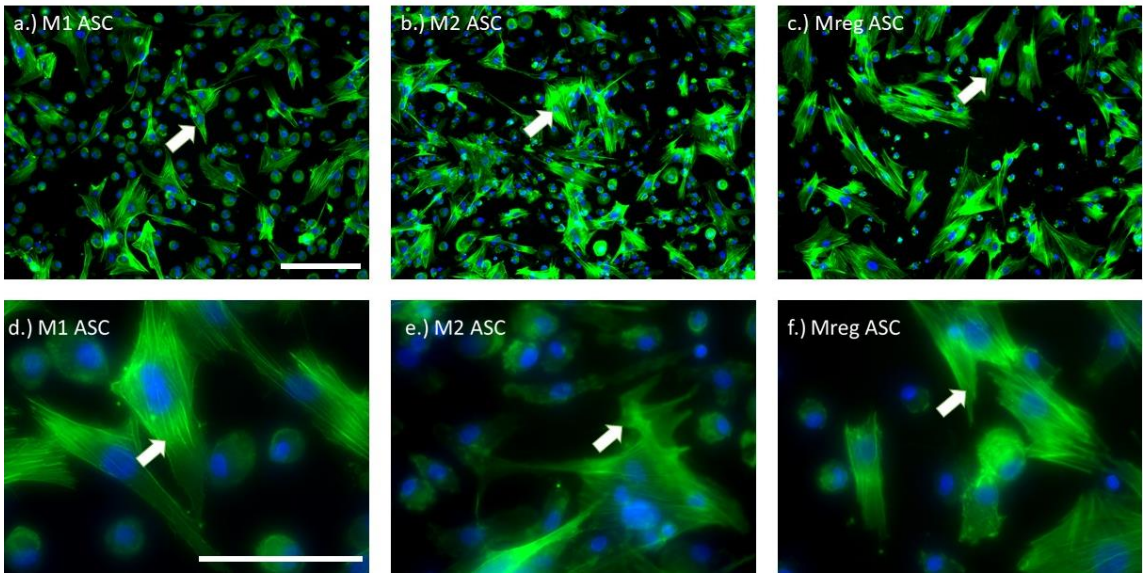

Figure 13. *Morphology of different macrophages in co-culture with ASC after 6 days. a) M1ASC b) M2ASC and c) MregASC. Scale bar: 200µm. d) M1ASC e) M2ASC and f) MregASC. Arrows pointing the ASC in the images. Scale bar: 100 µm*

5.2 Flowcytometry Analysis

5.2.1 Macrophages

The polarization of different macrophages M1, M2 and Mreg was further investigated with flow cytometry analysis.

a. M1 macrophages

The population of M1 cells was studied using CD markers CD206, CD163, CD11c, CD86 and HLA-DR (Figure 14). M1 type macrophages were selected for the analysis based on their size and granularity (Figure 15a and b). Dead cells were excluded from the analysis based on BD Horizon™ Fixable Viability 510 staining. On average 83.6% of the M1 macrophage population expressed CD206, 78.0% CD163 and 96.7% CD86 (Figure 14). Representative histogram images shown below are from a single experiment (Figure 15 c, d, e, f, and g).

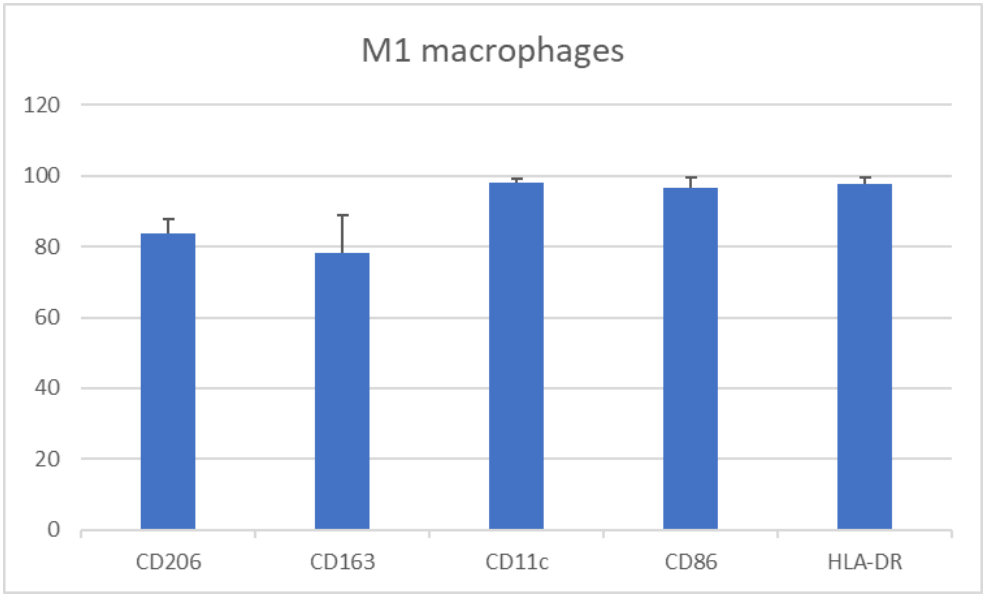

Figure 14. *CD marker expression profile of M1 macrophages in monoculture*

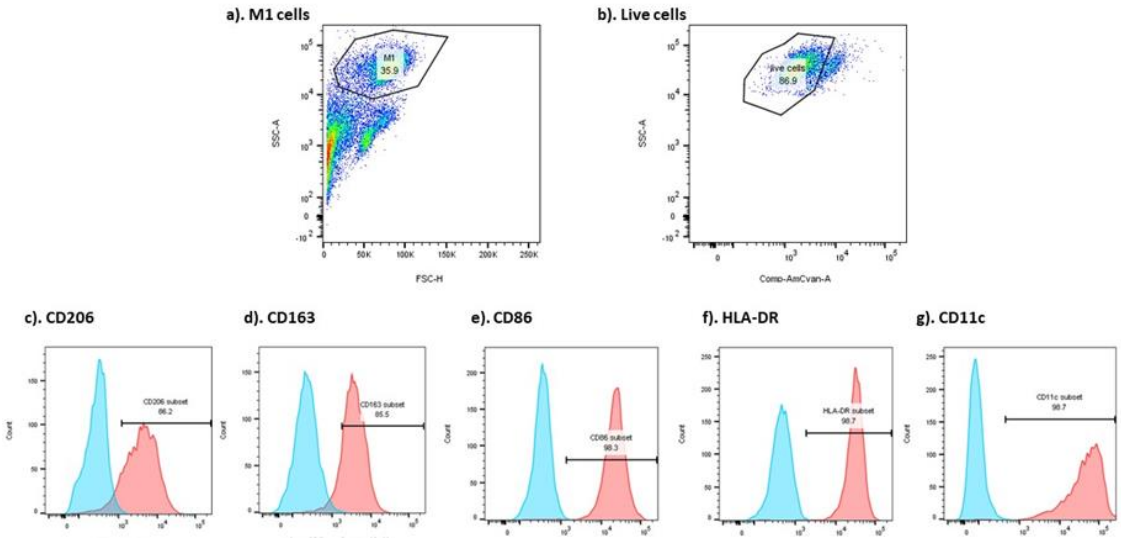

Figure 15. *FACS analysis of M1 type macrophages. a) Gating of M1 macrophage sample. b) Exclusion of dead population of M1 from other cells. c, d, e, f and g) expression of macrophage specific CD markers in red and unstained controls in blue*

b. M2 Macrophages

Similarly, the macrophages polarized into M2 phenotype were also studied using the same CD markers (Figure 16). On average, 97.6% of M2 macrophage population expressed CD206, 93.7% CD163 and 91.9% CD86 (Figure 16). Representative histogram images shown below are from a single experiment (Figure 17 c, d, e, f, and g).

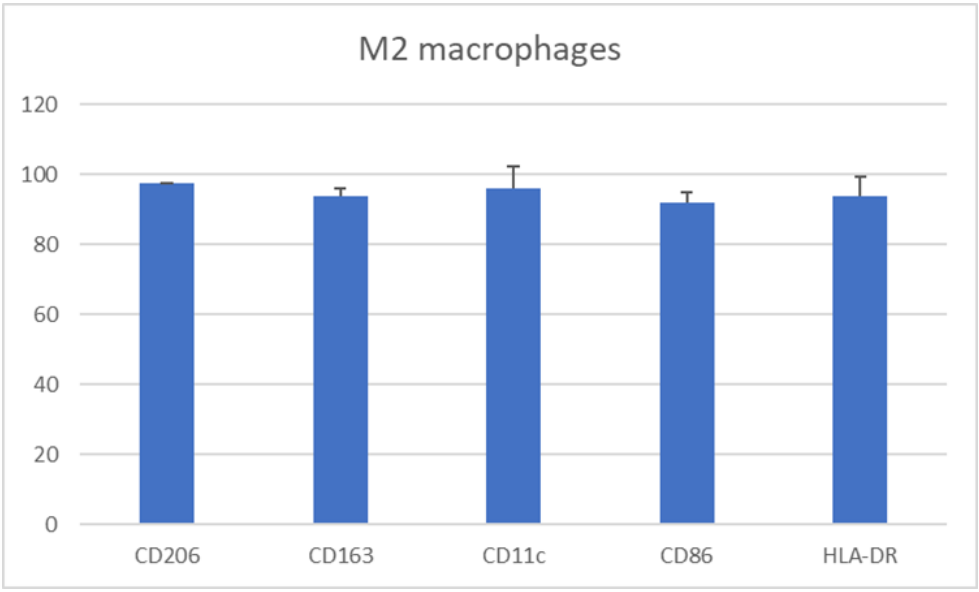

Figure 16. *CD marker expression profile of M2 macrophages in monoculture*

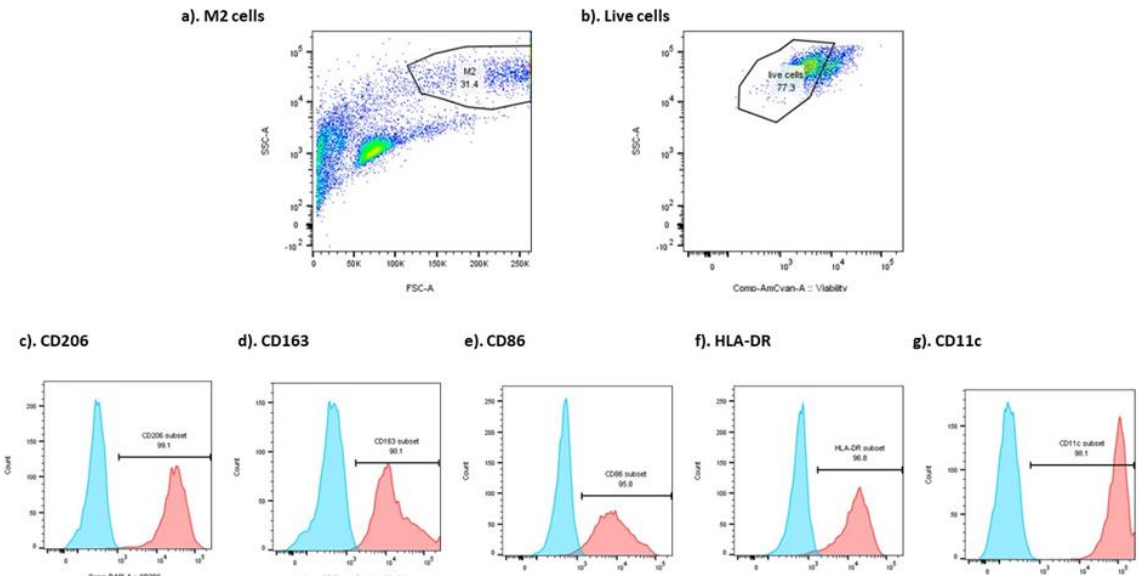

Figure 17. *FACS analysis of M2 type macrophages. a) Gating of M2 macrophage sample. b) Exclusion of dead population of M2 from other cells. c, d, e, f and g) expression of macrophage specific CD markers in red and unstained controls in blue*

c. Mreg Macrophages

Also, the macrophages polarized to Mreg phenotype were studied using the same CD marker profile (Figure 18). It was observed that on average 69.3% of Mreg macrophage population expressed CD206, 69.8% CD163 and 76.4% CD86 (Figure 18). Variation was observed in the result of two parallel samples. Representative histogram images shown below are from a single experiment (Figure 19 c, d, e, f, and g).

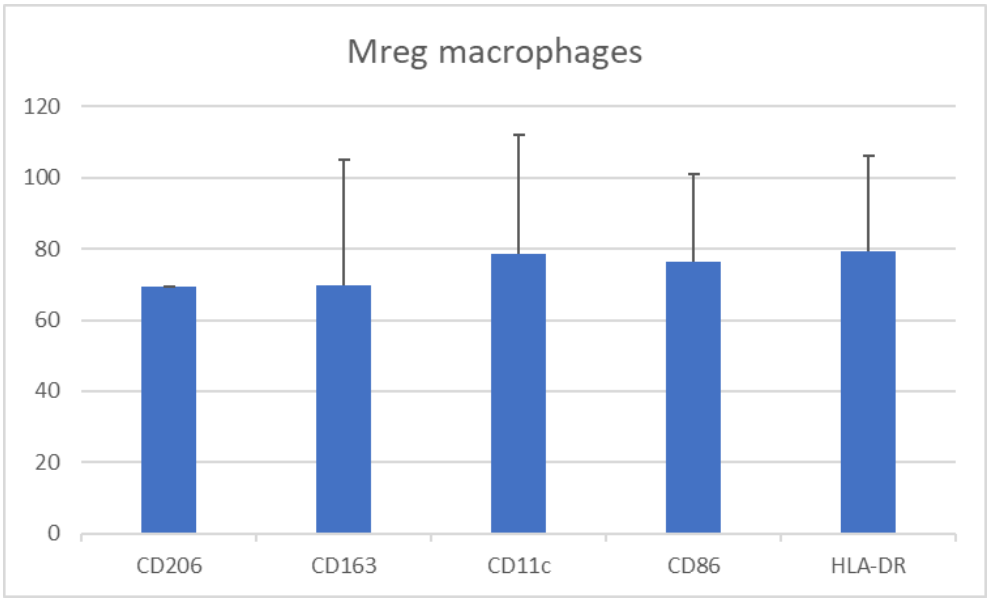

Figure 18. *CD marker expression profile of Mreg macrophages in monoculture*

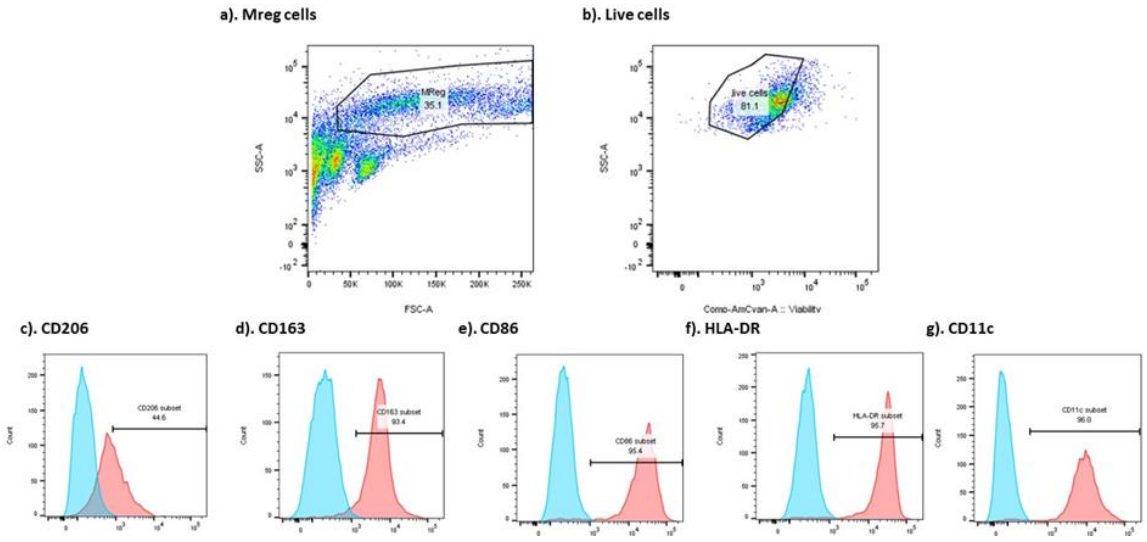

Figure 19. *FACS analysis of Mreg type macrophages. a) Gating of Mreg macrophage sample. b) Exclusion of dead population of Mreg from other cells. c, d, e, f and g) expression of macrophage specific CD markers in red and unstained controls in blue*

5.2.2 Adipose stem cells

a. Donor 1 and 2

Mesenchymal stem cell marker expression profile was analysed from ASC isolated before and after the weight-loss, in lean and obese state for both donors 1 and 2. Flow cytometric analysis of donor 1 was performed at passage 3 while that of donor 2 at passage 5-6. No change was observed in the stemness of ASC before and after weight loss as both obASC and lnASC were highly expressing the MSCs markers i.e., CD90, CD73 and CD105. On average, low expression of CD36 was observed in obASC compared with slightly increased expression that was observed in lnASC with high variation among the donors. The expression of CD142 was lower in obASC compared with slightly increased expression that was observed in lnASC with large standard deviation (Figure 20).

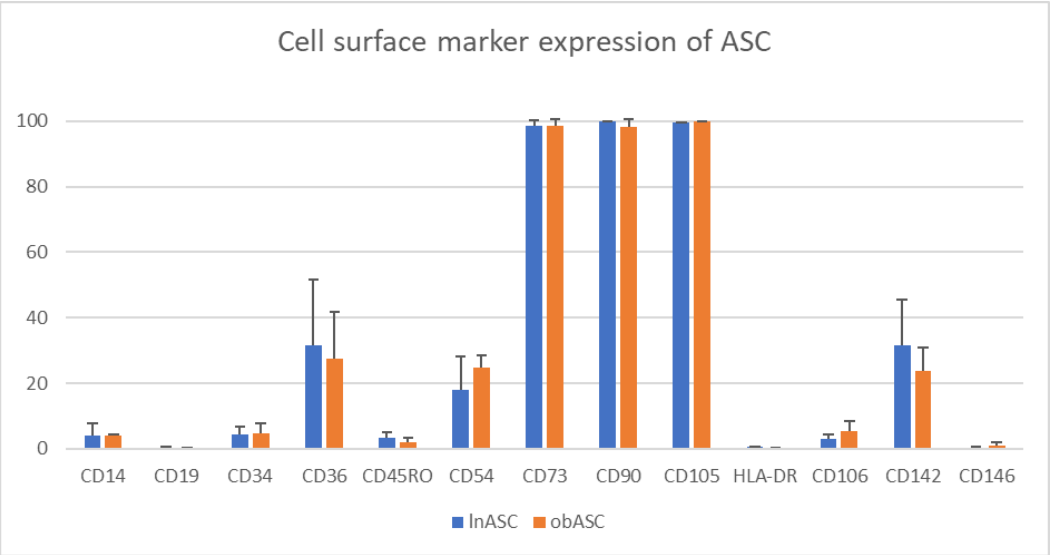

Figure 20. *Comparison between different CD marker expression for lnASC and obASC during FACS analysis with two different ASC donors*

5.2.3 Co-cultures of macrophages with ASC

a. M1 macrophages with lnASC and obASC

The expression of macrophage markers was studied in co-culture with lnASC and obASC (Figure 21). The ASC were excluded from the analysis based on their CD90 expression (Figure 22). In co-culture with lnASC, 31.7% of M1 macrophage population expressed CD206, 46% CD163 and 79.1% CD86 (Figure 21). In co-culture with obASC, 22.45% of M1 macrophage population expressed CD206, 34.25% CD163 and 76.2% CD86 (Figure 21). Representative histogram images shown here are results of macrophage immunophenotype in co-culture with ASC donor 1 (Figure 23). On the average, the expression of markers specific for M1 type macrophages CD86, CD11c and HLA-DR, decreased in co-culture with both lnASC and obASC. The average low expression of CD86 was observed in lnASC i.e., 79.1% and even lower in obASC i.e., 76.2% compared with M1 monoculture i.e., 96.7% (Figure 21).

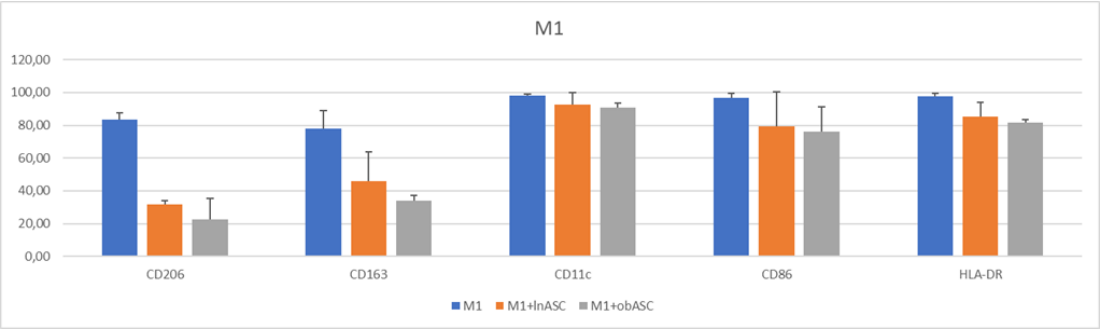

Figure 21. *Comparison between different CD marker expression for M1 macrophages during FACS analysis in mono and co-culture with two different ASC donors*

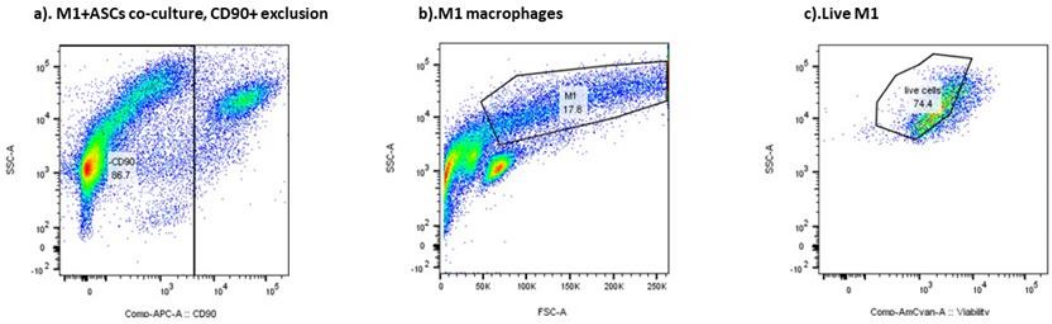

Figure 22. *Gating strategy and CD90 exclusion*

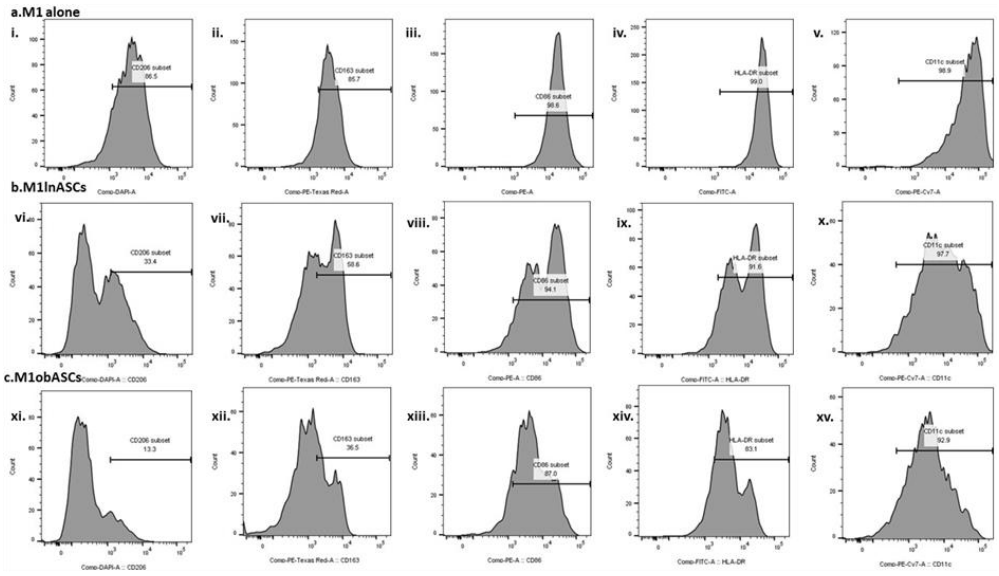

Figure 23. *FACS analysis of M1 macrophages in mono and co-culture with lnASC and obASC. a) Histograms of CD marker expression for M1 macrophages in monoculture. b) Histograms of CD marker expression for M1 macrophages with lnASC. c) Histograms of CD marker expression of M1* macrophages with obASC. i), vi) and xi) CD206, ii), vii) and xii) CD163, iii), viii) and xiii) CD86, iv), ix) and xiv) HLA-DR and v), x) and xv) CD11c

b. M2 macrophages with lnASC and obASC

The expression of different CD markers of M2 macrophages was also studied in co-culture with lnASC and obASC (Figure 24). In co-culture with lnASC, on average, 92.7% M2 macrophage population expressed CD206, 92.05% CD163 and 88.1% CD86 (Figure 24). In co-culture with obASC, 94.5% M2 macrophage population expressed CD206, 93.3% CD163 and 86.65% CD86 (Figure 24). Representative histogram images shown here are results of macrophage immunophenotype in co-culture with ASC donor 1 (Figure 26). On the average, the expression of M2 type macrophages was similar in mono and co-cultures.

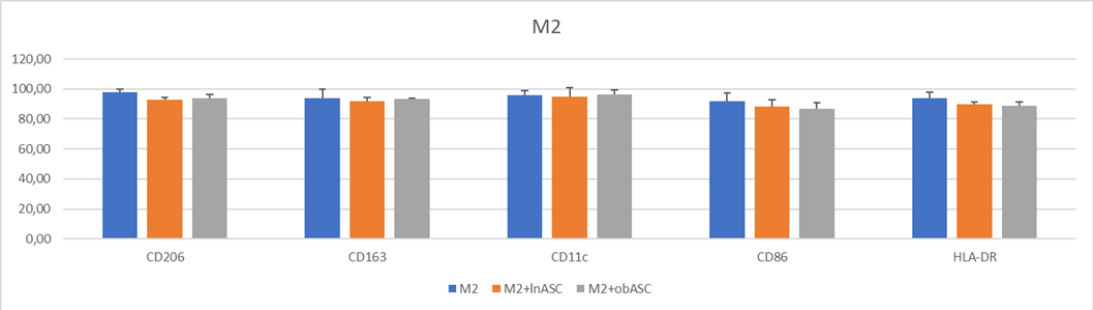

Figure 24. *Comparison between different CD marker expression for M2 macrophages during FACS analysis in mono and co-culture with two different ASC donors*

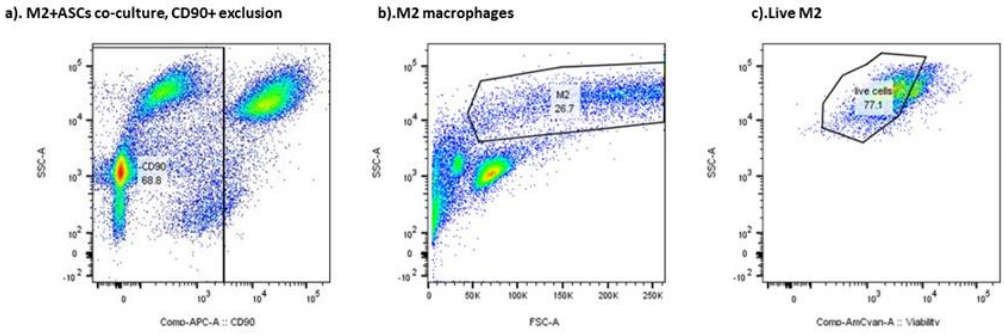

Figure 25. *Gating strategy and CD90 exclusion*

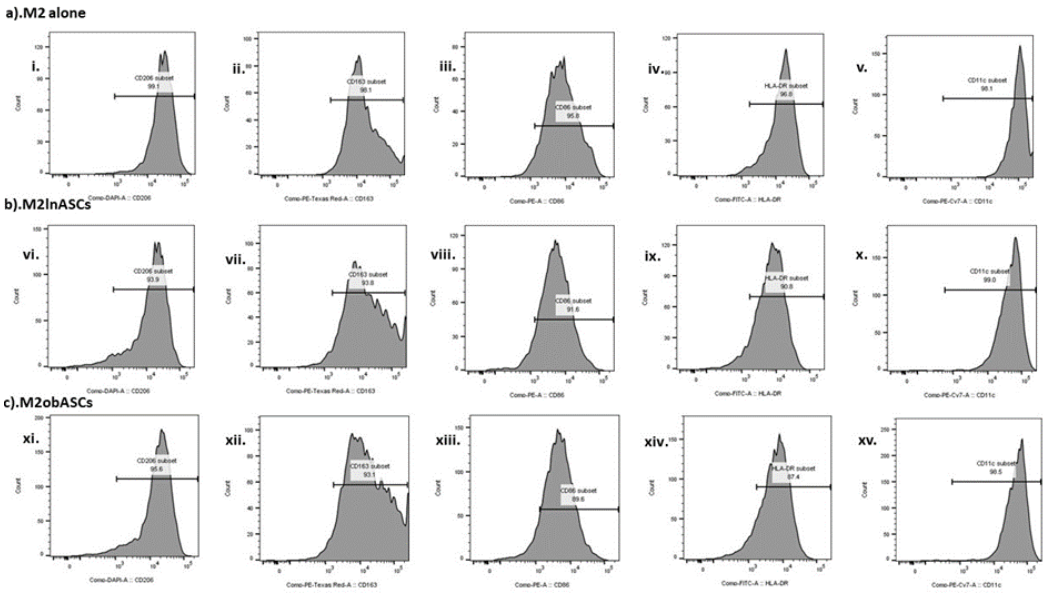

Figure 26. *FACS analysis of M2 macrophages in mono and co-culture with lnASC and obASC. a) Histograms of CD marker expression for M2 macrophages in monoculture. b) Histograms of CD marker expression for M2 macrophages with ln ASC. c) Histograms of CD marker expression for M2 macrophages with obASC. i), vi) and xi) CD206, ii), vii) and xii) CD163, iii), viii) and xiii) CD86, iv), ix) and xiv) HLA-DR and v), x) and xv) CD11c*

c. Mreg macrophages with lnASC and obASC

The expression of different CD markers of Mreg macrophages was also studied in co-culture with lnASC and obASC (Figure 27). In co-culture with lnASC, 67.7% of Mreg macrophage population expressed CD206, 84.5% CD163 and 85.1% CD86 (Figure 27). Similarly, in co-culture with obASC, 69.9% of Mreg macrophage population expressed CD206, 60.7% CD163 and 83.1% CD86 (Figure 27). Representative histogram images shown here are results of macrophage immunophenotype in co-culture with ASC donor 1 (Figure 29). A varied expression was observed with different markers in mono and co-cultures of Mreg macrophages with lnASC and obASC. On the average, a higher expression was observed for CD163 and 86 in co-cultures with lnASC as compared to obASC. Variation was observed in the result of two parallel samples.

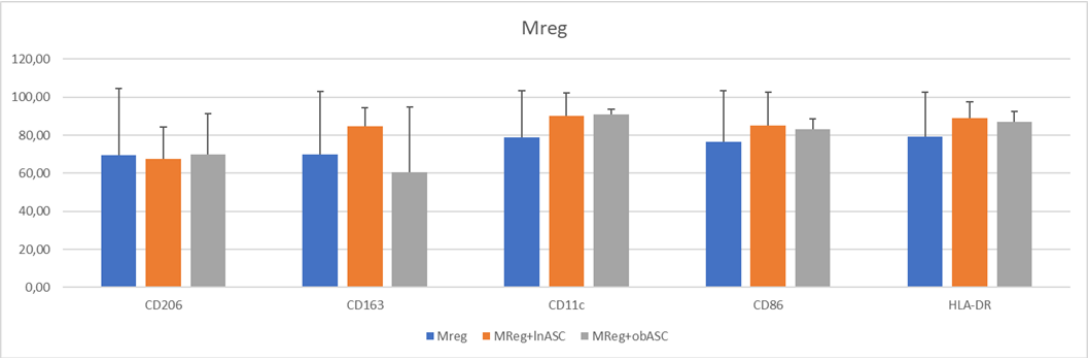

Figure 27. *Comparison between different CD marker expression for Mreg macrophages during FACS analysis in mono and co-culture with two different ASC donors.*

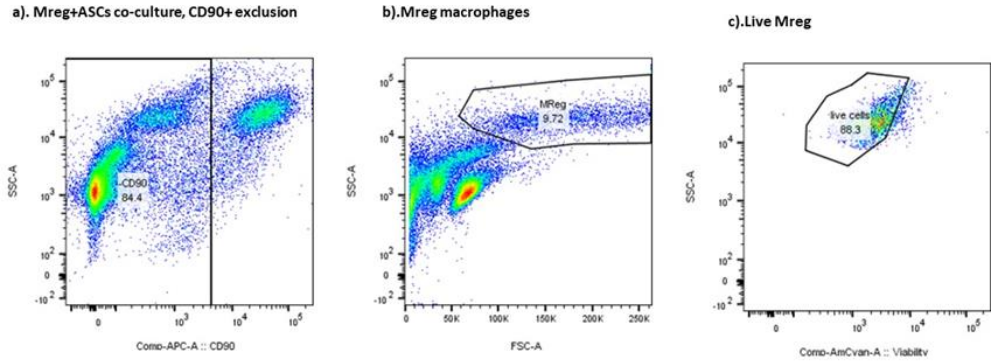

Figure 28. *Gating strategy and CD90 exclusion*

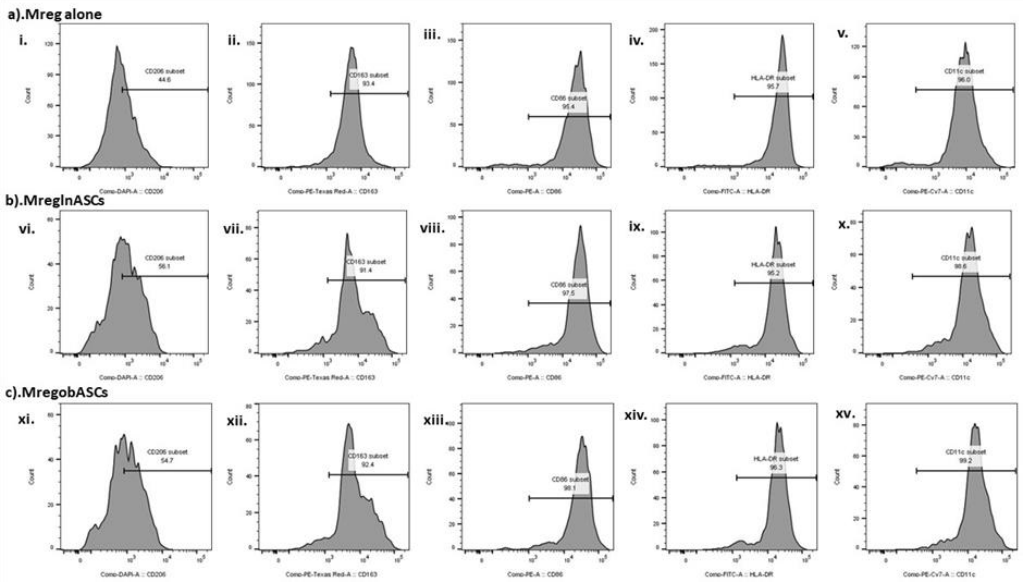

Figure 29. *FACS analysis of Mreg macrophages in mono and co-culture with lnASC and obASC. a) Histograms of CD marker expression for Mreg macrophages in monoculture. b) Histograms of CD marker expression for Mreg macrophages with lnASC. c) Histograms of Mreg macrophages with obASC. i), vi) and xi) CD206, ii), vii) and xii) CD163, iii), viii) and xiii) CD86, iv), ix) and xiv) HLA-DR and v), x) and xv) CD11c*

5.3 Cytokine Analysis

The cytokine secretion from polarized macrophages, lnASC and obASC in mono and co-cultures was further investigated.

5.3.1 Pro-inflammatory cytokines

a. Interleukin-6

The secretion of IL-6 was significantly higher in M1 co-cultures with lnASC (p=0.012) and in M1 co-cultures with obASC (p=0.004) compared with monocultures of M1 macrophages (Figure 30). Similarly, secretion of this cytokine was significantly higher in Mreg co-cultures with lnASC (p=0.024) and in Mreg co-cultures with obASC (p=0.008) compared with monocultures of Mreg macrophages (Figure 30). IL-6 secretion was low in M1, M2 and Mreg monocultures (Figure 30). High secretion of IL-6 was also observed in monocultures of obASC in M1 and Mreg conditions compared to other studied monocultures of ASC (appendices).

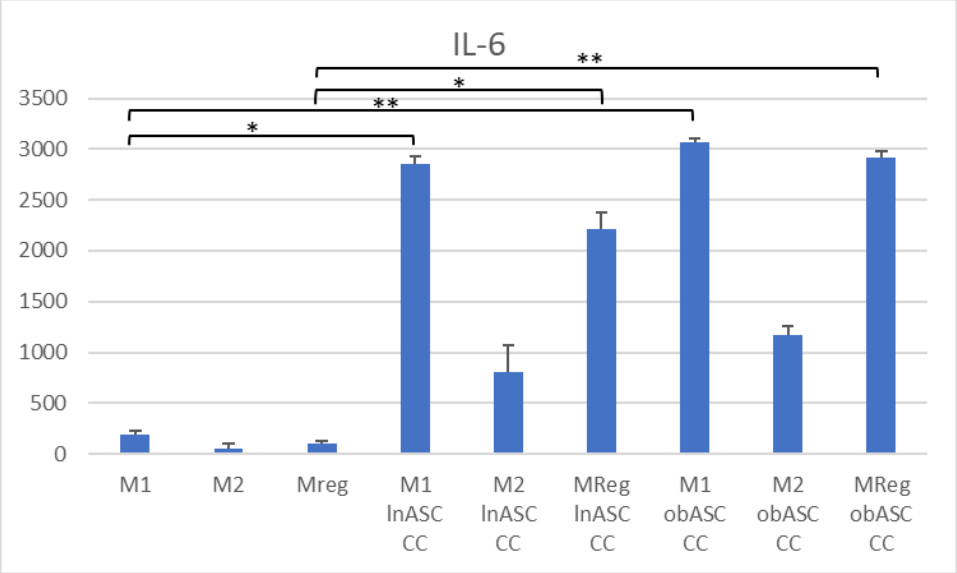

Figure 30. *Comparison of IL-6 secretion between M1, M2 and Mreg macrophages and their parallel co-cultures with lnASC and obASC from two different donors*

b. Tumour necrosis factor-alpha (TNFα)

It was observed that TNFα was highly secreted in M1 monocultures as compared with other studied monocultures (Figure 31). Also, an increased secretion of this cytokine was observed in co-cultures of M1 macrophages with lnASC and obASC compared with co-cultures of Mreg macrophages with lnASC and obASC (Figure 31). High standard deviation showed the variation amongst the donors.

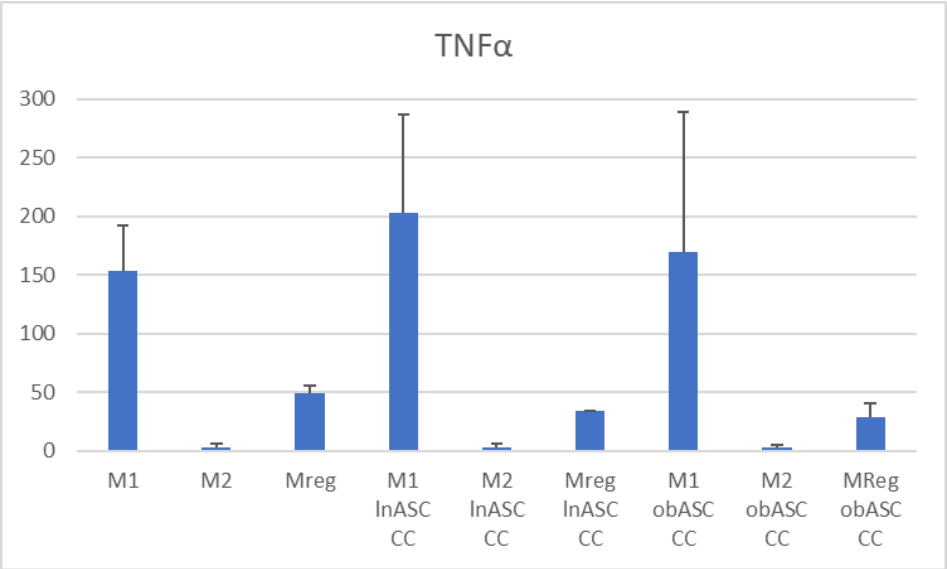

Figure 31. *Comparison of TNFα secretion between M1, M2 and Mreg macrophages and their parallel co-cultures with lnASC and obASC from two different donors*

c. Interleukin-1B

The secretion of IL-1B was significantly higher in M1 monocultures compared with M2 monocultures (p=0.024) (Figure 32). Also, high secretion of IL-1B was observed in co-cultures of M1 type macrophages with both lnASC and obASC as compared with other studied co-cultures (Figure 32). High secretion of this cytokine was also observed in monocultures of lnASC in M1 condition compared with other studied monocultures of ASC (appendices).

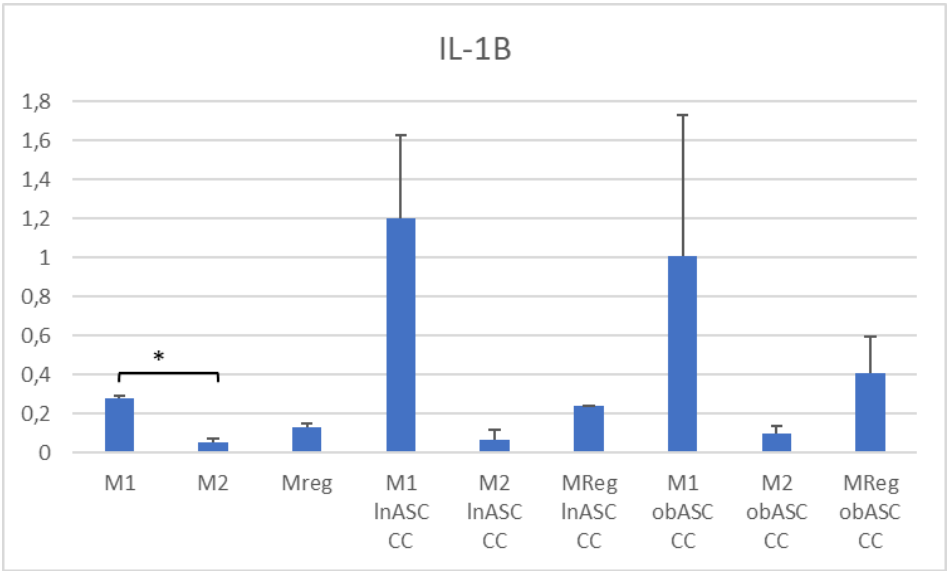

Figure 32. *Comparison of IL-1B secretion between M1, M2 and Mreg macrophages and their parallel co-cultures with lnASC and obASC from two different donors*

d. Interleukin-12p70

The secretion of IL-12p70 was significantly higher in Mreg monocultures compared with M1 monoculture (p=0.048) and with M2 monoculture (p=0.04) (Figure 33). The secretion significantly increased in M1 co-cultures with both lnASC (p=0.048) and obASC (p=0.02) compared with M1 monoculture (Figure 33). Also, high secretion of IL-12p70 was observed in co-cultures of obASC with Mreg conditions compared with other studied co-cultures (Figure 33). High secretion of IL-12p70 was also observed in monocultures of obASC in M1 and Mreg conditions compared with other studied monocultures of ASC (appendices).

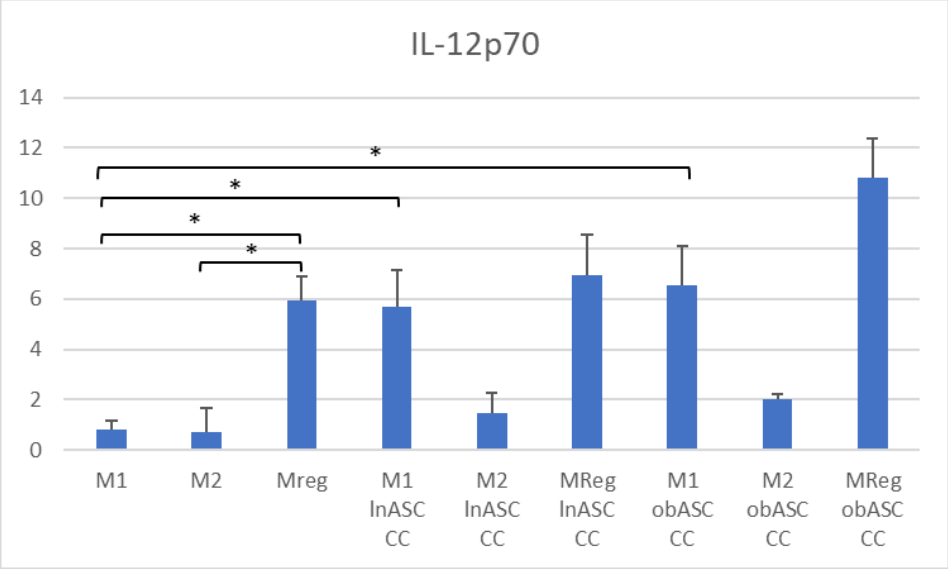

Figure 33. *Comparison of IL-12p70 cytokine secretion between 3 different types of macrophages and their parallel co-cultures with lnASC and obASC from two different donors*

e. Monocyte chemoattractant protein 1 (MCP-1/CCL2)

The secretion of MCP-1 (CCL2) was significantly higher in Mreg monocultures compared with M1 monocultures (p=0.04) and with M2 monocultures (p=0.036) (Figure.34). The secretion significantly increased in M1 co-cultures with both lnASC (p=0.04) and obASC (p=0.016) compared with M1 monocultures (Figure 34). Also, secretion of this cytokine was highest in co-cultures of obASC with Mreg conditions compared with other studied co-cultures (Figure 34). High secretion of MCP-1 was also observed in monocultures of obASC in M1 and Mreg conditions compared with other studied monocultures of ASC (appendices).

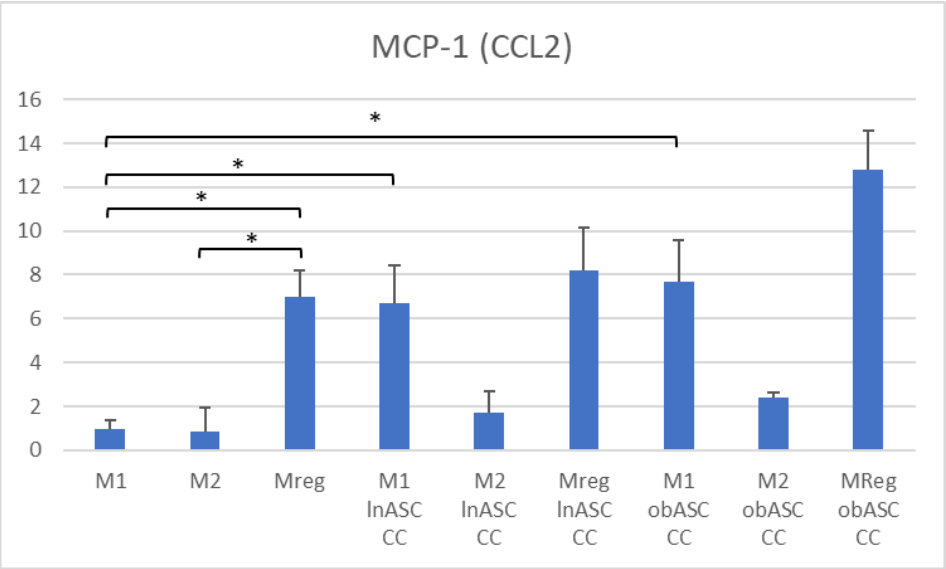

Figure 34. *Comparison of MCP-1 (CCL2) secretion between 3 different types of macrophages and their parallel co-cultures with lnASC and obASC from two different donors*

5.3.2 Anti-inflammatory cytokines

a. Thymus- and activation-regulated chemokine (TARC/CCL17)

The secretion of TARC was significantly higher in M2 monocultures compared with M1 monocultures (p=0.008) and with Mreg monocultures (p=0.004) (Figure 35). The secretion was significantly higher in Mreg co-cultures with obASC compared with Mreg monocultures (p=0.08) (Figure 35). Increased secretions of TARC were observed in all studied conditions of M2 (Figure 35 and appendices). High standard deviation showed that there was variation between donors (Figure 35 and appendices).

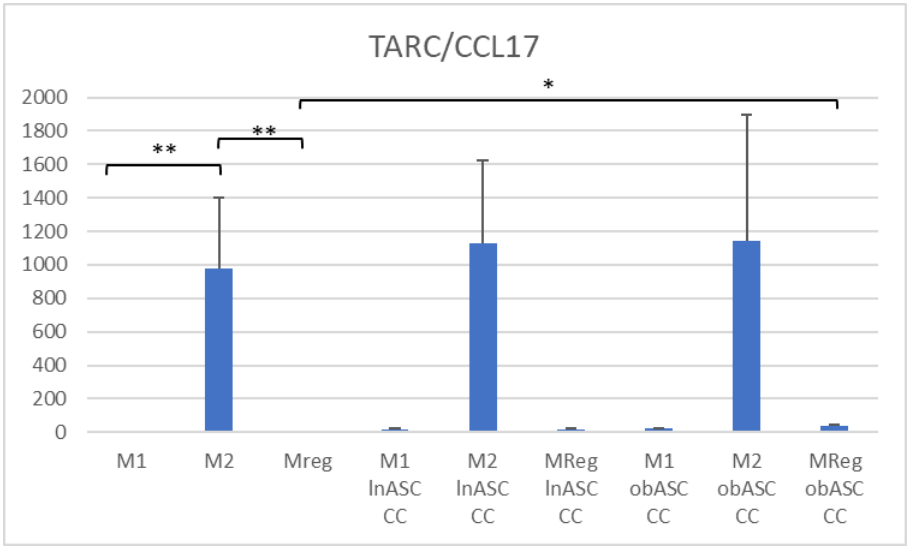

Figure 35. *Comparison of TARC (CCL17) secretion between 3 different types of macrophages and their parallel co-cultures with lnASC and obASC from two different donors*

b. Interleukin-1RA

The secretion of IL-1RA was significantly higher in M1 co-cultures with lnASC (p=0.004) and obASC (p=0.008) compared with M1 monocultures (Figure 36). High secretion was observed in ASC cultures but there was variation amongst donors, which was not dependent on donor weight or condition (Figure 36 and appendices).

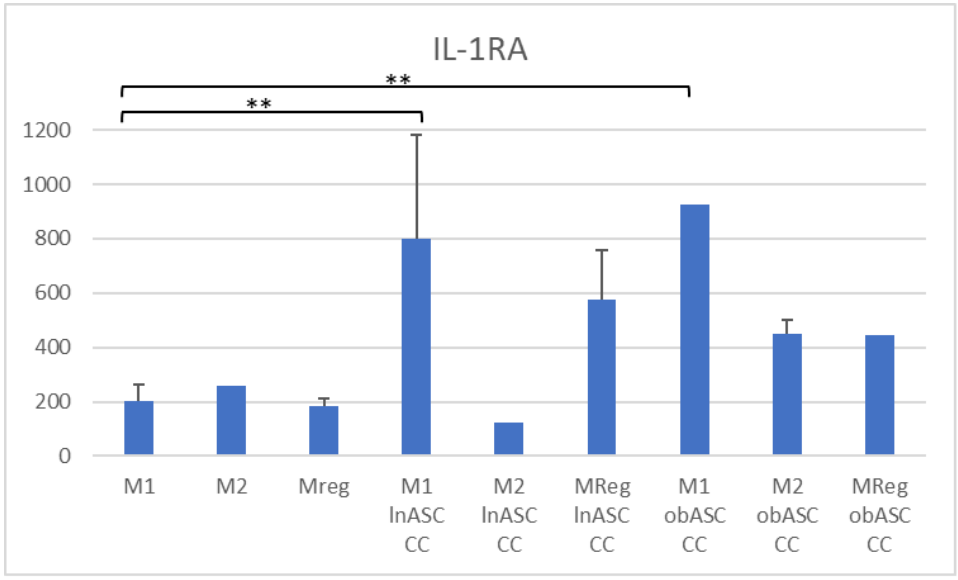

Figure 36. *Comparison of IL-1RA secretion between 3 different types of macrophages and their parallel co-cultures with lnASC and obASC from two different donors*

5.4 Metabolic Analysis

5.4.1 Cytokines of conditioned medium

The M1 conditioned medium, which was utilized during the study of metabolic capacity of lnASC and obASC, was investigated by measuring the cytokines secreted in the medium. It was observed that pro-inflammatory cytokines TNFα and IL-6 were highly secreted (Figure 37a). Also, low secretion of IL-12p70, IL1B, IL-4 MCP-1 and TARC/CCL17 was observed (Figure 37b).

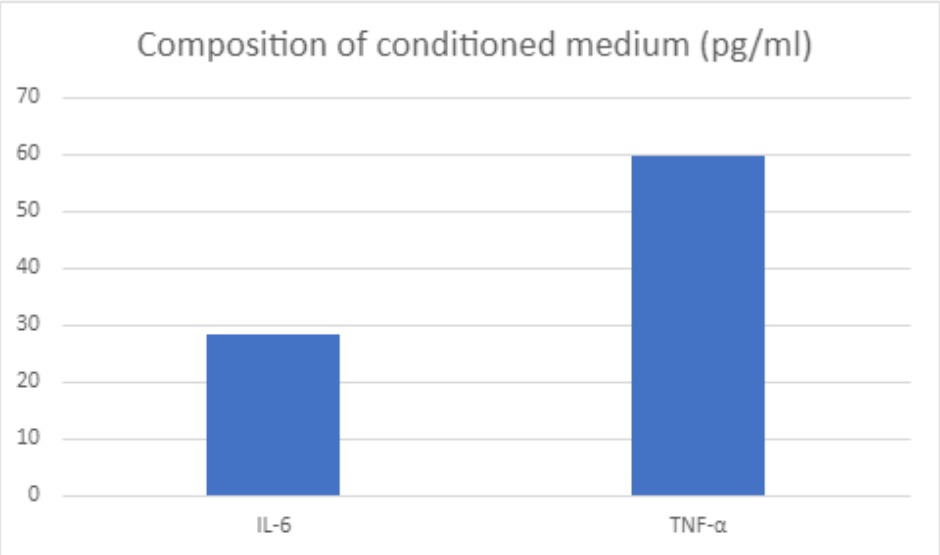

Figure 37a. *Cytokine secretions in the M1 conditioned medium*

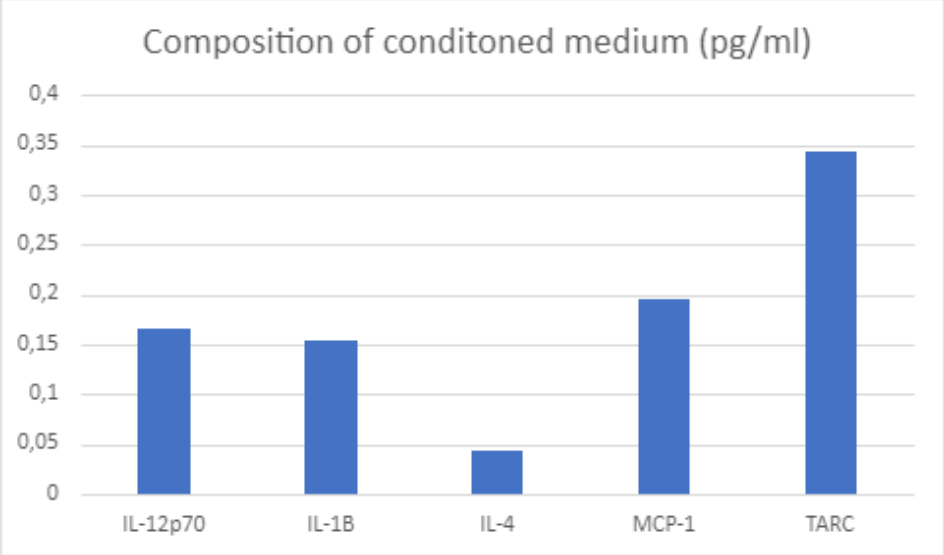

Figure 37b. *Cytokine secretions in the M1 conditioned medium*

5.4.2 Adipose stem cells

The mitochondrial respiratory capacity of lnASC and obASC was investigated with seahorse XF24 Cell Mito Stress Test by directly measuring the oxygen consumption rate (OCR) of cells under inflammatory conditions and control conditions. During the analysis, measured parameters were basal respiration, ATP production, proton leak, maximal respiration, spare respiratory capacity, and non-mitochondrial respiration based on the seahorse assay parameter equation (Table 6). Three measured parameters are presented in this study i.e., basal respiration, maximal respiration, and spare respiratory capacity.

Table 6. *Seahorse assay parameter equations*

Parameter value	Equation
Non-mitochondrial oxygen consumption	Minimum rate measurement after Rotenone/antimycin A injection
Basal respiration	(Last rate measurement before first injection) – (Non-mitochondrial respiration rate)
Maximal respiration	(Maximum rate measurement after FCCP injection) – (Non-mitochondrial respiration)
H^+ (Proton) leak	(Minimum rate measurement after Oligomycin injection) – (Non-mitochondrial respiration)
ATP production	(Last rate measurement before Oligomycin injection) - (Minimum rate measurement after Oligomycin injection)
Spare respiratory capacity	(Maximal respiration) – (Basal respiration)
Spare respiratory capacity %	(Maximal respiration) / (Basal respiration) × 100
Accurate responses	(Last rate measurement before Oligomycin injection) – (Last rate measurement before acute injection)
Coupling efficiency	(ATP production rate) / (Basal respiration rate) × 100

Mitochondrial respiration capacity of both donors i.e., donor 1 and 2 was measured at two different time points i.e., 24 hrs and 48 hrs (Figure 38). Increased cellular respiratory capacity of lean donors was observed in both time points under inflammatory conditions as compared to obese donor, while decreased cellular respiratory capacity of lean donor 6 was observed at 48hrs time point in comparison to obese donor (Figure 38).

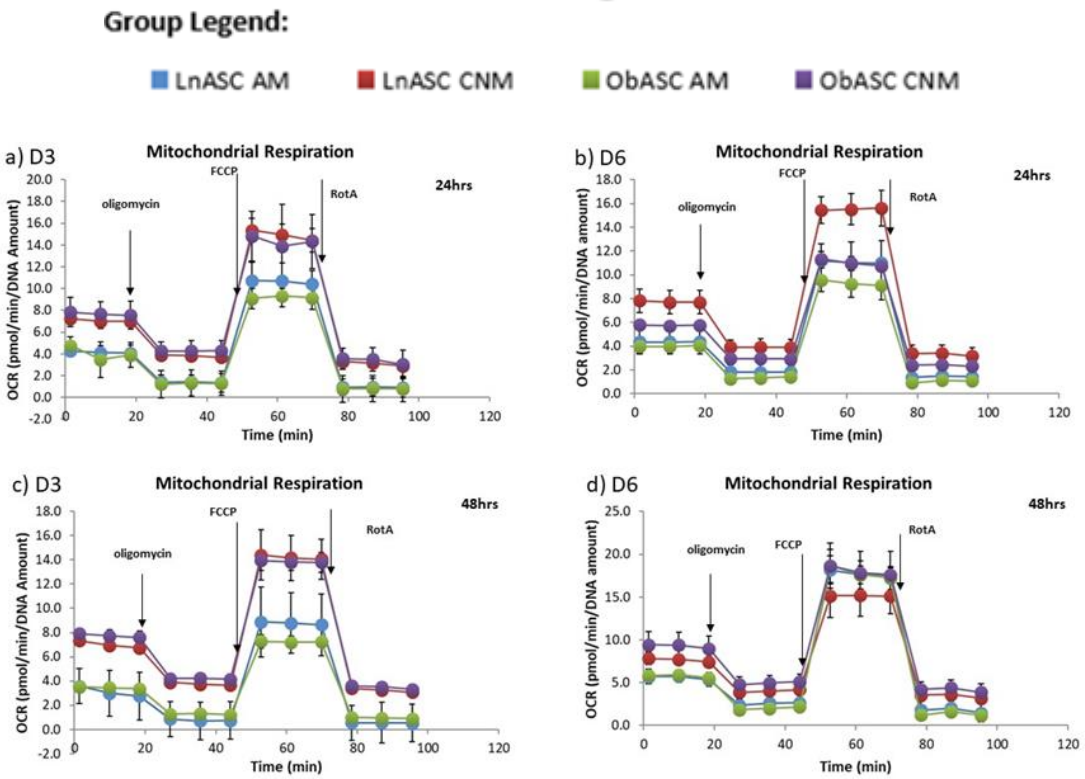

Figure 38. *Mitochondrial respiratory capacity of donor 1 and 2 at 24 and 48hrs time point. a) Donor 1 at 24hr b) Donor 2 at 24hr c) Donor 1 at 48hr and d) Donor 2 at 48 hrs. lean ASC in activation media (LnASC AM), lean ASC in conditioned media (LnASC CNM), obese ASC in activation media (ObASC AM), and obese ASC in conditioned media (ObASC CNM)*

5.4.3 Effect of inflammation on mitochondrial function

At 24hrs time point increased basal respiration of lnASC was observed in conditioned media as compared to lnASC in control media. Increased maximal respiration and spare respiratory capacity of lnASC was observed in conditioned media as compared to lnASC in control media. Similarly, at the same time point, increased basal, maximal, and spare respiratory capacity of obASC in conditioned media was observed as compared to obASC in control media (Figure 39).

On average, at 48hrs time point increased basal and maximal respiration of both lnASC and obASC was observed in conditioned media as compared to lnASC and obASC in control media. It was also observed that spare respiratory capacity of lnASC in conditioned media decreased compared to lnASC in control media while increase of spare respiratory capacity was observed in obASC in conditioned media compared to obASC in control media (Figure 40). A variation in spare respiratory capacity was observed among the donors and the conditions. Variation amongst the donors was observed at both time points, especially at 48hrs time point.

5.4.4 Effect of donor weight on mitochondrial functions

On average, at 24hrs time point an increase in basal, maximal, and spare respiratory capacity of lnASC was observed as compared to obASC (Figure 39). On the other hand, at 48hrs time point the basal and maximal respiration increased in obASC while a little decrease in spare respiratory capacity was observed compared with lnASC (Figure 40). Variation amongst the donors was observed especially at 48hrs time point.

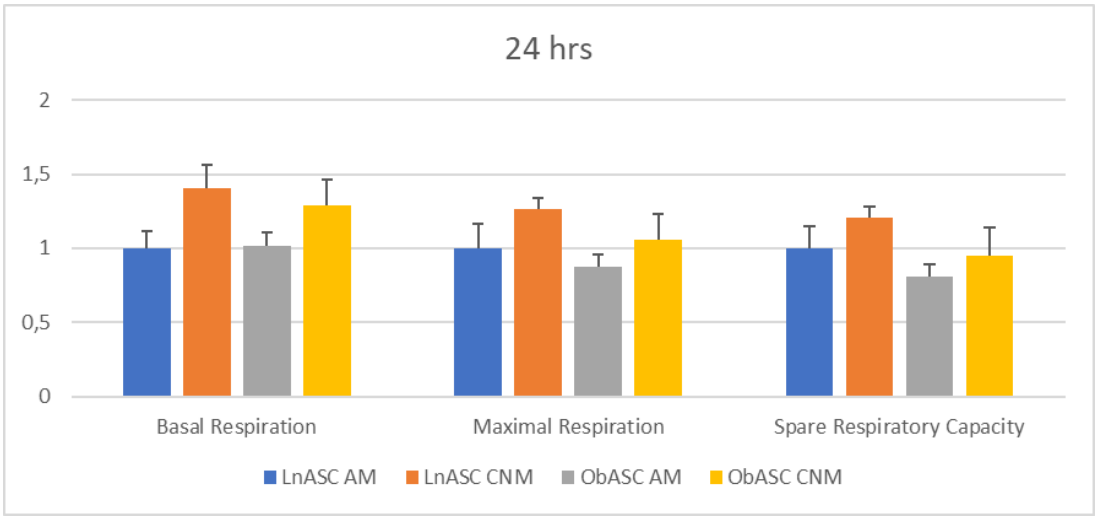

Figure 39. *Comparison between three different parameters of OCR of lnASC and obASC at 24hrs time point*

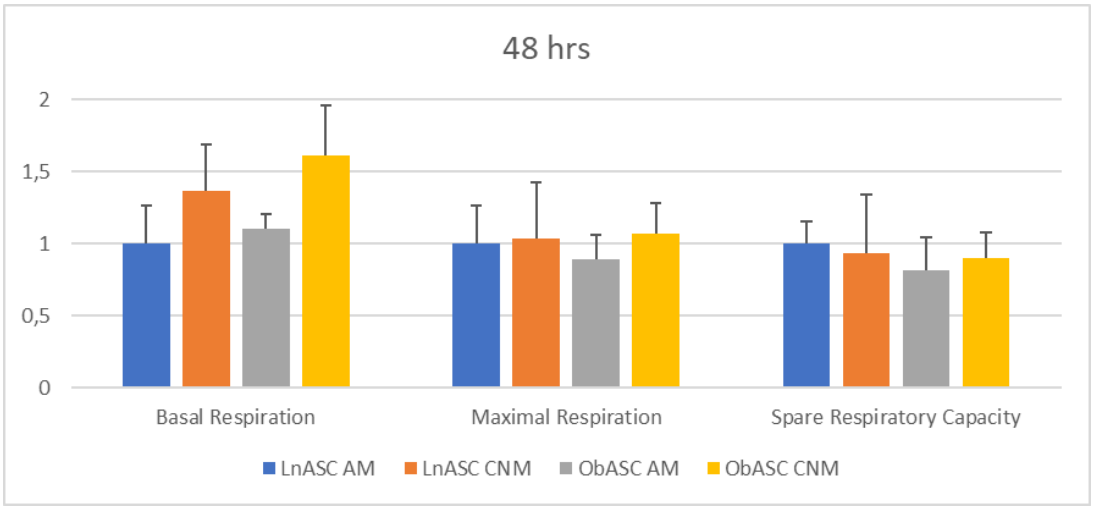

Figure 40. *Comparison between three different parameters of OCR of lnASC and obASC at 48hrs time point*

6. DISCUSSION

Earlier research conducted on the topic of obesity and metabolic disorders covers AT dysfunction and the role of inflammation providing the knowledge on malfunctional properties of ASC. Based on the previous work, this study analysed the effect of obesity-induced inflammation on the immunomodulatory and mitochondrial respiratory capacity using ASC derived from donors before and after weight loss. A major characteristic of obesity is the chronic, low grade inflammation (Lumeng 2013). Chronic nutrient excess causes the expansion of AT and dysfunction of adipocytes (Spalding et al. 2008). Immunomodulatory and immunosuppressive properties of ASC are affected by donor's metabolic phenotype (Serena et al. 2016).

6.1 Morphological Characteristics

Macrophages are the heterogenous population of innate immune cells that are present in tissues, mucosal surfaces, and body cavities. The functionality of macrophages is highly dependent on their lineage differentiation and plasticity (Orecchioni et al. 2019). Macrophages commonly exist in two different types: classically activated M1 type which are pro-inflammatory and alternatively activated M2 type which are anti-inflammatory in nature (Cassetta et al. 2011). In this study, these two subsets of macrophages in addition to another type of macrophage called Mreg were polarized from PBMCs. Based on the observations during fluorescence microscope imaging, different morphological features were observed in all three types. In line to this result, Waldo et al showed that macrophages differentiated with GM-CSF maintained a spherical shape showing features of M1 cells while macrophages differentiated with M-CSF were elongated in shape representing M2 cells (Waldo et al. 2008). On the contrary, Bertani et al observed the morphology of M1 as spindle shaped and M2 macrophages as more spread with large multinucleated cells in epifluorescence microscopy (Bertani et al. 2017). Rostam et al showed that M1 type macrophages are small, round, or irregular shaped cells which sometimes exhibits elongated spindle shaped appearance while M2 type macrophages are large, flattened and expanded in morphology (Rostam et al. 2017). Also, the Mreg macrophages shows a spindle shaped adhesive morphology clearly different from the round and non-adhesive M1 macrophages (Suzuki et al. 2016). This suggests that morphological differences of M1 and M2 macrophages in this study are different from the previous studies of Bertani et al and Rostam et al as we observed round M1 and spindle

shaped M2 while spindle shaped Mreg macrophages were quite similar to previous studies of Suzuki et al. Different morphologies have been observed with M1 and M2 macrophages, some are in line with this study while others have variations. This could be due the direct polarization towards three different types of macrophages from PBMCs rather than from naive macrophages (M0). Moreover, in this study lower intensity of phalloidin was also observed in M2 type macrophages especially around the nucleus which could possibly be because of the elongation of the cytoskeleton. Previous work showed that M1 type macrophages have tightly packed, dotted texture of actin while M2 macrophages are the cells with soother actin staining and distributed localised spots (Rostam et al. 2017).

In this study, high number of M2 macrophages were observed in some co-cultures with ASC as compared to co-culture of M1 and Mreg type macrophages. It is well studied that ASC possess immense immunomodulatory, predominately anti-inflammatory capacity in direct contact with immune cells. They can skew the M1 towards M2 macrophages (Stojanovic & Najman 2019). From this observation, we could speculate that higher number of M2 macrophages that were observed close to ASC could be a consequence of the growth factors of ASC supporting M2 proliferation.

6.2 Surface Marker Expression

6.2.1 Adipose stem cells

According to the International Society for Cellular Therapy (ISCT), ASC are defined based on their plasticity, plastic adherence, undifferentiated state maintenance, self-renewal ability, and multi linear differentiation (Zuk 2013). In 2006, the phenotypic identification of ASC was first published by Dominici et al (Dominici et al. 2006) and in 2013 was published by Bourin et al (Bourin et al. 2013). In this study, flow cytometric results suggests that there was no change in the characteristic immunophenotype of ASC before and after weight loss as both obASC and lnASC were highly expressing the MSCs markers i.e., CD90, CD73 and CD105. On average, low expression of CD36 in obASC was observed while increased expression was observed in lnASC with variation between both donors. It can be speculated that, high CD36 expression in lnASC indicates the regain of adipogenic differentiation capacity after the weight loss. Gao et al studied that CD36 is a cellular marker defining human adipocyte progenitor (Gao et al. 2017). Previous studies also shows that expression of CD142 in subpopulation of ASC could act as adipogenesis-regulatory cells, which can suppress adipocyte formation in a paracrine manner (Schwalie et al. 2018). In this study, a slightly increased expression of CD142 was

observed in lnASC as compared to obASC having variation between both donors. These speculations are based on few CD marker expressions and the results need to be verified by more extensive studies.

6.2.2 Macrophages

A high level of phagocytic activity has been expressed by M1 like polarized macrophages and the prime CD markers to characterize these macrophages are CD64 and CD80, while the nature of M1 stimulus defines the level of expression for these markers (Tarique et al. 2015). Previously studies showed that CD163 belonging to a cysteine-rich scavenger receptor family member is distinctively expressed by M2 like macrophages (Verreck et al. 2006). M2 like macrophages are characterized based on the expression of CD64 and CD209 marker (Tarique et al. 2015). Also, Hyvärinen et al showed that Mreg type macrophages show high expression for CD86, intermediate for CD163 and low for CD206 marker (Hyvärinen et al. 2018). In this study, similar trend of different CD marker expression for three subtypes of macrophages was observed compared with the previous studies. One exception was observed for CD86 which is a typical M1 marker, but its expression was relatively high in all three subtypes of macrophages i.e., 96.7% in M1, 91.9% in M2 and 76.4% in Mreg macrophages. Also, the expression of M2 markers CD206 and CD163 increased after M2 polarization compared to M1 polarized macrophages. The expression of M1 markers CD86, CD11c and HLA-DR slightly decreased after M2 polarization compared to M1 polarized macrophages. Moreover, expression of Mreg markers was similar to what was reported by Hyvärinen et al (Hyvärinen et al. 2018). The expression of different markers decreased in Mreg macrophages having variation between two parallel samples. Malyshev & Malyshev studies ruled out that there could be no possibility to achieve fully polarized M1/M2 macrophages *in vitro* that are derived from human PBMCs (Malyshev & Malyshev 2015).

6.2.3 Immunomodulatory responses of ASC

The capacity to modulate immune responses is one reason for the success of using ASC in the treatment of different inflammatory diseases and autoimmune disorders. ASC possess immense immunomodulatory, predominately anti-inflammatory capacity in direct contact with immune cells. They can skew the M1 cells towards M2 cells (Stojanovic & Najman 2019). In this study, on average, the expression of CD markers in M1 type macrophages i.e., CD86, CD11c and HLA-DR was decreased in co-culture with both lean and obese derived ASC while the expression of M2 macrophages remained similar in mono and co-culture with lean and obese derived ASC. A mixed expression of different

CD marker expression was observed in mono and co-cultures of Mreg with lean and obese derived ASC. Variations in expression of CD206,163 and 86 was observed between both donors.

Obesity-induced inflammation affects the angiogenic, antiapoptotic and immunomodulatory properties of ASC (Badimon & Cubedo 2017). In a previous study, Serena et al showed that the differentiation and immunomodulatory capacity of ASC is highly dependent on the donor's weight (Serena et al. 2016). ASC derived from obese donors have reduced immunosuppressive properties compared with ASC derived from lean donors. Their capacity to supress M1 cells and to activate M2 cells become impaired and less effective. Serena et al also showed that lnASC can induce anti-inflammatory and immunomodulatory effects which was not in line with our study. In this study ASC did show immunomodulation but independent of the donor's weight. We can speculate that our results are partly contradictory compared with Serena et al because of the different study design. Serena et al cultured the M0 macrophages in the conditioned medium of lean and obese derived ASC to measure the immunosuppressive properties of ASC. On the contrary, this study contains the direct co-cultures of different types of polarized macrophages with lean and obese derived ASC.

There were no significant differences observed amongst the mono and co-cultures of macrophages due to small sample size. More donors must be included in the study to show the statistically significant differences.

6.3 Cytokine Analysis

Macrophages differentiated from PBMCs when polarized into subtypes can produce cytokines and chemokines e.g., polarized M1 cells secrete IL-1B, TNFα, IFN-g while polarized M2 cells secrete IL-13, CCL17, and CCL18 (Tarique et al. 2015). Also, it is known that the secretome of ASC is enriched in cytokines, growth factors, angiogenic factors, and adipokines which supports the regenerative capacity of ASC (Dubey et al. 2018).

6.3.1 IL-6

The secretion of IL-6 was significantly increased in the co-cultures of lnASC with M1 and Mreg macrophages. Interestingly, co-cultures of obASC with M1 and Mreg macrophages exhibit a significantly increased secretion of IL-6 compared with monocultures of the same conditions. It is known that the elevated levels of leptin in obesity stimulates the secretion of IL-1, IL-6, IL-12, and TNFα by innate immune cells (Carbone et al. 2012). The ASC in a niche of subcutaneous WAT of obese individuals commit themselves to

adipocyte differentiation showing upregulation of inflammatory gene expression related to the loss of their differentiation and proliferative capacity (Onate et al. 2013). In line with previous research, IL-6 secretion was high in the monocultures of ASC cultured in M1 and Mreg condition and was increased in co-cultures of M1 and Mreg macrophages with ASC as compared with M2 co-cultures and monocultures. This result might suggest that our polarized Mreg macrophages show pro-inflammatory phenotype. Trayhurn studied that the hypoxia in obese AT stimulates the secretion of cytokines e.g., TNF- α, IL-6, IL-10, and CCL-2 (MCP-1) causing the infiltration of pro-inflammatory macrophages (Trayhurn 2013). Moreover, the highly significant secretion of this cytokine in co-cultures of obASC with M1 and Mreg type macrophages suggest that obASC shows pro-inflammatory nature.

6.3.2 Interleukin-1B

It was observed that IL-1B secretion was increased in co-cultures of M1 with lean and obese derived ASC compared to other studied co-cultures. The secretion was significantly high in M1 monocultures compared with M2 monocultures. It is known that cells of the innate immune system secrete IL-1B which is a potent pro-inflammatory cytokine produced without any signal sequence (Lopez-Castejon & Brough 2011). The phagocytic activity of macrophages is promoted by many pro-inflammatory cytokines e.g., TNFα, IL-1B and GM-CSF etc (Ren & Savill 1995). Based on the previous work this result also suggests the pro-inflammatory nature of polarized M1 macrophages.

6.3.3 IL-12p70

IL-12p70 is considered as a pro-inflammatory cytokine responsible for inflammation (Konenkov et al. 2018). In this study, on average, high secretion of IL-12p70 was observed in all mono and co-cultures of Mreg. The secretion was significantly increased in monocultures of Mreg macrophages compared with M1 and M2 monocultures. The secretion significantly increased in M1 co-cultures with both lnASC and obASC compared with M1 monoculture. Also, increased secretion was observed in obese Mreg co-cultures as compared to lean Mreg co-cultures. From previous studies it has been shown that obASC secrete more pro-inflammatory cytokines. Increased liberation of pro-inflammatory cytokines has been shown by obese derived ASC (Pérez et al. 2013). IL-12p70 is a heterodimeric cytokine consisting of alpha (IL-12p35) and beta (IL-12p40) chains and belongs to cytokine family IL-12. Alpha chain shares the homology of IL-6 cytokine while beta chain shares the homology with soluble cytokine receptor chains such as IL-6RA (Collison & Vignali 2008). It is also known from the studies of Fernandez-Botran that

soluble cytokine receptors e.g., IL-6RA can regulate cytokine activity *in vivo* and have immunoregulatory implications (Fernandez-Botran 1991). From this observation it can be suggested that IL-12p70 is a regulatory cytokine rather than inflammatory. On the other hand, it can also be hypothesized that polarized Mreg in this study exhibits pro-inflammatory nature.

6.3.4 Monocyte chemoattractant protein 1 (MCP-1/CCL2)

Significantly high secretion of MCP-1 was observed in monocultures of Mreg macrophages compared with M1 and M2 monocultures. Also, the secretion significantly increased in co-cultures of M1 macrophages with both lnASC and obASC compared with M1 monocultures. Increased secretion of this chemokine was observed in obese Mreg co-cultures compared to lean Mreg co-cultures. It has been discussed in previous works that obASC release enhanced levels of pro-inflammatory cytokines (Pérez et al. 2013). MCP-1 chemokine regulates monocyte chemotaxis and plays a critical role during inflammation (Bianconi et al. 2018). Takahashi et al observed that obese individuals have elevated plasma concentrations of MCP-1 compared to lean individuals (Takahashi et al. 2003). The ASC of obese individuals are dysfunctional and not able to maintain the tissue homeostasis, rather than that their impaired nature causes detrimental effects due to the secretion of pro-inflammatory cytokines such as MCP-1 and TNFα (Pérez et al. 2013). This observation also suggests that polarized Mreg macrophages in this study were having pro-inflammatory phenotype.

6.3.5 IL-1RA

A significantly higher secretion of IL-1RA was observed in M1 co-cultures with lnASC and obASC compared with M1 monocultures. Increased secretion of IL-1RA was observed in monocultures of ASC compared with macrophage monocultures. Also, high levels of secretion were observed in co-cultures of lnASC and obASC with Mreg macrophages. In relation to this study, MSCs secretes IL-1RA in an inflammatory milieu, which polarizes the M1 cells towards M2 (Luz-Crawford et al. 2016). This result suggests the immunosuppressive capacity of ASC may be increased under the inflammatory environment. It was also observed from previous work of Jiang & Xu that the microenvironment of MSCs can switch the macrophages between pro and anti-inflammatory phenotype i.e., insufficient inflammation can promote the MSCs to create a pro-inflammatory environment (Jiang & Xu 2020).

In this study, the results of the cytokine analysis and their statistics suggest that obese co-cultures were secreting more pro-inflammatory cytokines compared with lean co-cultures (Figure 41). The secretion of pro-inflammatory cytokines in obese co-cultures was higher with M1 and Mreg macrophage which are pro-inflammatory in nature. From the previous studies in humans, elevated gene expression of IL-6, TNFα, IL-1 and MCP-1 have been shown in obese individuals (Chylikova et al. 2018).

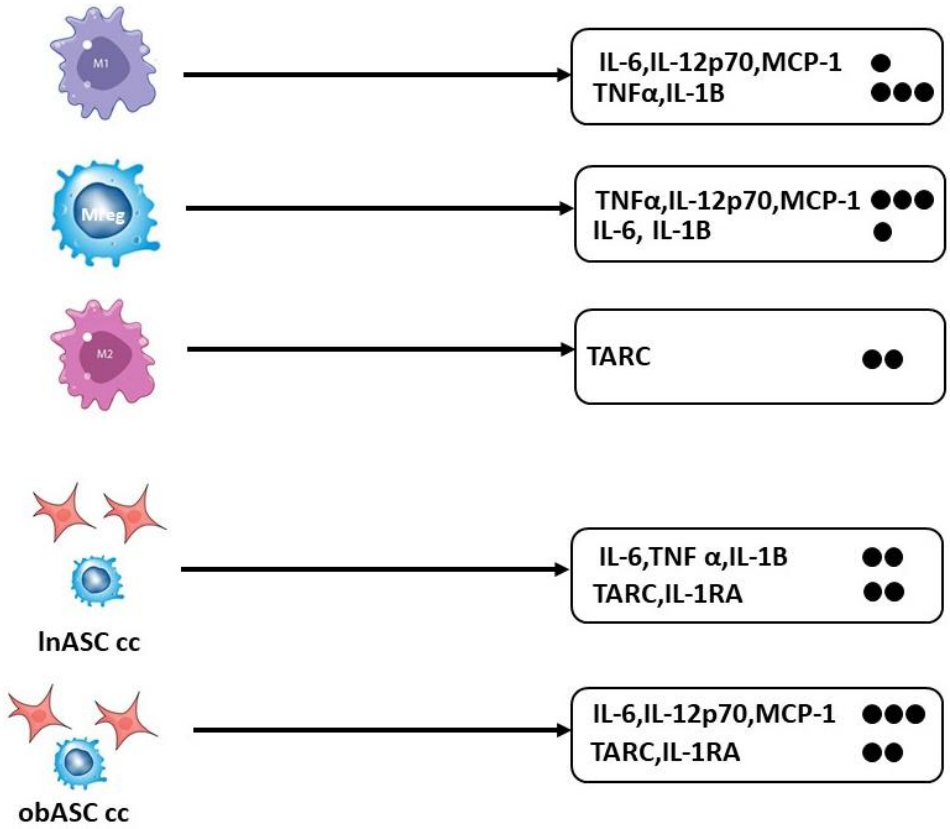

Figure 41. *Schematic presentation of cytokine secretion from mono and co-culture of different macrophages with lean and obese ASC. ● depicts low, ●● moderate and ●●● high secretion of cytokines*

6.4 Metabolic Analysis

In obesity, the inflammatory processes give rise to the production of ROS causing oxidative stress upon the adipocytes (de Mello et al. 2018). The excessive nutrient uptake leads the mitochondria to release more ATP which at the same time generates excessive ROS. Therefore, metabolic imbalance affects the capacity of mitochondria to generate and sustain enough ATP levels which causes the abnormal function of mitochondria. Underwood et al showed that obesity increases the inflammation, oxidative stress, and mitochondrial activity in WAT (Underwood et al. 2020). It is known that decrease in mitochondrial oxidative capacity is associated with obesity. Insulin resistance, metabolic alterations and low-grade inflammation during obesity is related to downregulation of

mitochondrial functions and oxidative metabolic pathways in subcutaneous AT (Heinonen et al. 2015). It is also observed from previous studies of Pérez et al that ASC isolated from obese human donors have decreased mitochondrial content and functions with impaired respiration capacity compared to lean donors (Pérez et al. 2015). In this study, an increase in basal, maximal respiration and spare capacity of both lnASC and obASC was observed under inflammatory conditions during measured time points as compared to control media. Bondia-Pons et al studied that in obesity phenomena like inflammation and oxidative stress are interacting side by side causing mitochondrial alteration and overproduction of ROS (Bondia-Pons et al. 2012).

During the study, the metabolic activity of ASC based on donor weight was also observed which suggests that lnASC have increased cellular respiration capacity as compared to obASC at least regarding the maximal respiration and spare respiratory capacity, although there was variation between the donors. Our results suggest that ASC have increased cellular respiration capacity after the weight loss.

7. CONCLUSIONS

In this study effects of obesity on immunomodulatory and metabolic capacity of ASC were analysed. Furthermore, it was demonstrated that differentiated macrophage types can be polarized from frozen PBMCs *in vitro*, i.e., M1, M2 and Mreg macrophages with characteristic morphology, phenotype, and cytokine secretion.

The pro-inflammatory capacity of Mreg type macrophages was studied. It was observed that Mreg macrophages are pro-inflammatory and regulatory in nature. The expression of pro-inflammatory CD markers i.e., CD86, CD11c and HLA-DR was higher compared to anti-inflammatory CD markers. Also, they secrete more pro-inflammatory cytokines compared to anti-inflammatory cytokines.

The secretion of pro-inflammatory cytokines particularly, IL-6, IL-12p70 and MCP-1 was observed to be higher in ASC from obese donors. Also, the secretion of these cytokines was significantly higher in co-cultures of M1 type macrophages with both lean and obese derived ASC comparing to M1 monocultures.

It was also studied that the ASC after weight loss have increased cellular respiration capacity. Moreover, an inflammatory environment rapidly increases the cellular respiration capacity of ASC *in vitro*.

Our expectation from the study was to observe a reduction in cellular respiration of ASC obtained from obese donors under an inflammatory condition. However, the results obtained from this study were unexpected to certain degree as we observed an increase in cellular respiration with M1 conditioned media. Although the reaction was quite clear and interesting, we could speculate that it is an acute reaction to increased inflammation and M1 conditioned medium might not fully mimic the chronic low-grade inflammation that is present in obese AT. In this study, the increase in cellular respiration under inflammatory conditions might be the first acute response to the inflammation. A better model to mimic low grade inflammation is required to achieve desired results during seahorse study.

The model of this study may need different approaches and some further optimization. For example, during macrophage polarization with co-culture, ASC could be added at an earlier stage of the polarization process to have stronger effect on macrophage differentiation with a longer co-culture time. Also, more samples will be needed to find the significant differences and specific data to study the immunomodulatory capacity of ASC in obese state.

Hence, more experiments with higher number of ASC obtained from obese and lean donors and optimized study protocols will provide further insight of obesity induced inflammation and its effects on immunomodulatory functions of ASC.

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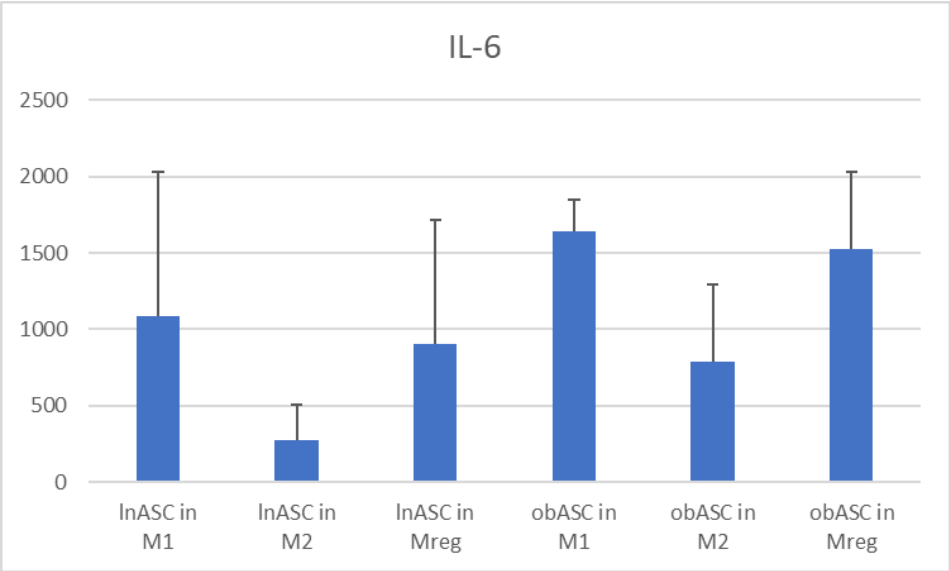
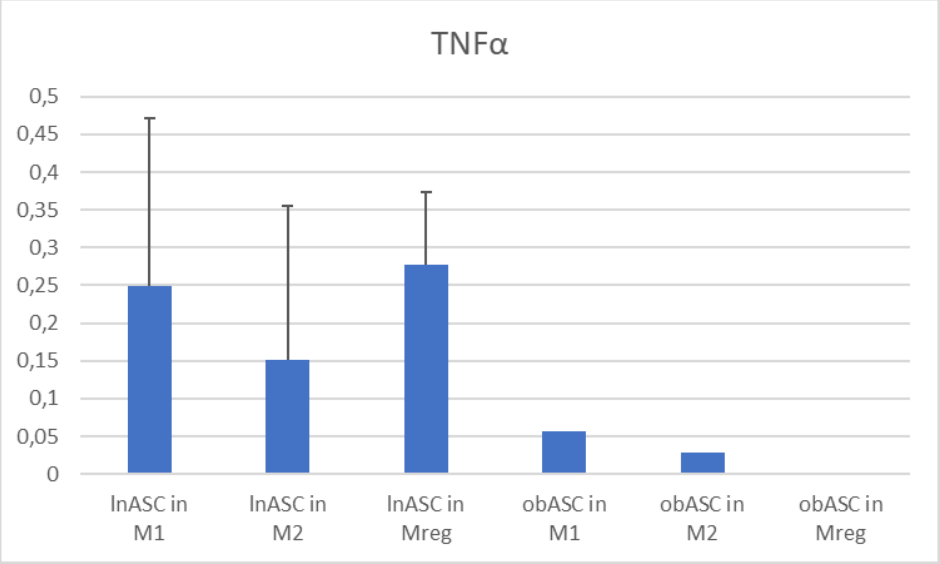
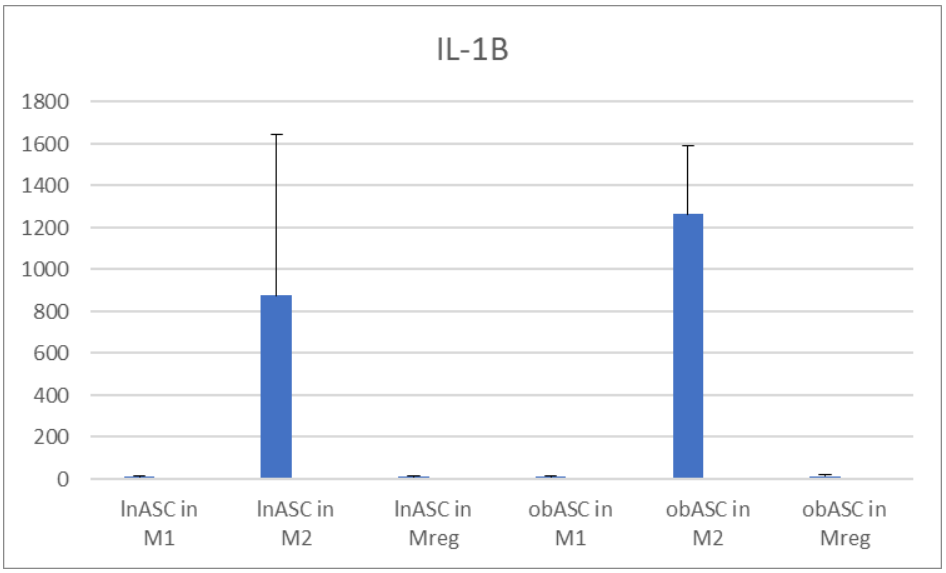
APPENDICES



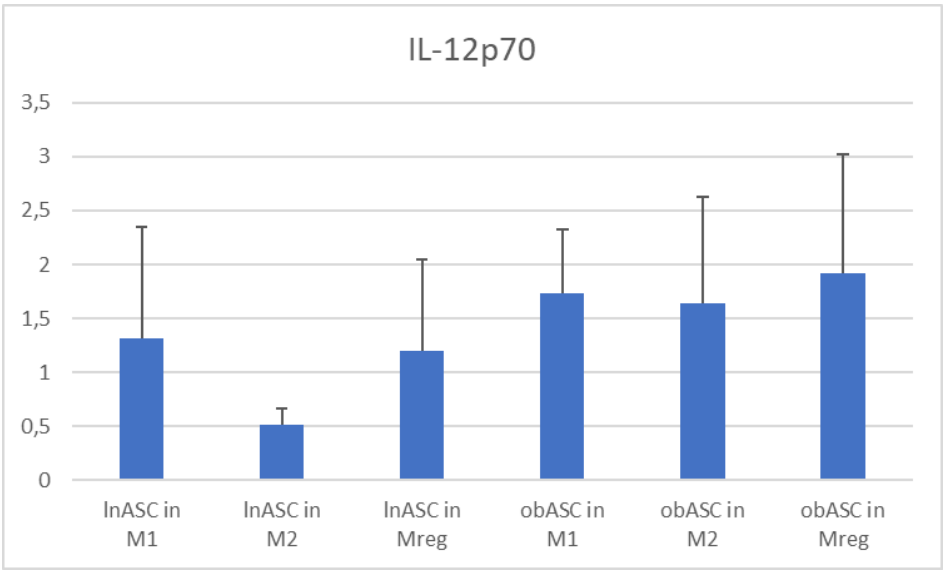
Appendix fig 1. *Comparison of IL-6 secretion between monocultures of lnASC and obASC in different conditions*



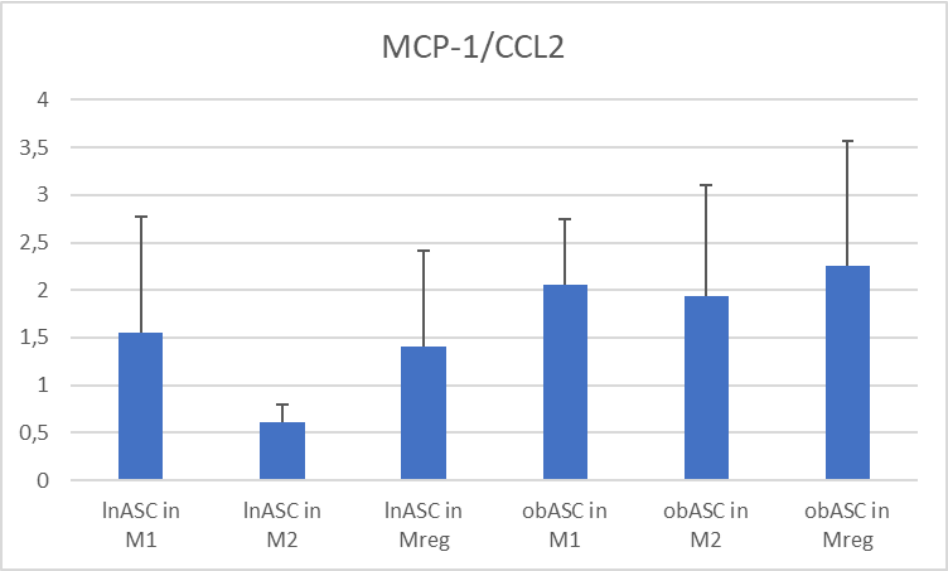
Appendix fig 2. *Comparison of TNFα secretion between monocultures of lnASC and obASC in different conditions*



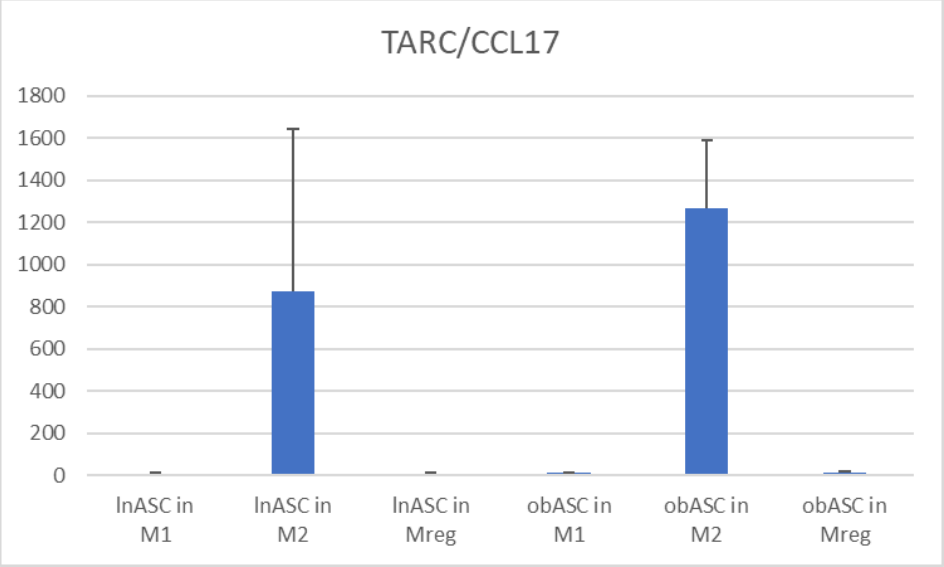
Appendix fig 3. *Comparison of IL-1B secretion between monocultures of lnASC and obASC in different conditions*



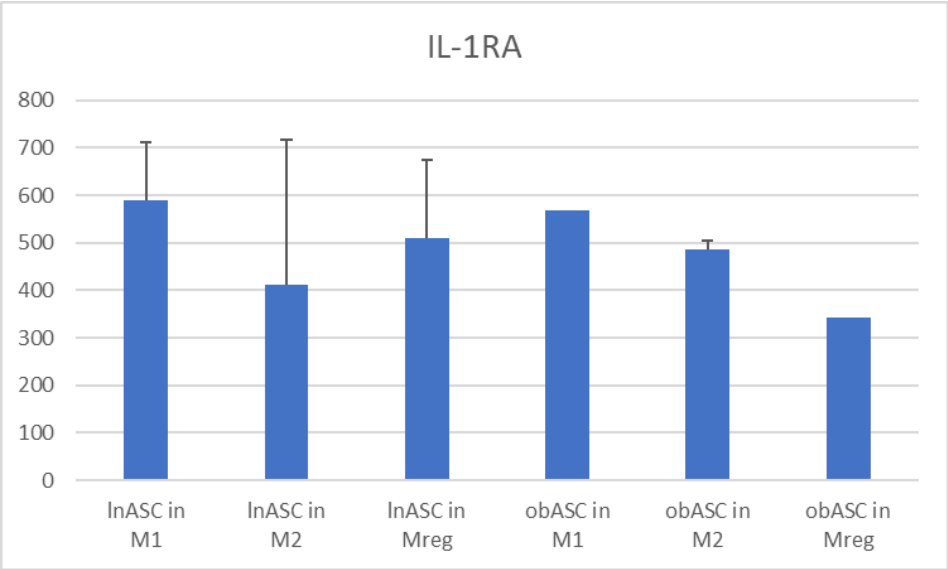
Appendix fig 4. *Comparison of IL-12p70 secretion between monocultures of lnASC and obASC in different conditions*



Appendix fig 5. *Comparison of MCP-1/CCL2 secretion between monocultures of lnASC and obASC in different conditions*



Appendix fig 6. *Comparison of TARC/CCL17 secretion between monocultures of lnASC and obASC in different conditions*



Appendix fig 7. *Comparison of IL-1RA secretion between monocultures of lnASC and obASC in different conditions*