Activation of Intracellular Signalling Pathways in Adipose Stem Cell-mediated Immunosuppression

Laura Oksa

Master's Thesis

BioMediTech

University of Tampere

28.04.2016

Pro Gradu –tutkielma

Paikka: TAMPEREEN YLIOPISTO
BioMediTech (BMT)
Tekijä: OKSA, LAURA KAROLIINA
Otsikko: Rasvakudoksen kantasolujen sisäisten signaalireittien aktivoituminen immuunivasteen muodostumista ehkäisevissä reaktioissa.
Sivumäärä: 65
Ohjaajat: FT Mimmi Patrikoski, FT Sari Vanhatupa
Tarkastajat: Apulaisprofessori Heli Skottman, FT Mimmi Patrikoski
Päiväys: 28.04.2016

Tiivistelmä

Tutkimuksen tausta ja tavoitteet: Rasvakudos on monikykyisten kantasolujen lähde. Rasvakudoksen kantasoluilla on erinomainen kyky jakaantua ja erilaistua eri solutyypeiksi. Rasvakudoksen kantasolut eivät myöskään aiheuta voimakasta immunologista vastetta sekä niillä on kyky säädellä elimistön immuunireaktiota. Rasvakudoksen kantasolujen immunologisia ominaisuuksia on tutkittu jo aikaisemminkin, mutta tarkemmat solunsisäiset mekanismit ovat edelleenkin epäselviä. Tämän työn tarkoituksena oli tutkia rasvakudoksen kantasolujen kykyä säädellä immuunireaktioita sekä suorassa että epäsuorassa yhteisviljelmässä veren perifeeristen mononukleaarisolujen kanssa. Lisäksi tutkittiin solunsisäisiä signaalireittejä (STAT1, STAT3, NF-κB ja Smad1/5) rasvakudoksen kantasoluissa ja veren mononukleaarisoluissa yhteisviljelmän aikana.

Tutkimusmenetelmät: Rasvakudoksen kantasolujen kykyä alentaa immuunivastetta tutkittiin neljän luovuttajan soluilla. Immuunivasteen säätelyä tutkittiin rasvakudoksen kantasolujen ja veren mononukleaarisolujen suorilla ja epäsuorilla yhteisviljelmillä (two-way mixed lymphocyte reaction; MLR). Rasvankudoksen kantasolujen tehokkuutta säädellä immuunivastetta tutkittiin BrdU ELISA –määrityksellä. Signaalireittien aktivaatiota tutkittiin Western Blot -analyysin avulla MLR-näytteistä.

Tutkimustulokset: Kolme neljästä käytetystä rasvakudoksen kantasolulinjasta alensi huomattavasti immuunivastetta veren mononukleaarisoluissa. Suorassa yhteisviljelmässä immuunivasteen säätely oli voimakkaampaa verrattuna epäsuoriin yhteisviljelmiin. Rasvakudoksen kantasolut hillitsivät voimakkaammin toista veren perifeeristen mononukleaarisolujen yhdistelmää (MLR2) verrattuna toiseen yhdistelmään (MLR1). Signaalireittien aktivaatioissa oli suurta vaihtelua eri kantasolulinjojen välillä. Kaksi signaalireittiä (NF-κB ja Smad1/5) aktivoituivat vain suorissa reaktioissa kun taas STAT3- ja STAT1-reitit aktivoituivat myös epäsuorissa reaktioissa. NF-κB:n fosforylaatio inhiboitui ja Smad1/5 aktivoitui vain toisen luovuttajan soluilla suorissa reaktioissa. Fosforyloidun STAT3:n tuotto inhiboitui suorissa reaktiossa, mutta pääsääntöisesti aktivoitui epäsuorissa reaktioissa. STAT1 aktivoitui vaihtelevasti sekä veren mononukleaarisoluissa että rasvakudoksen kantasoluissa.

Johtopäätökset: Rasvakudoksen kantasoluilla on kyky vähentää immuunivastetta veren mononukleaarisoluissa sekä suorassa että epäsuorassa yhteisviljelmässä. Solujen immuunisäätelyn taustalla olevien signaalimekanismien määritykseen ja varmentamiseen tarvitaan lisää toistoja käyttäen useampia rasvakudoksen kantasolulinjoja ja veren perifeeristä mononukleaarisolulinjoja.

Master's Thesis

Place: UNIVERSITY OF TAMPERE
BioMediTech (BMT)
Author: OKSA, LAURA KAROLIINA

Title: Activation of Intracellular Signaling Pathways in Adipose Stem Cell –mediated immunosuppression

Pages: 65
Supervisors: PhD Mimmi Patrikoski, PhD Sari Vanhatupa
Reviewers: Associate professor Heli Skottman, PhD Mimmi Patrikoski
Date: 28.04.2016

Abstract

Background and aims: Adipose tissue is a source of multipotent adipose stromal/stem cells (ASCs). ASCs have excellent proliferation and multilineage differentiation capacity, low immunogenicity and promising immunosuppressive capacity. The immunosuppressive properties of ASCs have been previously studied but detailed intracellular mechanisms are still unknown. The aim of this study was to investigate the suppressive potential of ASCs in direct versus indirect co-culture with peripheral blood mononuclear cells (PBMCs) and to identify the signaling pathways (STAT3, STAT1, NF-κB and Smad1/5) that are activated during ASC-mediated immunosuppression.

Methods: Four different ASC donors were used to study the immunosuppressive capacity of ASCs. Mixed lymphocyte reactions (MLR) using direct and indirect co-cultures of ASCs and PBMCs were used to study the immunosuppressive capacity of ASCs. Immunosuppression was analyzed using BrdU-ELISA. Activation of intracellular signaling pathways were analyzed using Western Blot –analysis from direct and indirect MLR samples.

Results: Strong immunosuppression on PBMC proliferation was obtained with three ASC donors. ASCs in direct co-culture with PBMCs had stronger immunosuppressive capacity compared to indirect co-cultures. ASCs cultured with MLR2 combination possessed stronger immunosuppressive capacity compared to MLR1 combination. There was variation in the activation of intracellular signaling pathways between different donors. Two signaling pathways (NF-κB and Smad1/5) were activated only in direct reactions compared to pathways STAT3 and STAT1, which were activated also in indirect reactions. NF-κB phosphorylation was inhibited in ASCs in direct reactions. Activation of Smad1/5 was donor-specific and it was activated with other ASC donor and inhibited with another ASC donor. In direct reactions STAT3 phosphorylation was inhibited and in indirect reactions STAT3 was mainly activated. STAT1 was phosphorylated in PBMCs with three ASCs donors and in two ASC donors in indirect reactions.

Conclusion: ASCs have immunosuppressive capacity when co-cultured with PBMCs in both direct and indirect cultures. More studies with more ASC and PBMC donors are needed to obtain more detailed information about intracellular signaling behind the immunosuppression of ASCs.

Acknowledgements

This Master's thesis was conducted in Adult Stem cell group, at the institute of Biosciences and Medical Technology (BioMediTech), University of Tampere.

I would like to express my gratitude to our group leader, docent, Susanna Miettinen, for giving me the opportunity to accomplish this study under her professional supervision. I also want to express my gratitude to my supervisor Mimmi Patrikoski. I want to thank Mimmi for her excellent guidance in laboratory work, scientific writing and all the support that she has given me during this project. I have learned a lot from you.

I want to express my gratitude to whole Mese-group for advice, support and encouraging atmosphere of the "Mese" office room. Special thanks to Miia Juntunen, Anna-Maija Honkala and Sari Kalliokoski for technical assistance and advises.

I also want to thank my family and friends. Thank you for all the encouragement and support you have given me. Especially, thank you for my little brother Lauri and friends around the ice hockey, you know how to cheer me up.

Table of contents

Abbreviations

ALK	activin-like kinase receptor
ASC	adipose-derived stem cell
BM	basic medium
BM-MSC	bone marrow-derived mesenchymal stem cell
c-myc	myc proto-ongogene protein
CB-MSC	cord blood-derived mesenchymal stem cell
CXCL	CXC chemokine ligand
DC	dendritic cell
ECL	enhanced chemiluminescene detection
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular signal-regulated kinase
ESC	embryonic stem cell
GVHD	graft-versus-host-disease
HFSC	humat fat deriven stem cell
HLA	human leukocyte antigen
HS	human serum
ICAM-1	intracellular adhesion molecule 1
IDO	idoleamine 2,3-deoxygenase
IFATS	Internationa Federation of Adipose Therapeutics and Science
IFN-γ	interferon γ
IFN-γ-R1	interferon γ receptor 1
IL	interleukin
IL-6R	interleukin 6 receptor
iPSC	induced pluripotent stem cell
ISCT	International Society for Cellular Therapy
JAK	Janus kinase
Klf-4	Kruppel-like factor4
MLR	mixed lymphocyte reaction
MSC	mesenchymal stem cell
NF-κB	nuclear factor κ B
NK	natural killer cell
Oct 3/4	octamer-binding transcription factor 3/4
p/s	penicillin/streptomycin
PBMC	peripheral blood mononuclear cell
PGE2	prostaglandin E2
PI3K	phosphoinositide 3-kinase
PLA	processed lipoaspirate
R-Smad	receptor-regulated Smad
SOCS3	suppressor of cytokine signaling 3
SOD	superoxide dismutase
Sox2	sex determining region Y box 2
STAT	signal transducer ad activator of transcription

SVF	stromal vascular fraction
TGF-β	transforming growht factor β
Th	T-helper cell
TLR	Toll-like receptor
TMB	tetramethylbenzidine
TNF-α	tumor necrosis factor α
Treg	T-regulatory cell
TβG-II	transforming growht factor β type II receptor
VCAM-1	vascular cell adhesion protein 1

1 INTRODUCTION

During the last couple of decades, regenerative medicine has evolved tremendously with advances in stem cells research. However, the availability of cells remains a challenge in the usage of stem cells in regenerative medicine (Wankhade, et al. 2016). Previously human adipose tissue was perceived as worthless and unwanted, but during the last 15 years adipose tissue has emerged as a premiere cell source for regenerative medicine (Kapur, et al. 2015). The global interest in the use of adipose tissues as a cell source has growth during last decease probably due to easy isolation (Kapur, et al. 2015, Katz, et al. 1999, Zuk, et al. 2001).

Human adipose stromal/stem cells (ASCs) are multipotent cells that have the potential to differentiate into adipogenic, chondrogenic and osteogenic cells (Zuk, et al. 2001, Zuk, et al. 2002). Moreover, ASCs have shown to have potential to differentiate into neuronal cells, hepatocytes and pancreatic islet cells (Tsuji, et al. 2014, Zuk, et al. 2002, Zuk. 2010). Additionally, ASCs have low immunogenicity and promising immunosuppressive potential (McIntosh, et al. 2006, Niemeyer, et al. 2007). These characteristics of ASCs make them an attractive cell source for clinical treatments and applications such as allogeneic cell therapy (Atoui and Chiu. 2012, Baer. 2014, McIntosh, et al. 2006, Niemeyer, et al. 2007, Patrikoski, et al. 2014).

Promising results have been published considering the immunomodulative functions of ASCs. Previous studies have shown that allogeneic ASCs do not produce severe immune reaction *in vivo* (Kuo, et al. 2011). The anti-inflammatory cytokine milieu of ASCs has been shown to modulate the function of immune cells (Kuo, et al. 2011, McIntosh, et al. 2013). Also clinical trials for treatment of autoimmune diseases with ASCs have been reported (Fang, et al. 2007a, Garcia-Olmo, et al. 2009). The immunomodulative properties of ASCs have been studied worldwide but the specific intracellular mechanisms behind those are still unknown.

In this thesis the immunosuppressive capacity of ASCs is studied in direct and indirect mixed lymphocyte reaction (MLR) cultures with peripheral blood mononuclear cells (PBMCs). Four ASC donors and two MLR combinations were used to study the immunosuppressive capacity. Activation of intracellular pathways in ASC –mediated immunosuppression was also studied. This thesis provides a preliminary data from intracellular signaling in ASC –mediated immunosuppression.

2 REVIEW OF THE LITERATURE

2.1 Stem Cells

Stem cells are the self-renewing progenitors of several tissues. They have the ability to provide undifferentiated stem cells and the capacity to differentiate into one or more committed descendants (Choumerianou, et al. 2008). The multipotency and the capacity to self-renew make stem cells attractive candidates for regenerative medicine, tissue engineering and replacement therapies (Choumerianou, et al. 2008). Additionally, human stem cells provide understanding of embryonic development, disease progression and pharmaceutical and toxicological research (Choumerianou, et al. 2008, Wobus and Boheler. 2005).

There are many types of stem cells but all stem cells share similar features (Brignier and Gewirtz. 2010). Stem cells are classified according to their origin to embryonic, germinal or somatic (fetal or adult) stem cells (Choumerianou, et al. 2008). The most common source of adult stem cells, somatic stem cells of adult tissues, is the bone marrow but these cells can be obtained also from adipose tissue or less-mature sources such as the umbilical cord blood, the placenta and fetal tissues. Cells can be classified into totipotent, pluripotent and multipotent stem cells according to their differentiation capacity (Figure 1). Totipotent cells are the most primitive cells and they have the capacity to develop into a complete embryo. Totipotent cells appear after fertilization and the totipotency disappear by the time embryo reach the 8-cell stage (Brignier and Gewirtz. 2010). The cells from inner cell mass of the blastocyst, termed as pluripotent cells, are capable of differentiating into cells of all three embryonic germ layers: ectoderm, mesoderm and endoderm. The reprogrammed somatic cells are called induced pluripotent stem cells (iPSCs). By the time and after division pluripotent cells lose the capacity to form entire organs (Brignier and Gewirtz. 2010) and cells become multipotent stem cells that can differentiate only into specific cell lines (Choumerianou, et al. 2008). Multipotency is the property of adult stem cells that are able to self-renew during the lifetime of an organism generating new differentiated daughter cells (Brignier and Gewirtz. 2010). In fact, stem cells are not only involved in embryogenesis but also in maintenance of normal homeostasis of adult tissues (Rao and Mattson. 2001).

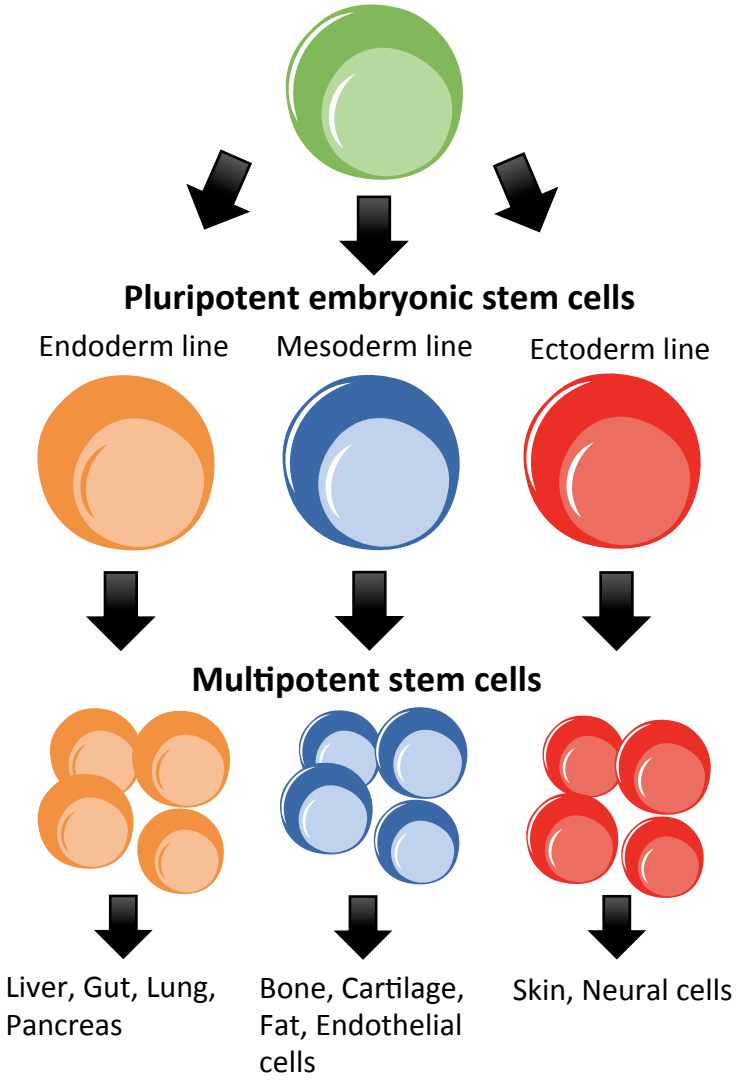

Figure 1. Hierarchy of stem cells. Stem cells have the ability self-renew and differentiate into specialized cell types. Totipotent stem cells exist only for a short time after fertilization before cells become embryonic stem cells (ESCs), which can be derived from inner cell mass of blastocyst. Pluripotent stem cells are able to form multipotent stem cells of three germ lines (endo-, ecto- and mesoderm), which are further able to differentiate cells of specific tissues.

2.1.1 Embryonic and induced pluripotent stem cells

The first embryonic stem cell line was derived from mouse blastocyst in 1981 (Evans and Kaufman. 1981, Martin. 1981) and 17 years later, in 1998, the first human embryonic stem cell (hESC) line was isolated by James Thomson and co-workers from blastocyst (Thomson, et al. 1998). After the first isolation, hESCs have been under extensive research but also surrounded by ethical and political issues (Thomson, et al. 1998). In Finland, hESCs can be isolated only from excessive embryos from in vitro fertilization with the donor's written

consent. The production of embryos exclusively for research purposes shall be forbidden; only *in vitro* fertilized embryos, which would otherwise be discarded, can be used (Medical Research Act, Laki Lääketieteellisestä tutkimuksesta 488/1999, referred 2.9.2015). However, hESCs research policies vary enormously between different countries (Wobus and Boheler. 2005).

Human ESCs are derived from the inner cell mas of blastocyst after five days from fertilization. Cells are cultured on top of fibroblast layer to support the hESCs and to help the hESCs to form tight stem cell colonies (Mallon, et al. 2006). Human ESCs have several attractive features, which make them promising for the regenerative medicine and will help to understand the mechanisms of embryonic development and disease progression (Choumerianou, et al. 2008). Human ESCs are pluripotent cells having the capacity to differentiate into all cell types of the human body. The ESCs lines express high levels of telomerase activity that maintain the telomere length of the cells and plays an important role in hESCs replicative life-span. Human ESCs are able to maintain their pluripotency during the culturing due to their efficient self-renewal ability (Choumerianou, et al. 2008, Jensen, et al. 2009, Thomson, et al. 1998, Wobus and Boheler. 2005).

Although hESCs provide a great promise for regenerative medicine, there are also problems related to use of hESCs. It is necessary to pay attention to questions related to directed differentiation, immune responses and oncogenic properties of hESCs before clinical applications can be considered (Choumerianou, et al. 2008, Wobus and Boheler. 2005). To ensure patient safety in clinical application, it is also recommended to use non-animal culturing methods to prevent cross-species contaminations such as foreign pathogens, concerning the use of all stem cells not only hESCs. However, the properties of hESCs make them valuable tools for pharmacological tests and *in vitro* models development. The data obtained from animal tests, e.g. testing drug pharmacokinetics, may not be optimal because the effects between humans and animals can be different and thus, the data may not serve fully reliable for humans (Choumerianou, et al. 2008).

Nowadays it is possible to generate pluripotent stem cells form a patient's own cells by the defined factors (Takahashi and Yamanaka. 2006). These iPSCs and hESCs share similar morphology, proliferation, surface antigens, gene expression, epigenetic status of pluripotent cell-specific genes and telomerase activity. Similar to hESCs, iPSCs are capable of differentiating into all cell types of the three germ layers and to form teratomas *in vivo*

(Takahashi, et al. 2007). Takahashi and Yamanaka have been demonstrated four factors, Oct3/4, Sox2, c-Myc and Klf4, which can be used to induce somatic cells into iPSCs by reprogramming the cells back into an embryonic/pluripotent state (Takahashi and Yamanaka. 2006). However, hESCs are isolated from embryos, which make hESCs face multiple ethical questions. iPSCs are reprogrammed from donor's somatic cells and do not face same ethical problems than ESCs, but still provide a useful tools to study same targets than hESCs (Takahashi, et al. 2007).

Although the several advantages of hESCs and hiPSCs they also have disadvantages (Fong, et al. 2010). HESCs may have problems related to immunorejection when the cells are not patient's own, but immunorejection can be avoided by reprogramming patient's own somatic cell to hiPSCs (Fong, et al. 2010). However, the pluripotency of hESCs and hiPSCs can cause chaotic differentiation and teratomas Transcription factors c-Myc and Klf-4 that are used in reprogramming the somatic cells to hiPSCs are oncogenes and if these factors are overexpressed, tumors can be formed (Fong, et al. 2010).

2.1.2 Adult stem cells

Adult stem cells are multipotent cells, which have the potential to differentiate into one or several cell types that maintain and repair damaged and old tissues. Compared to ESCs and iPSCs, adult stem cells have limited self-renewal capacity restricted to specific tissue or organ. Nevertheless, there are less ethical and safety issues related to the use of adult stem cells compared with the use of pluripotent stem cells (Brignier and Gewirtz. 2010, Choumerianou, et al. 2008).

The hematopoietic stem cells and bone marrow stromal cells were the first discovered stem cells that were isolated from bone marrow (BECKER, et al. 1963, Friedenstein, et al. 1968, Till and McCulloch. 2012). Since then, adult stem cells have been isolated from several tissues, and in theory, adult stem cells can be isolated from almost all tissues of an adult individual and can be divided into three categories according to the germ layer of which they originate: ectodermal, mesodermal or endodermal stem cells (Choumerianou, et al. 2008).

2.2 Mesenchymal stem cells

Mesenchymal stem cells (MSCs) were first isolated and characterized from bone marrow in 1974 by Friedenstein (Friedenstein, et al. 1974). Afterwards, MSCs have been isolated from

different tissues, e.g., bone marrow, peripheral blood, umbilical cord blood, amniotic membrane and adult connective, adipose and dental tissues with similar properties (Dominici, et al. 2006, Tatullo, et al. 2015). MSCs have the capacity for multipotential differentiation and also for immunomodulation by sensing and controlling the inflammation (Brignier and Gewirtz. 2010, Dominici, et al. 2006, Eggenhofer, et al. 2014). MSCs repair injured and old tissues by regeneration (Caplan. 2015). Inflammation occurring during the injury serves a condition for MSCs to start the tissue regeneration on site of injury.

The Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) has been develop the minimal criteria to define human MSC. According of these criteria, MSC must be plastic-adherent when cultured in standard culture conditions, must express surface marker CD105, CD73 and CD90; lack the expression of CD45, CD34, CD14 or CD11b, CD79 or CD19 and HLA-DR surface molecules and must differentiate to osteoblast, adipocytes and chondroblasts in vitro (Dominici, et al. 2006). According to ISCT guidelines, MSCs could alternatively be called as multipotent mesenchymal stromal cells, because the lack of the total stemness compared to pluripotent stem cells (Brignier and Gewirtz. 2010, Dominici, et al. 2006).

MSCs isolated from different sources have similar characteristics, although variations exist in abundance, differentiation potential, phenotype and immunomodulatory capabilities (Hanley. 2015). Bone marrow-derived mesenchymal stem cells (BM-MSC) are the most studied MSCs and most of clinical trials have used BM-MSCs (Hanley. 2015). However, the BM-MSCs need multiple passages and numerous cell doublings to reach the sufficient cell amount for clinical trials. Also the age of bone marrow donor have effects on the amount of stem cells in bone marrow. Compared to bone marrow the adipose tissue contains 2,500 times more MSCs (Brignier and Gewirtz. 2010, Hanley. 2015). ASCs have been reported to have better immunosuppressive and proliferation capacity than BM-MSCs but the osteogenic and chondrogenic differentiation potential may be lower compared to BM-MSCs (Hanley. 2015, Lotfy, et al. 2014, Najar, et al. 2010). Besides of bone marrow and adipose tissue, cord blood is one potential source of MSCs, but it has the lowest frequency of MSCs at around 0,00003% of all cells in the cord blood unit (Hanley. 2015). However cord blood-derived MSCs (BC-MSC) have been reported an excellent proliferation capacity (Hanley. 2015).

Since 1990s, use of MSCs have become more popular and their use in clinical therapies has start to evolve (Caplan. 1991, Caplan. 2015). The multipotency, immunomodulatory effect

and diversity of sources of MSCs made them an interest target of clinical therapies. Nowadays over 500 clinical trials studying MSCs are ongoing and these trials use MSCs to treat medical disorders utilizing the immunomodulatory or regenerative potential of MSCs (Caplan 2015).

2.3 Adipose stem cells

Katz, Zuk and co-workers were first who reported that the stromal vascular fraction (SVF) isolated from lipoaspirates contained cells referred as processed lipoaspirate cells (PLAs) (Katz, et al. 1999, Zuk, et al. 2001). PLAs were reported to have multilinage differentiation potential into adipogenic, osteogenic, chondrogenic and myogenic cells (Zuk, et al. 2001). After the discovery of ASCs, they have been one of the most popular adult stem cell populations in the stem cell field (Zuk. 2010). After it was recognized that adipose tissue is not only an energy reservoir, it has been intensively studied as a cell source for tissue engineering, regenerative medicine and for immunomodulatory purposes (Baer. 2014, Lotfy, et al. 2014). ASCs can be obtained from adipose tissue by a minimal invasive method, which results in a high number of cells, compared to the BM-MSCs (Tsuji, et al. 2014). Zuk *et al.* introduced a widely used method for isolation of ASCs from adipose tissue in 2001 (Zuk, et al. 2001). Adipose tissue is first mechanically disrupted followed by enzymatic digestion by using collagenase and centrifugation to obtain SVF pellet from adipose tissue. Red blood cells are lysed from SVF, which is then filtered to remove cellular debris. With further SVF culturing only plastic adherent ASCs are obtained and other cells of SVF will be disposed during the medium changes (Baer. 2014, Zuk, et al. 2001).

ASCs are of mesodermal origin so they have the capacity to differentiate into adipogenic, osteogenic and chondrogenic cells (Zuk, et al. 2001, Zuk, et al. 2002). Additionally, it is reported that ASCs have the potential to differentiate into ectodermal and endodermal origin such as neuronal cells or epidermal cells and hepatocytes and pancreatic islet cells (Baer and Geiger. 2012, Tsuji, et al. 2014, Zuk. 2010). Because ASCs may have the potential to differentiate into cells of all three germ layers (meso-, ecto- and endoderm), it is speculated that the term pluripotent stem cells would be correct for ASCs rather than multipotent (Baer and Geiger. 2012, Zuk. 2010). However, the pluripotency of ASCs is not accepted in the scientific community because the morphology of ASCs varies form pluripotent stem cells and they are not able to form teratomas (Baer and Geiger. 2012). ASCs can be directed to

differentiate toward a desired direction using linage-specific induction media *in vitro* (Tsuji, et al. 2014).

It is characterized that ASCs are rather heterogenic than homogenous cell population (Baer and Geiger. 2012, Baer. 2014, Zuk, et al. 2002). Currently, there is no unique surface marker for ASCs, but these cells express mesenchymal markers. ASCs express CD13, CD29, CD44, CD63, CD73, CD90 and CD105 and lack the expression of hematopoietic markers CD14, CD31, CD45 and CD144 (Tsuji, et al. 2014). In 2013, International Federation for Adipose Therapeutics and Science (IFATS) published a revised statement for minimal phenotypic criteria to characterize the uncultured SVF and the adherent stromal/stem cell population from adipose tissue (Baer. 2014, Bourin, et al. 2013). This statement requires ASCs to be positive for CD44, CD73, CD90 and CD105 and negative for CD45 and CD31. Still more studies are needed to identify unique surface markers for ASCs (Baer. 2014). Expression of CD34 surface marker show positive expression during the first passages of ASCS but CD34 expression decreases after further passaging (Baer and Geiger. 2012, Baer. 2014, Tsuji, et al. 2014). Although ASCs are heterogenic cell population, it is considered that passaging select cell population with more homogenous cell surface markers compared to SVF (Tsuji, et al. 2014).

ASCs isolated from different donors have variation in characteristics, which are affected by age, body mass index, gender, ethnicity and medical history including preexisting diseases, smoking or alcohol abuse (Baer and Geiger. 2012). It has been shown that cells from younger donors (<40 years) have better viability, proliferation and differentiation capacity of ASCs (Choudhery, et al. 2014). Osteogenic and chondrogenic differentiation is negatively affected by increasing age of donor whereas adipogenic differentiation potential is positively affected by increasing age. Also, ASCs from younger donors catalyzes the conversion of superoxide radicals (O^{2-}) to hydrogen peroxide and further to oxygen and water more efficiency than ASCs from older donors (>50 years). Shortly, the superoxide dismutase (SOD) activity protects cells from aging and age-related variations such as mutations by converting the harmful superoxide radicals to oxygen and water. The SOD antioxidant activity is higher in ASCs from young donors (Choudhery, et al. 2014). Also body mass index (BMI) has been shown to negatively correlate with the number of the stromal cells per adipose tissue gram and also differentiation capacity of ASCs is affected by BMI (Baer and Geiger. 2012). Conflicting result has been published on the effects of gender on the differentiation potential of ASCs. In 2008, Aksu *et al*. reported that ASCs from male donors differentiate more rapidly

and efficiently into osteogenic cells compared with ASCs from female donors (Aksu, et al. 2008). However, in 2014 Yang *et al.* published an opposite results, where they showed no difference in osteogenic differentiation potential between ASCs from male and female donors (Yang, et al. 2014). Also tissue harvest location will affect to the characteristics of ASCs. ASCs harvested from subcutaneous fat have been shown better proliferation and adipogenic differentiation capacity than ASCs isolated from visceral or omental fat (Baglioni, et al. 2012). Additionally, cells derived from donors with inflammatory diseases such as severe ischemic heart disease, diabetes and renal failure have documented reduced proliferation and differentiation capacities (Atoui and Chiu. 2012). Alcohol abuse has been shown to decrease the osteogenic and adipogenic differentiation of ASCs (Huff, et al. 2011).

2.4 Clinical applications

In February 2016, a total of 191 clinical trials studying ASCs were found on the www.ClinicalTrials.gov database. However, all 191 trials did not use expanded ASCs but at least in 43 clinical trials cells of SVF were used. 139 clinical trials were progressed into phase I or II, whereas only 13 were progressed into phase III or IV. For immunological disorders, containing also Crohn's disease and osteoarthritis, 37 clinical trials studied the use of ASCs. The clinical trials at www.ClinicalTrials.gov site were carried out by both companies and universities.

Although ASCs have shown therapeutic potential, the number of clinical trials studying ASCs for immunological/inflammatory diseases is relatively low. ASCs have been used to treat inflammatory diseases such as graft-versus-host-disease (GVHD), severe sepsis, Crohn's disease and rheumatoid arthritis. As an example, Garcia-Olmo *et al.* have reported promising results of phase II trial where ASC have used to treat perianal fistulas associated with Crohn's disease (Garcia-Olmo, et al. 2009, Garcia-Olmo, et al. 2015). One half of ASCs were injected under the epithelium along the fistulas and the other half were mixed to fibrin glue and injected directly to the fistula (Garcia-Olmo, et al. 2009). In further study for 7 patients from 10, ASCs were mixed to fibrin glue and for 3 patients ASCs alone were used (Garcia-Olmo, et al. 2015). The treatment proved to be safe and more effective than the old treatment using fibrin glue alone without ASCs (Garcia-Olmo, et al. 2009, Garcia-Olmo, et al. 2015). The immunomodulation capacity of ASCs is likely involved in the therapeutic effects of cells that were used for the treatment of Crohn's disease, as described by Garcia-Olmo.

Additionally, Fang and co-workers have also reported promising results of clinical case studies to prevent the steroid-resistance acute GVHD by using allogeneic ASCs (Fang, et al. 2007a, Fang, et al. 2007b, Fang, et al. 2007c). Two children received 1 x 10^6 allogeneic ASCs per kilogram by intravenous infusion (Fang, et al. 2007b). Both children responded to the treatment and after 12 months both were alive and well (Fang, et al. 2007b). Seven adult patients also received 1-2 x10^6 allogeneic ASCs per kilogram by intravenous infusion and successful results were reported in 5 of 7 patients (Fang, et al. 2007a, Fang, et al. 2007c). It is suggested that the outcomes of successful treatments may be explained by shifting of pro-inflammatory cytokine milieu to anti-inflammatory milieu (Lin, et al. 2012).

Riordan *et al.* have reported a clinical case study where three multiple sclerosis patients were treated with combination of autologous adipose-derived SVF, allogeneic CD34+ cells and allogeneic ASCs (Riordan, et al. 2009). Two of the patients received cells with intravenous infusion within 10 days period and one of the patients received the cells within 9 days period. All of the patients tolerated the cell infusions well and no significant side effects were observed. The study showed improvements in patient's conditions and the need for regular medications was decreased. However, the MRI images revealed that lesions in the brains were similar after the treatment (Riordan, et al. 2009).

Furthermore, TiGenix NV is a leading European cell therapy company that has focused to development of cell therapies with allogeneic ASCs for treatment of inflammatory and autoimmune diseases (http://www.tigenix.com). In March 2015, the company completed a phase I trial where safety and efficacy of allogeneic ASCs were studied for treatment of sepsis using intravenous infusion of 0,25 - 4 x10^6 ASCs/kg for 32 healthy male volunteers, who were challenged with a bacterial endotoxin to elicit an inflammatory response inducing sepsis-like clinical symptoms (NCT02328612; www.clinicaltrials.gov). Currently, a phase I/II clinical trial is ongoing evaluating the use of allogeneic ASCs for the treatment of chronic GVHD (NCT01222039, www.clinicaltrials.gov). This study will investigate the safety and feasibility of intravenously injected allogeneic ASCs 1-3 x10^6 cells/kg used with combination of a gradually decreasing dosage of the conventional treatment.

2.5 Immunological properties of ASCs

MSC, including ASCs, have low immunogenicity and a promising immunosuppressive potential, which make ASCs an attractive cell source for clinical treatments and applications such as allogeneic cell therapy (Atoui and Chiu. 2012, Baer. 2014, McIntosh, et al. 2006, Patrikoski, et al. 2014). Allogeneic MSCs may not be fully immunopriviledged, but the rate of immune detection of MSCs is low and determined by the balance between cell's relative expression of immunogenic and immunosuppressive factors (Ankrum, et al. 2014).

2.5.1 ASC have low immunogenity

The ASCs have a low immunogenicity due to a lack of major histocompatibility complex (MHC) class II molecule expression and low expression of T- and B-cell co-stimulatory molecules CD40, CD80 and CD86 that are required for complete T-cell activation (Leto Barone, et al. 2013, McIntosh, et al. 2006, Niemeyer, et al. 2007). It has been shown that ASCs do not stimulate a proliferative response of allogeneic T-cells when used as stimulator cells in a one-way lymphocyte reaction (MLR) assay (McIntosh, et al. 2006, Niemeyer, et al. 2007, Puissant, et al. 2005). Low immunogenicity of ASCs is promising concerning the use of allogeneic ASCs in future cell therapies without severe immune reactions (Leto Barone, et al. 2013, McIntosh, et al. 2013). The ability to use allogeneic ASCs in cell therapy would be beneficial for clinical demands, and thus, donors with optimal characteristics, such as young healthy donors, could be chosen for each cell therapy. *In vivo* studies have also shown that allogeneic ASCs do not produce immune reaction (Jeong, et al. 2014, Kuo, et al. 2011, McIntosh, et al. 2013). Kuo *et al.* and Jeong *et al.* have shown similar results of allotransplant survival (Jeong, et al. 2014, Kuo, et al. 2011) and demonstrated that ASCs modulate immune systems, significantly prolong allotransplant survival times and changes in anti-inflammatory cytokine expression that leads to altered T-cell functions (Kuo, et al. 2011).

It is demonstrated that immunogenicity of ASCs decreases along cell passaging and SVF cells may have stronger immunogenicity compared with cells at higher passages (Leto Barone, et al. 2013, McIntosh, et al. 2006, Wang, et al. 2015). Wang *et al.* demonstrated that a secretion of several key immunoinhibitors, such as interleukin-10 (IL-10) and hepatocyte growth factor (HGF), is decreased at higher ASCs passages (Wang, et al. 2015). Furthermore, the IFN-γ secretion was found to be higher in cultures where PBMCs were co-cultures with ASCs with

higher passage number (Wang, et al. 2015). During passaging only minor changes occur in immunophenotype of ASCs, while the cells appear to lose their immunomodulatory properties (Wang, et al. 2015). SVF's may have more immunogenic properties, which may be caused by cell subpopulations with different properties (McIntosh, et al. 2006). Additionally, it is also suggested that differentiation of ASCs may change the immunogenicity of cells (McIntosh, et al. 2013). Although osteogenic and adipogenic differentiation of ASCs may not affect to immunogenicity, chondrogenic differentiation may decrease the immunogenicity of the differentiated ASCs (Kim, et al. 2014, McIntosh, et al. 2013, Niemeyer, et al. 2007). Immunogenicity of ASC population undergoing differentiation may depend on the ratio of differentiated and undifferentiated cells as undifferentiated cells have stronger immunogenicity and differentiated cells may have lower immunosuppressive properties (McIntosh, et al. 2013).

2.5.2 Immunosuppressive capacity of ASCs

ASCs have immunosuppressive properties due to both cell-cell interactions and inflammatory cytokine expression (McIntosh, et al. 2006). Immunosuppression is a complex phenomenon, which has been explained using a following theory. ASCs have been demonstrated to have immunosuppressive functions, by inhibiting the production of inflammatory cytokines, such as interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α), and stimulating the production of anti-inflammatory cytokines, such as transforming growth factor beta (TGF-β), idoleamine 2,3-dioxygenase (IDO) and prostaglandin E2 (PGE2) (Ankrum, et al. 2014, Atoui and Chiu. 2012, Leto Barone, et al. 2013, Patrikoski, et al. 2014). ASCs have been also shown to stimulate the production of antigen specific T-regulatory cells (Tregs) (Leto Barone, et al. 2013). The concentrations of TNF-α, IFN-γ and IL-6 are increased under inflammatory conditions that further induce the immunosuppressive potential of ASCs (Crop, et al. 2010, Leto Barone, et al. 2013). Additionally, TNF-α has been shown to increase the immunosuppresive capacity of ASCs by significantly increasing the PGE2 production of ASCs. PGE2 is a product of arachidonic acid metabolism and acts as a powerful immunosuppressant, inhibiting T-cells proliferation, dendritic cells (DCs) maturation and production of TNF- α and IL-2 (Ghannam, et al. 2010). IFN-γ have an effect on immunomodulatory functions of ASCs by directly inducing the production of IDO that further affects the tryptophan metabolism in T-cells by degrading tryptophan and inhibiting the T-cell proliferation and natural killer cells (NK cells) activity (Atoui and Chiu. 2012, Leto

Barone, et al. 2013). TGF-β have several effects, e.g. it suppress T-cell proliferation, inhibits the maturation of macrophages, NK cells and DCs, activates the differentiation of Tregs and induces the production of cytokines such as TNF-α and IFN-γ (Melief, et al. 2013, Yoshimura, et al. 2010). IL-6 has both anti- and pro-inflammatory effects and it has also functions in regulation of metabolic, regenerative and neural processes (Melief, et al. 2013, Scheller, et al. 2011). Thus, depending on context, IL-6 can either support or suppress the inflammation. Anti-inflammatory effects of IL-6 are inhibition of monocyte and DCs maturation and T-cell proliferation (Ghannam, et al. 2010). When activated T-cells produce TNF-α and IFN-γ, it activates ASCs to produce cytokines (e.g. IL-6, INF-γ, TNF-α and TGF-β), which suppress the proliferative response of T-cells. Thus, the feed-back-loop inhibition effectively reduces the amount of TNF-α and IFN-γ produced by activated T-cells (Leto Barone, et al. 2013).

Di Nicola *et al.* were the first to demonstrate that immunosuppression is stronger in direct MLR co-culture of ASCs and PBMCs compared to indirect co-cultures, where ASCs and PBMCs are separated by a semipermeable membrane that allow the movement of cytokines but prevent the cell-cell contacts (Di Nicola, et al. 2002). The study was performed with BM-MSCs but MSCs derived from different adult tissues have reported to have similar immunomodulatory properties (Ankrum, et al. 2014, Leto Barone, et al. 2013, Yoo, et al. 2009). BM-MSCs have shown to express integrins, intracellular adhesion molecule 1 (ICAM-1, CD54) and vascular cell adhesion protein 1 (VCAM-1, CD105) and other adhesion molecules, by which they form high affinity bonds between BM-MSCs and T-lymphocytes (Haddad and Saldanha-Araujo. 2014). Because ASCs do not express VCAM-1 on the cell surface, the ASC-mediated immunosuppression may be ICAM-1- but not VCAM-1–mediated. Furthermore, it has been shown that activated PBMCs are bound to ASCs, which supports the importance of direct cell-cell interaction in immunosuppression (Quaedackers, et al. 2009).

2.6 Signaling pathways in ASC-mediated immunosuppression

Immunomodulation of ASCs includes several signaling pathways, but in this work, only specific pathways are described. These signaling pathways are complex and not well-known, in fact, there are no published studies on the signaling pathways related to ASC-mediated immunomodulation. However, several cytokines such as IL-6, TNF-α, IFN-γ and TGF-β that are secreted during immunosuppression (Patrikoski, et al. 2014) activate signaling pathways, which lead to anti-inflammatory functions, such as inhibition of proliferation, differentiation

and activation of T-cells and regulation of pro- and anti-inflammatory cytokine, chemokine and adhesion molecule expression.

IL-6 is a cytokine that have both pro- and anti-inflammatory functions depending on the context (Scheller, et al. 2011). It is secreted by monocytes, T-cells, fibroblasts, endothelial cells and several other cell types during the inflammation (Schaper and Rose-John. 2015). Depending the function of IL-6 it can activate either classical or trans-signaling pathways (Figure 2) (Schaper and Rose-John. 2015, Scheller, et al. 2011, Wolf, et al. 2014). The anti-inflammatory functions of IL-6 are mediated through activation of classical signaling pathway (Figure 3). During immunosuppression, IL-6 activates the classical signaling pathway by binding the IL-6 receptor (IL-6R) in surface membrane of T-cells, monocytes and neutrophils (Schaper and Rose-John. 2015, Scheller, et al. 2011, Wolf, et al. 2014). The IL-6/IL-6R– complex binds to two molecules of type I transmembrane signal transducer protein gp130, which results the signal initiation and activation JAK/STAT, ERK and PI3K signal pathways (Schaper and Rose-John. 2015, Wolf, et al. 2014). Classical IL-6 mediates activation of the signal transducer and activator of transcription 3 (STAT3), which is one of the most important signaling pathway for immunosuppression because it is negative regulator of inflammatory responses (Liu, et al. 2015). Classical STAT3 activation decreases maturation of DCs, inhibits activation and proliferation of lymphocyte and inhibits activation of macrophages (Park 2004, Luig 2015, Najar 2009, Scheller 2011Luig, et al. 2015, Najar, et al. 2009, Park, et al. 2004, Scheller, et al. 2011). IL-6R is expressed only on a limited number of cell types, such as immune cells and hepatocytes, resulting the immunosuppressive functions. Most cell types are not able to express the IL-6R, whereas all cells display gp130 on the cell surface. The cells that only express gp130 proteins on their surface can respond to a complex of IL-6 bound to soluble form of IL-6 receptor and induce process called *trans-signaling*. This complex binds to two gp130 molecules on cell membrane, which activates STAT3 signaling pathway. The pro-inflammatory functions of IL-6 are activated by trans-signaling pathway, which regulates T-cell differentiation via STAT3 activation. (Liu, et al. 2015, Scheller, et al. 2011, Wolf, et al. 2014) By inhibiting this pathway it is possible to modulate immunosuppressive properties of MSCs (Liu, et al. 2015).

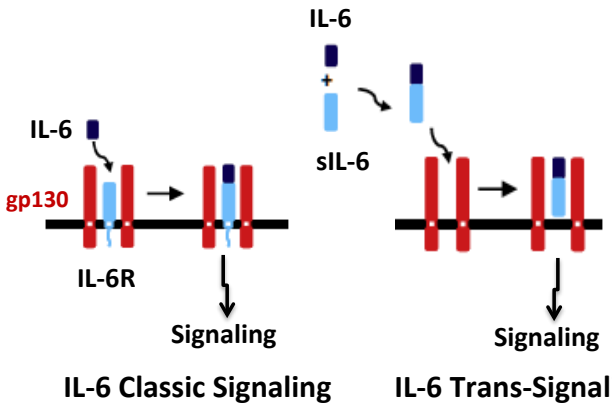

Figure 2. IL-6 mediated signaling. In IL-6 Classic Signaling, IL-6 activates the signaling pathway by binding the IL-6 receptor (IL-6R) in surface membrane. This complex binds to two gp130 proteins, resulting the initiation of signal pathway. In IL-6 Trans-Signaling, IL-6 activates the signaling pathway by binding the soluble IL-6 receptor (sIL-6R). This complex binds to two gp130 proteins, resulting the initiation of signal pathway. Figure modified from Scheller *et al.* The pro- and anti-inflammatory properties of the cytokine interleukin-6 (Scheller, et al. 2011).

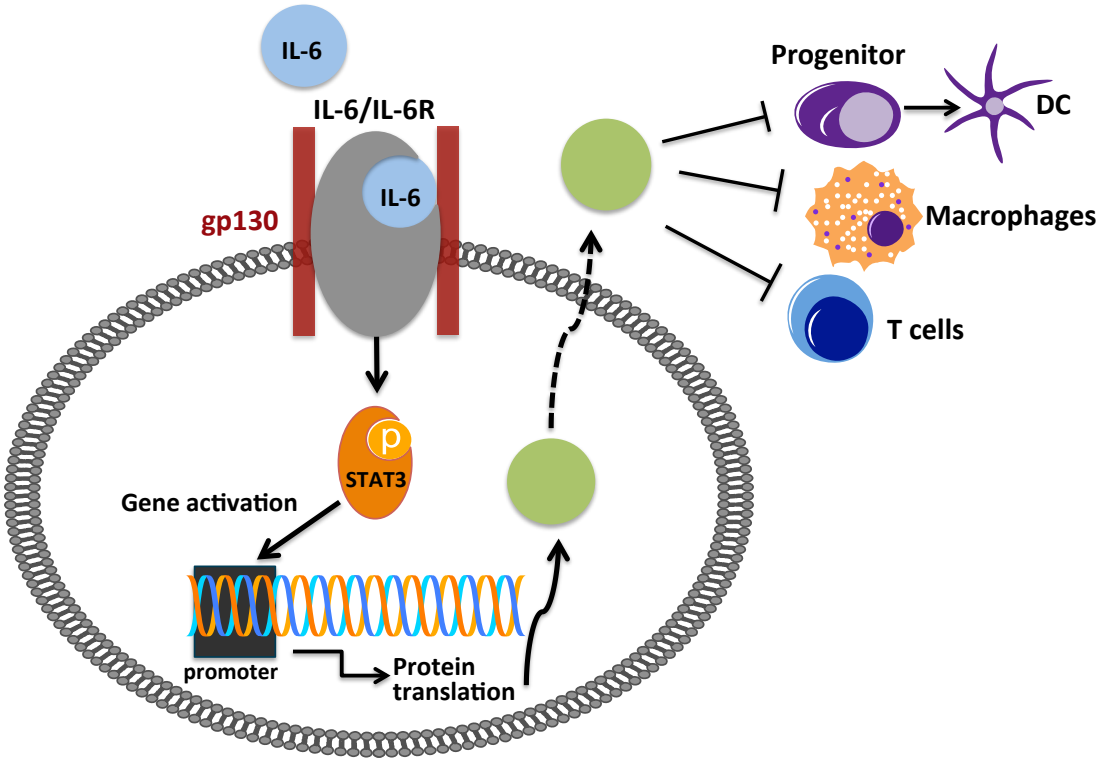

Figure 3. STAT3 activation via classical interleukin-6 (IL-6) signaling pathway. IL-6 functions as an anti-inflammatory cytokine when IL-6 binds to IL-6 receptor (IL-6R) on cell surface. This is followed by STAT3 phosphorylation, gene activation and finally protein translation that leads to inhibition of dendritic cells (DCs) maturation, T-cell activation and proliferation and macrophages activation.

TNF-α is a pro-inflammatory cytokine that is secreted from activated T-cells during inflammation (Pistoia and Raffaghello. 2014). Together with IFN-γ they are the key cytokines to activate the immunomodulative functions on MSCs (Prasanna, et al. 2010). TNF-α has immunosuppressive functions activating the nuclear factor kappa B (NF-κB). Dorronso *et al.* were first to show that TNF-α –mediated NF-κB activation in MSCs primarily leads to inhibition of T-cell proliferation and it may have some minor effects on expression of T-cell activation markers CD69 and CD25 (Dorronsoro, et al. 2014). Activated T-cells secrete TNF-α that binds to tumor necrosis factor receptor 1 (TNFR1) located on surface of MSCs activating NF-κB pathway. Activation of NF-κB pathway leads to expression of PGE2 that inhibits the proliferation of T-cells, maturation of DC cells and expression of IDO that further inhibits T-cell proliferation as shown in Figure 4 (Dorronsoro, et al. 2014, Yagi, et al. 2010). Additionally, NF-κB pathway activation inhibits the TNF-α production on T-cells and reprograms of macrophages (Dorronsoro, et al. 2014, Yagi, et al. 2010).

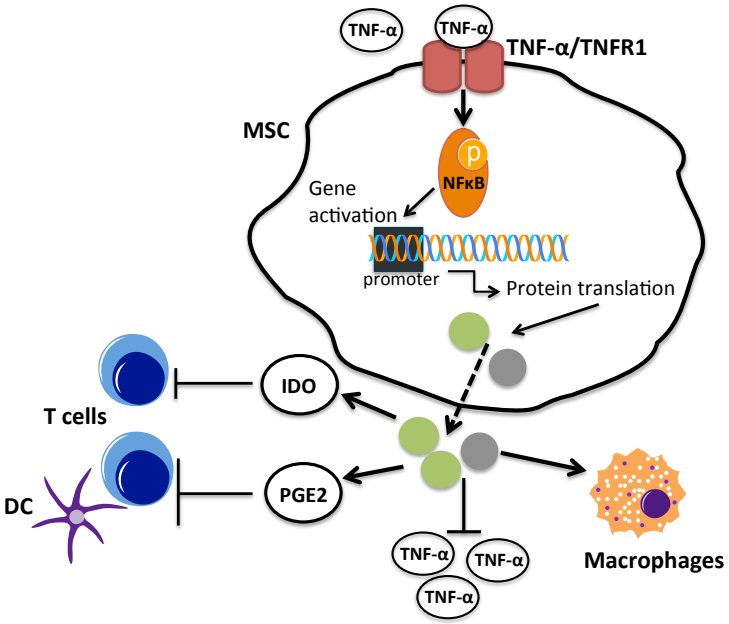

Figure 4. NF-κB activation via TNF-α. NF-κB phosphorylation is preceded by TNF-α binding to tumor necrosis factor receptor 1 (TNFR1) on the surface of MSC. Phosphorylated NF-κB activates the expression of idoleamine 2,3-dioxygenase (IDO) and prostaglandin E2 (PGE2), which inhibit T-cell proliferation and T-cell and dendritic cells (DC) differentiation. NF-κB phosphorylation also reprograms macrophages and inhibits the production of TNF-α on MSCs.

In addition to TNF-α, IFN-γ is an important pro-inflammatory cytokine that activates the immunomodulative functions on MSCs. It is secreted primarily by activated T-cells during inflammation. IFN-γ has known to induce immunosuppressive functions by the activation of

signal transducer and activator of transcription 1 (STAT1) and NF-κB pathways shown in Figure 5 (Liu, et al. 2015, Xiao, et al. 2015, Yagi, et al. 2010). STAT1 pathway is activated when IFN-γ binds to IFN-γ-receptor type 1 (IFN-γ-R1). The activation of STAT1 pathway further enhances the expression of SOCS3 (suppressor of cytokine signaling 3). SOCS3 expression inhibits STAT3 production by IL-6 trans-signaling, which is essential for the T-cell production and differentiation (Liu, et al. 2015). When IFN-γ binds to Toll-like receptor (TLR) on surface of MSCs, NF-κB pathway is activated. Activation of NF-κB pathway inhibits T-cell proliferation by expression of PGE2 and by controlling the expression of inflammatory cytokines and chemokines, such as IL-6, IL-8 and CXCL10 (Yagi, et al. 2010). Besides the activation of signaling pathways, IFN-γ also induces MSCs to produce IDO that is a key player in immunosuppression of T-cells (Prasanna, et al. 2010). IDO is not typically expressed by MSCs but in inflammatory environment IFN-γ activates the IDO production and inhibits T-cell proliferation and decrease of NK cells activity (Leto Barone, et al. 2013, Prasanna, et al. 2010).

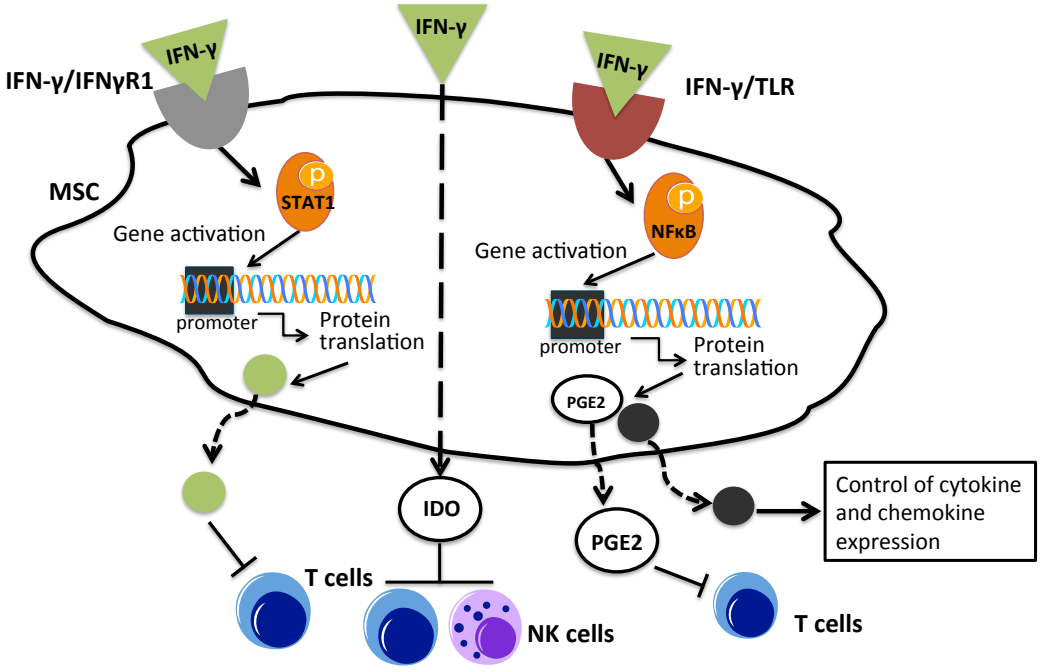

Figure 5. Immunosuppressive factors activated by IFN-γ. IFN-γ is able to activate both STAT1 and NF-κB pathways. IFN-γ binds to IFN-γ-receptor type 1 (IFN-γ-R1) on surface of MSC, activating STAT1 that is followed by gene activation and finally protein translation that leads to inhibition of T-cell production and differentiation. When IFN-γ binds to toll-like receptor (TLR), it activates NF-κB that is followed by control of cytokine and chemokine expression and production of prostaglandin E2 (PGE2) that finally inhibits T-cell proliferation. Additionally, IFN-γ is able to directly activate the IDO production, which inhibits T-cell proliferation and natural killer (NK) cells activity.

Transforming growth factor-β (TGF-β) is a pleiotropic cytokine that have both anti-inflammatory and pro-inflammatory effects depending on the cell source (Rodriguez, et al. 2015, Yoshimura, et al. 2010). TGF-β is secreted by activated T-cells and mesenchymal stem cells, especially by ASCs (Ock, et al. 2016, Patrikoski, et al. 2014, Rodriguez, et al. 2015). In mammals, three different isoforms of TGF-β (TGF-β1, -β2 and -β3) have been identified, of which TGF-β1 and TGF-β2 has been reported to have immunomodulative properties (Rodriguez, et al. 2015, Wrzesinski, et al. 2007, Yoshimura, et al. 2010). TGF-β has several immunosuppressive effects on immune cells; it inhibits proliferation of different types of immune cells, inhibits differentiation of helper T-cells (Th cells) into effector T-cells, induces the maturation of Tregs, inhibits the maturation of immune cells such as macrophages, NK cells and DCs and modulate the production of cytokines (Figure 6). The most important immunosuppressive functions are production of Treg cells and inhibition of T-cell differentiation (Gu, et al. 2012, Taylor. 2009, Wrzesinski, et al. 2007, Yoshimura, et al. 2010). Some of the immunosuppressive effects of TGF-β are mediated via activation of Smad signaling and inhibition of IL-6 trans-signaling (Liu, et al. 2015, Massague, et al. 2005). The signaling pathways mediated through TGF-β and its family members (e.g. bone morphogenetic proteins; BMPs) are complex and all mechanisms behind the immunosuppressive functions are not well-known (Massague, et al. 2005).

Principally, Smad 1 and 5 are substrates for BMP receptors but it is proven that also TGF-β is able to activate Smad1/5 (Massague, et al. 2005, Nurgazieva, et al. 2015). TGF-β binds to type I and II serine/threonine kinase receptors, promoting the formation of a hetero-tetrameric receptor complex (Massague, et al. 2005, Miyazono, et al. 2010, Vanhatupa, et al. 2015). Type II serine/threonine receptor phosphorylates a serine/threonine-rich GS region of type II serine/threonine receptor. After phosphorylation, type I receptor serves docking site for receptor-regulated Smads (R-Smads: Smad1/2/3/5/8) and phosphorylates the R-Smads. Phosphorylated Smads form complex with Smad4, which translocates into the nucleus and regulates transcription of target genes through interaction with transcription factors and transcriptional coactivators (Massague, et al. 2005, Miyazono, et al. 2010). In humans, seven different type I receptors and five different type II receptors are found, which form different receptor complexes that activate different type of Smads. Smad1/5 is phosphorylated by TGF-β, when it binds to TGF-β type II receptor (TβR-II) and activin receptor-like kinase 5 (ALK5) receptors (Massague, et al. 2005, Miyazono, et al. 2010).

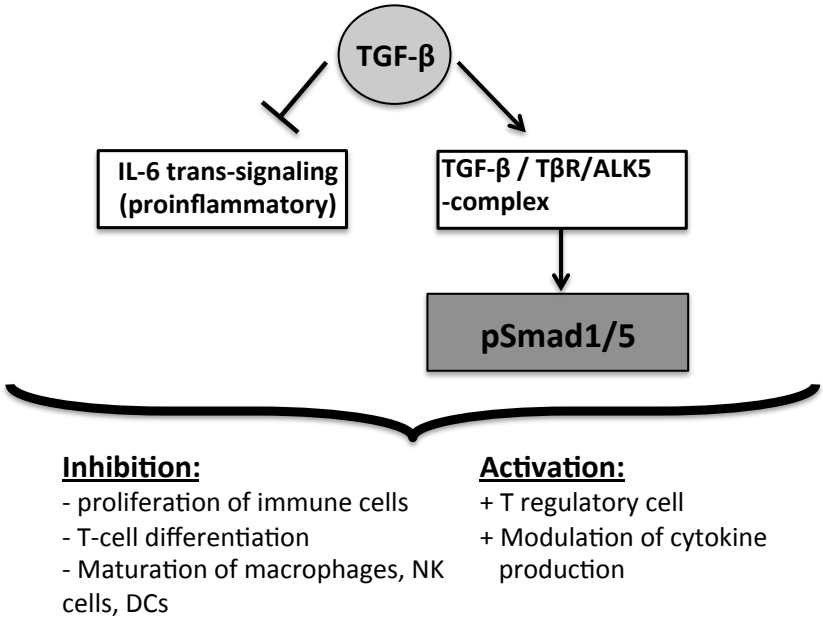

Figure 6. TGF-β in immunosuppression. TGF- β is able to inhibit pro-inflammatory effects of IL-6 by inhibiting the IL-6 trans-signaling pathway. TGF- β binds to TGF-β type II receptor (TβR-II)/activin receptor-like kinase 5 (ALK5) receptor complex that is followed by Smad1/5 activation. TGF-β has multiple functions in immunosuppression inhibiting and activating different type immune cells and cytokine production.

3 AIMS OF THE RESEARCH

The aim of this study was to analyze the immunosuppressive potential of ASCs in direct versus indirect co-culture with PBMCs and to identify the signaling pathways that are activated during ASC-mediated immunosuppression.

The specific aims of this thesis were:

1. To analyze the influence of direct versus indirect contact between ASCs and PBMCS on the immunosuppressive capacity of ACSs
2. To identify the activation of intracellular signaling pathways (STAT1, STAT3, NF-κB and Smad1/5) that were selected based on the secretion of certain cytokines (IFN-γ, IL-6, TNF-α and TGF-β) during ASC-mediated immunosuppression.

4 MATERIALS AND METHODS

4.1 Ethical consideration

The collection of adipose tissue and peripheral blood was approved by the ethics committee of the Pirkanmaa Hospital District in Tampere (ethical approval R03058). The ASCs were obtained from Tampere University Hospital in the Department of Plastic Surgery and Private Clinic Laser Tilkka (Helsinki, Finland) and the buffy coat samples from the Finnish Red Cross Blood Service. The ASCs were isolated from adipose tissue samples obtained from four female donors (ages 28, 31, 34 and 52 years). All analyses described below were performed separately with four ASC donor cell lines.

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

Allogenic human PBMCs were isolated from buffy coat samples (n=5) by density gradient centrifugation using Ficoll-Paque PLUS (density 1.077 g/mL; GE Healthcare, Little Chalfont, U.K., http://www.gehealthcare.com) according the manufacturer's instruction. After isolation the cells were aliquoted and cryopreserved in the nitrogen gas phase until co-cultures in MLRs.

4.3 Adipose stem cells isolation and cell culture

Isolation of ASCs was performed by mechanical and enzymatic procedure described previously by Zuk and co-workers (Zuk, et al. 2001). Briefly, the adipose tissue samples were cut into small fragments and then digested with collagenase type I (Gibco by Life Technologies™,Thermo Fisher Scientific, Waltham, Massachusetts, http://www.thermofisher.com/) at +37°C water bath under shaking conditions. The digested tissue was centrifuged and the fat layer was removed. The resulting pellet containing ASCs was filtered and washed to separate ASCs from the surrounding tissue. Finally, isolated ASCs were resuspended in Dulbecco's Modified Eagle medium (DMEM)/F-12 1:1 (Life Technologies™, Thermo Fisher Scientific) supplemented with 3 % human serum (human serum type AB; GE Healtcare, Pasching, Austria), 1 % L-Glutamine (GlutaMAX I; Life Technologies™, Thermo Fisher Scientific), 1 % antibiotics (p/s; 100 U/mL penicillin, 100 U/mL streptomycin, Lonza, Basel, Switzerland, www.lonza.com). This medium composition will be referred to as basic medium (BM).

Isolated ASCs were maintained and expanded in T-75 polystyrene flask (Nunc; Roskilde, Denmark, http://thermoscientific.com) in BM. For technical reasons, ASCs that were cultured in direct MLR assay were maintained and expanded in DMEM/F-12 1:1 medium supplemented with 3 % human serum (human serum type AB male; Biowest, Nuaillé, France, www.biowest.net), 1 % L-Glutamine (GlutaMAX I; Life Technologies™, Thermo Fisher Scientific), 1 % antibiotics (p/s; 100 U/mL penicillin, 100 U/mL streptomycin, Lonza). This medium composition will be referred to as Biowest basic medium (Biowest BM). ASCs were detached using TrypLE Select (Life Technologies™, Thermo Fisher Scientific), an animal origin free recombinant enzyme for dissociating mammalian cells. The expanded cells were aliquoted and cryo-preserved in the nitrogen gas phase in freezing solution (HS supplemented with 10% dimethyl sulfoxide; DMSO Hybri-Max®, Sigma-Aldrich, St.Louis, USA, http://www.sigmaaldrich.com). For experiments cells were thawed and expanded in BM. The medium was changed two times a week until the cells reached confluency or until required cell number was reached. The experiments with ASCs were made in passage 2.

The cell morphology and viability of ASCs were examined by light microscopy imaging with Nikon TS100 light microscopy unit and Nikon DS-5M-L1 camera with 4x air objective (Nikon, Tokyo, Japan, www.nikon.com).

4.4 Flow cytometric surface marker expression analysis

Flow cytometry is a laser-based technology, which is used to measure the physical and biochemical characteristics of biological particles (Jaroszeski and Radcliff. 1999). It is used to measure characteristics of whole cells as well as cellular constituents (such as organelles and nuclei) that can be labeled with a wide range of commercially available dyes and monoclonal antibodies. The principle of flow cytometry is that a single-cell suspension flows past an excitation light source. As the laser beam strikes to individual cell two types of phenomena occur: light scattering and fluorescence emission. Light scattering generates information about the cell size and surface complexity, directly related to structural and morphological cell features. Fluorescence occurs if the cells are labeled with a fluorescent probes (e.g. monoclonal antibodies conjugated to fluorochromes) and certain cells also contain endogenous fluorophores generating auto-fluorescence (Monici. 2005).

Undifferentiated ASCs cannot be identified using a single or a few surface markers. However, ASCs are commonly characterized by their immunophenotype, which is identified by a large set of surface markers. BM-MSCs and ASCs express very similar surface markers but still there are minor differences between BMSCs and ASCs. It has been reported that the expression of some surface markers change during cell culturing and passaging. Even there may be differences between cell lines, the flow cytometric analysis of the surface markers is a routine characterization method for all the new ASC donors.

After primary cell culture (passage 1), ASCs were harvested and analyzed by flow cytometry (fluorescence-activated cell sorting; FACS) (FACSAria®; BD Biosciences, Erembodegem, Belgium, www.bdbiosciences.com). Used monoclonal antibodies were against CD3-PE, CD14-PE-Cy7, CD19-PE-Cy7, CD45R0-APC, CD54-FITC, CD73-PE, CD90-APC (BD Bioscience, San Jose, USA); CD11A-APC, CD80-PE, CD86-PE, CD105-PE (R&D Sytems, Minneapolis, USA, https://rndsystems.com);CD34-APS and HLA-DR-PE (ImmunoTools GmbH, Sriesoythe, Germany, www.immunotools.de/). Analysis was performed on 10 000 cells per sample and the positive expression was defined as the level of fluorescence greater than 99 % of the corresponding unstained cell sample.

4.5 Analyses of ASC immunology

Two-way Mixed Lymphocyte Reaction assays were used to determine the immunosuppressive properties of ASCs in direct and indirect co-cultures. MLR assays were cultured in BM.

4.5.1 Two-way MLR immunosuppression assay

Two-way MLRs were performed as previously described by McIntosh *et. al.* (McIntosh. 2011). PBMCs cross-reactivity and HLA dissimilarities were determined by pre-test. Two different MLR combinations were formed according to results of pre-test and each MLR combination contained PBMCs from two different donor (Figure 4). The equal amounts of cells were mixed to activate the proliferative response of each PBMC donors. A total number of 800 000 PBMCs were seeded per well on 24-well plate (Nunc™, Thermo Fisher Scientific). After mixing the MLRs, the stimulator ASCs were added to the reactions at densities of 30 000 cells per well either in direct or indirect co-cultures using semipermeable membrane inserts to prevent direct cell-cell contacts between ASCs and PBMCs (Figure 7). When using

insert, PBMCs were pipetted to the bottom of the wells and ASCs were pipetted into the inserts (pore size 0.4µ; ThinCert, Greiner Bio-One, Aesch, Switzerland, www.gbo.com). Control wells contained only MLR combinations without ASCs or ASCs alone were also seeded. Four parallel reactions for the proliferation assay and eight parallels for the signal pathways detection were pipetted from every treatment group, and the cultures were incubated at +37°C in 5 % CO_2 for 5 days in BM (indirect co-cultures) or in Biowest BM (direct co-cultures).

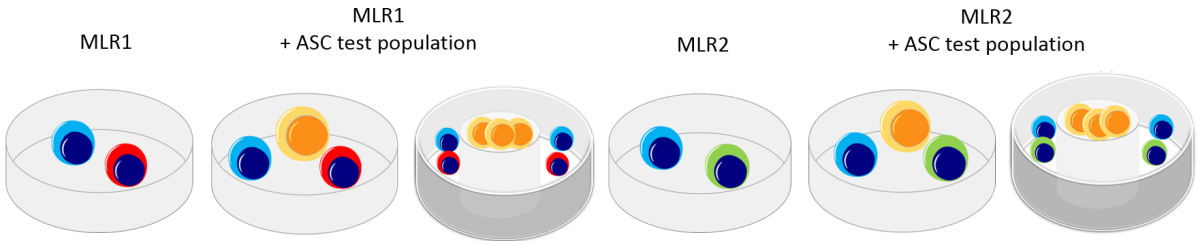

Figure 7. Schematic illustration of two-way MLR assay. Two different MLR combinations were formed from allogenic PBMCs (blue and red; blue and green). MLRs 1 and 2 were co-cultured in direct and indirect (ASCs in insert) with allogeneic ASCs (yellow) to evaluate the suppressive potential of ASCs.

4.5.2 Bromodeoxyuridine (BrdU) ELISA

On day 4 of the MLR culturing, 10 mM BrdU was added to direct MLR cultures. After adding the BrdU cells were incubated for additional 16 hours at +37°C. BrdU is a synthetic nucleoside that is an analog of thymine. It incorporates into the DNA of dividing cells instead of thymine during the 16 hours incubation. After 16 hours incubation, on day 5, PBMC proliferation was determined by BrdU enzyme-linked immunosorbent assay (ELISA) (Roche Diagnostics GmbH, Mannheim, Germany, www.roche.de/) according to the manufacturer's instructions. Briefly, cells were fixed, permeabilized and the DNA was denatured, followed by antibody binding to the incorporated BrdU. Anti-BrdU monoclonal antibody was incubated for 90 minutes followed by washes. After washes, the substrate tetra-methylbenzidine (TMB) was added which caused the blue color reaction. Finally, the reaction was terminated with sulfuric acid (H_2SO_4) and the formed color was determined at 450 nm with a microplate reader (Victor 1429 Multilabel Counter, Wallac, Turku, Finland, www.perkinelmer.com). The intensity of the color is proportional to the amount of dividing cells in the sample. Percentage immunosuppression was counted using formula: [1-(MLR+ASCs/MLR)].

4.6 Detection of activated intracellular signaling pathways using Western Blot analysis

Detection of activated intracellular signaling pathways during immunosuppression was studied using Western blot analysis.

For the Western Blot analysis, studying the activation of intracellular signaling pathways, MLR-reactions were plated in 24-well plate. In addition to MLR-reactions containing two PBMC donors and ASCs, controls wells were added containing ASC alone, MLR alone and medium alone. To achieve the needed protein concentration for western blot analysis, 8 wells per each treatment group were seeded.

After 5 days of MLR culture, cells were detached by scrapping and cell suspensions were collected into 50 ml falcon tubes. Cell suspensions containing PBMCs or cells from direct co-cultures were centrifuged (300g, 10 minutes) and cell pellets were lysed with 2x Laemmli's sample buffer (300 µl/pellet). ASCs were directly lysed with 2x Laemmli's sample buffer (300 µl/ 8 wells) in well-plates. Lysates were heated 5 min at 95°C heat block, centrifuged briefly and stored at -80°C.

Lysates were thawed by heating 5 min at 95°C heat block. For the analysis of the signaling proteins, 30 µl of cell lysates were loaded into 10 % SDS-PAGE gels. To assess the β-actin levels, the loaded cell lysate was 5 µl. The protein ladder used was Page™Ruler Plus Prestained Protein Ladder (Thermo Fisher Scientific™), the loaded amount of protein ladder was 4 µl. Gels were electrophoresed (Mini-Protean® Tetra System, Bio-Rad, Hercules, California, USA, http://www.bio-rad.com) with the run parameters 70 V ~20 min+ 140 V ~1 h. After the electrophoresis, the proteins were transferred from the gels to MetOH (EMD Millipore) activated PVDF membranes (Amersham Hybond™ 0,45 µm PVDF Blotting membrane, GE Healtcare and Amersham Hybond™ 0,2 µm PVDF Blotting membrane, GE Healtcare) with the run parameters 15 V, 300 mA, 1 h (Trans-Blot®SD semi dry transfer cell, Bio-Rad; Electrophoresis Power Supply ESP-601, Ge Healtcare).

The empty binding sites in the membranes were blocked with 5 % fat free milk powder (Valio Oy Lappinlahti, Finland, www.valio.fi) in 0,05 % Tween-TBS (Tween®20, Sigma-Aldrich) for 1 h at shaker and room temperature. After blocking the membranes, three 5 minutes washes were performed with 0,1 % Tween- TBS and 0,05 % Tween-TBS.

The target proteins were detected with the primary antibodies listed in Table 1. Primary antibodies were diluted in 5 % milk powder in 0,05 % Tween-TBS. Antibodies were incubated over night at +4°C, expect the Anti-β-actin that were incubated 1 h at room temperature.

Table 1. Primary antibodies in Western blot.

Antibody	**Host species**	**Dilution/ Concentration**	**Manufacturer**
Anti-pSTAT1	Rabbit	1:2000	R&D Systems
Anti-STAT1	Rabbit	1:800	Cell Signaling Technologies
Anti-pSTAT3	Rabbit	1:1000	R&D Systems
Anti-STAT3	Rabbit	1:1500	Cell Signaling Technologies
Anti-pNF-κB	Rabbit	1:500	R&D Systems
Anti-pSmad1/5	Rabbit	1:1000	Cell Signaling Technologies
Anti-β-actin	Mouse	1:2000	Santa Cruz Biotechnology
Anti-STAT1	Rabbit	1:800	Cell Signaling Technologies
Anti-STAT3	Rabbit	1:1500	Cell Signaling Technologies
Anti-Smad1	Rabbit	1:1000	Cell Signaling Technologies

After primary antibody incubations, membranes were washed three times (5 minutes) with 0,5 % Tween-TBS, 0,1 % Tween-TBS and 0,05 % Twee-TBS. The membranes were treated with HRP-conjugated (HRP = horseradish peroxidase) secondary antibodies goat anti-mouse IgG (dilution: 1:2000; Santa Cruz Biotechnology, Dallas, Texas, USA, http://www.scbt.com/) and anti-rabbit IgG (dilution 1:2000; Cell Signaling Technologies, Danvers, MA, USA, http://www.cellsignal.com/) to recognize the primary antibodies. Secondary antibodies were

incubated 1 h at room temperature. Finally, the membranes were washed three times as described previously and the membranes were stored at +4°C in TBS.

The protein bands were detected using enhanced chemiluminescence detection (ECL), which is based on oxidation of luminol generating chemiluminescense catalyzed by HRP. The detection reagents (Amersham™ ECL™ Prime Western Blotting Detection Reagent, GE Healthcare) were mixed 1:1 and incubated in dark for 5 min. The chemiluminescense was captured with Bio-Rad ChemiDoc™ XRS+ with Image Lab 5.2 Imaging system. Exposure times were from 1 second to 10 minutes. The images of the blots were edited with Microsoft Office PowerPoint version 2013 and the intensity of the bands of the blots were quantified with Image J software.

The phosphorylated STAT1, STAT3 and Smad1/5 values obtained from quantification were normalized using unphosphorylated STAT1, STAT3 and Smad1 values. Normalizations were performed using ImageJ analysis tool (U.S. National Intitutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/). Phosphorylated NF-κB were normalized using the values of β-actin.

4.7 Statistical analyses

Statistical analyses were performed with GraphPad Prism 5.01 (GraphPad Software, CA, USA, www.graphpad.com). The significance of the immunosuppression between direct and in-direct MLR reactions was compared with non-parametric statistic using t-test with Mann-Whitney post hoc test to analyze the specific sample pairs for significant differences. The obtained significances were corrected using Bonferroni adjustment in order to justify multiple comparisons. For example, the obtained p-values was multiple by the comparison made within the MLR control and MLR co-culture (MLR control vs. direct MLR co-culture, MLR control vs. indirect MLR-co-cultures, direct MLR co-culture vs. indirect MLR co-culture) and multiplied with the number of samples (x2). When e.g. p=0,005 was obtained with Mann-Whitney, the p value was multiplied with 2 giving the final p value 0,01. The results were considered significant when $p<0,05$. All experiments were repeated four times using different donor in each repeat (n=4). Four replicates of each sample were used in immunosuppression assays.

5 RESULTS

5.1 Adipose stem cells morphology and cell viability

The cell morphology and viability were examined by light microscopy imaging after 7 days culture (Figure 8). Lower proliferation rate was observed with ASC 2/15 donor compared with other ASC donors.

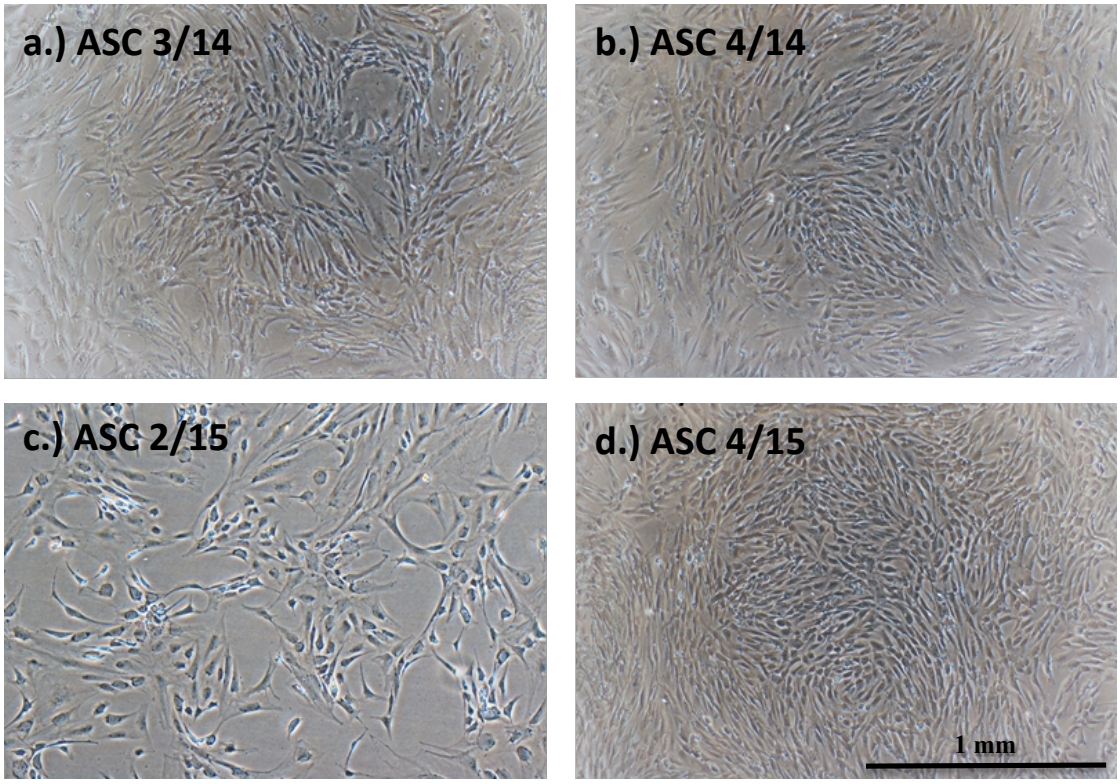

Figure 8. Morphology of ACSs after 7 day culture in 3% HS medium. a) ASC 3/14 b) ASC 4/14 c) ASC 2/15 d) ASC 4/15. Figures a, b and d were taken from 24-well plate and figure c from T75 flask.

5.2 Flow cytometric surface marker expression analysis

Cell surface marker expression analysis was performed for all used ASC donors to confirm the mesenchymal origin of the cells. Flow cytometric analysis was performed at passage 1. The results are combined from cells of four donors and average values with standard deviation (SD) are presented in Table 2. All studied cells of ASC donors expressed (>95%) stromal markers CD73, CD90 and CD105. ASCs lacked the expression (<2%) or had low expression (2-10%) of markers CD3, CD11a, CD14, CD19, CD45, CD80, CD86 and HLA-DR and showed moderate expression (10-30%) for surface markers CD34 and CD54.

One ASC donor (ASC 4/14) had an exceptional high expression (66,7%) of the hematopoietic progenitor and endothelial cell marker CD34 at passage 1, which was seen as high average expression and large SD. After further passaging of ASCs, the expression was decreased to typical level when new analysis was performed on passage 5.

Table 2. Surface marker expressions of ASCs at passage 1 (n=4). Expression of CD34 (*) was exceptional high in one of the ASC donors, which affected to the mean and the standard deviation of CD34 marker. Lack of expression (< 2%) and low expression (2-10%) are marked by -, moderate expression (10-30%) + and strong expression (>95%) ++.

Surface marker	Antigen	Mean	SD	Expression
CD3	T-cell surface glygoprotein	0,2	0	-
CD11a	Lymphocyte function-associated protein	1,1	0,4	-
CD14	Serum lipopolysaccharide binding protein	0,8	0,5	-
CD19	B lymphocyte-linage differentiation antigen	0,5	0,3	-
CD34	Sialomucin-like adhesion molecule	19,7*	31,3*	+
CD45	Leukocyte common antigen	2,3	0,8	-
CD54	Intercellular adhesion molecule 1	20,1	6,7	+
CD73	Ecto-5'-nucleotidase	98,9	0,3	++
CD80	B-lymphocyte activation antigen B7	0,6	0,2	-
CD86	B-lymphocyte activation anticen B7-2	0,8	0,4	-
CD90	Thy-1 (T-cell surface glygoprotein)	99,6	0,2	++
CD105	SH-2, endoglin	99,4	0,2	++
HLA-DR	Major histocompatibility class II antigens	0,5	0,3	-

5.3 Pre-test on PBMC reactivity

The pre-test was carried out to obtain the two strongest MLR combinations from four different PBMC donors (MLR1-MLR6). Cells from two different donors were combined (800 000 cells/ donor) and the proliferation of different combinations was measured after 5 days culture. Proliferation of PBMCs was the strongest in MLR2 and MLR3, and thus, they were selected for further studies (Figure 9).

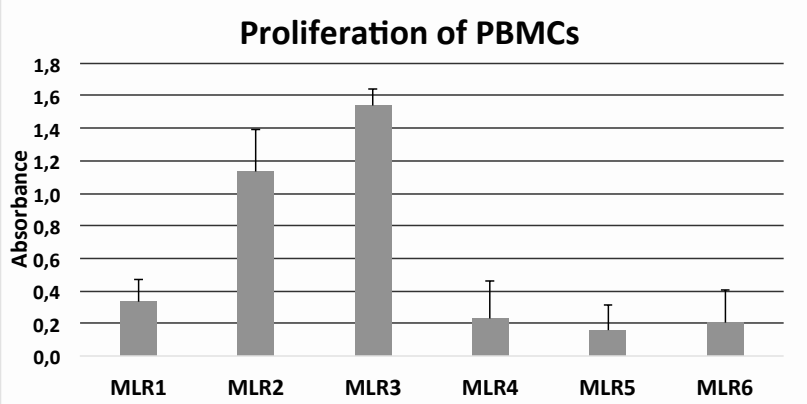

Figure 9. Pre-test to study the proliferation of PBMCs. Six different MLR combinations (MLR1-6) were mixed from cell of four different PBMC donors. The proliferation was strongest in MLR2 and MLR3 reactions.

5.4 Immunosuppression of adipose stem cells

Immunosuppression of ASCs was analyzed using BrdU-ELISA assay after 5 days MLR culture. Co-culture results in two-way MLRs were normalized subtracting the positive control values of MLRs without ASCs. The immunosuppression was analyzed separately for each ASC donor (Figure 10a). Suppressive capacity was observed with three ASC donors (ASC 3/14, ASC 4/14 and ASC 2/15) with both MLR combinations (MLR1 and MLR2) in both direct and indirect reactions. However, one ASC donor (ASC 4/15) could only suppress in direct contact when co-cultured with MLR2. Immunosuppression results of all four ASC donors were combined in Figure 10b that demonstrates that immunosuppression was statistically significant ($p<0.05$) only in reactions with MLR2. However, when the results of both MLR1 and MLR2 reactions were combined, a statistically significant ($p<0.05$) immunosuppression was observed in both direct and indirect MLR reactions (Figure 10c). Immunosuppression was stronger in direct reactions (17.3%) compared to indirect reactions (11.8%), although no statistically significant difference was observed in immunosuppression between direct and indirect reactions.

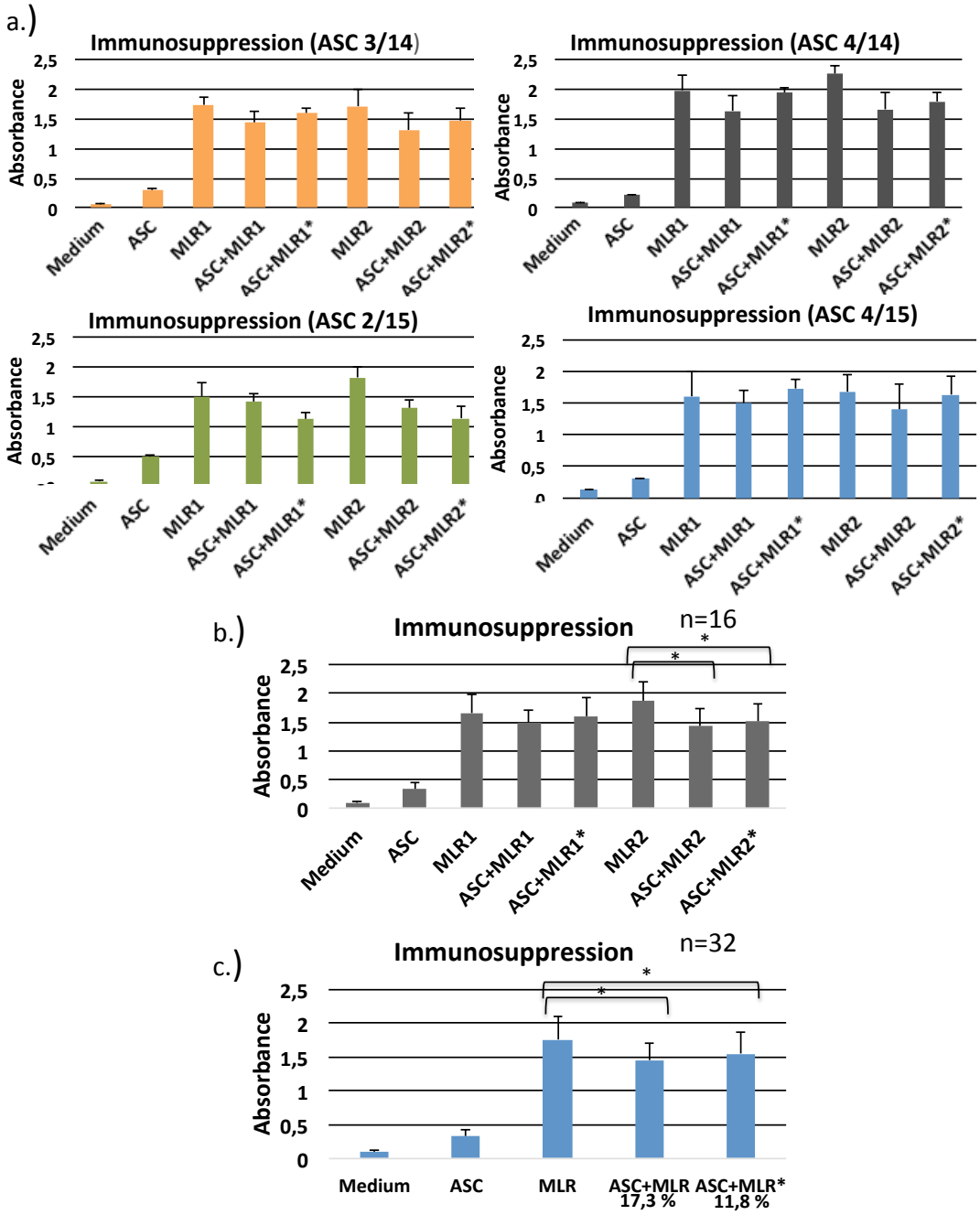

Figure 10. The immunosuppression potential of ASCs in direct and indirect MLRs. Immunosuppression assay was performed in passage 2 (ASC) and the cell ratio between PBMCs and ASCs was 27:1. a) The immunosuppression potential of each ASC donors. b) Combined immunosuppression of ASC donors. Four parallels from one MLR reaction and from four ASC donors were used, thus n=16. c) Combined immunosuppression of ASC when the results of MLR1 and MLR2 are combined. Four parallels from two MLR reactions and four ASC donors were used, thus n=32. Suppression percentage in direct reactions was 17.3 % and in indirect reactions 11.8 %. Statistical significance ($p<0.05$) marked by *. Abbreviations: ASC = Adipose stem cells, MLR1 = Lymphocytes from donors 1 and 2, MLR2 = Lymphocytes from donors 1 and 3, ASC+MLR1 or MLR2 = ASCs and MLR1 or 2 in *direct co-culture,* ASC+MLR1* or MLR2* = ASCs and MLR1 or 2 in *indirect co-culture* (*means co-culture where the ASCs are in insert and lymphocytes in the bottom of the well).

5.5 Activation of intracellular signaling pathways

Activation of signaling pathways was studied by Western blot analysis that was performed from direct and indirect MLR assays by using ASCs from four donors and two MLR combinations (MLR1 and MLR2) (Figure 11a, 12a, 13a and 14a). To obtain the normalized amounts of pSTAT1, pSTAT3 and pSmad1/5 the quantification values were normalized by using unphosphorylated protein values. For pNF-κB, normalization was performed using β-actin values. Activated protein levels of pSTAT1 and pSTAT3 were analyzed from both direct and indirect MLR assays, whereas pNF-κB and pSmad1/5 were analyzed from only direct MLR assays.

STAT1 was phosphorylated in PBMCs in direct co-culture with both ASC donors (ASC 3/14 and 4/14) (Figure 11a, 11c). In indirect MLR assays STAT1 was phosphorylated in lymphocytes of both MLR combinations when they were cultured with three ASC donors (ASC 3/14, 4/14 and 4/15). In co-culture with ASC 2/15, STAT1 was phosphorylated in lymphocytes of MLR2 combination but in lymphocytes of MLR1 combination STAT1 phosphorylation was inhibited. Phosphorylation was inhibited in ASC donor 3/14 with both MLR reactions. In ASC 4/14, the level of phosphorylation was the same in ASC control and in co-culture of ASCs and MLR2 reaction, but phosphorylation was decreased when ASCs were co-cultured with MLR1 reaction (Figure 11b, 11d). In summary, STAT1 was phosphorylated in PBMCs and to a lesser degree in ASCs in direct co-cultures. However, the direct co-culture of PBMCs and ASCs did not increase the STAT1 levels significantly in lymphocytes. In indirect reactions, STAT1 levels did not differ significantly in separately cultured PBMCs and ASCs, but in indirect co-culture, STAT1 phosphorylation was increased in lymphocytes. STAT1 phosphorylation in ASCs during indirect co-culturing was ASC donor- and MLR-combination-dependent.

STAT3 phosphorylation was the highest compared to other studied proteins. In direct reactions, phosphorylation was decreased in PBMCs and ASCs in all studied ASC donors (Figure 12a, 12c). In indirect reactions, STAT3 phosphorylation was activated when ASC 2/15 and 4/15 were co-cultured with MLR reactions. STAT3 was phosphorylated in ASCs 3/14 when co-cultured with MLR2 reaction, but phosphorylation was decreased when ASCs were cultured with MLR1 reaction. When ASC 4/14 cells were co-cultured with MLR1, the level of STAT3 phosphorylation was between ASC control and co-culture of ASCs and MLR1. However, when ASC 4/14 was co-cultured with MLR2 reaction, the phosphorylation

of STAT3 was inhibited. STAT3 phosphorylation was inhibited in PBMCs when ASC 4/14 and 4/15 were co-cultured with MLR reactions and when ASC 2/15 cells were co-cultured with MLR2 reaction. STAT3 was phosphorylated in PBMCs when ASC 3/14 cells were co-cultured with MLR and when ASC 2/15 cells were co-cultured with MLR1 (Figure 12b, 12d). To summarize, the STAT3 was phosphorylated in both PBMCs and ASCs when cells were cultured alone. In direct co-culture, the STAT3 phosphorylation was decreased significantly in both PBMCs and ASCs. In indirect reactions, the phosphorylation of STAT3 between PBMCs and ASCs alone did not differ significantly and the phosphorylation of STAT3 in ASCs in indirect co-cultures was ASC donor- and MLR-combination-dependent.

Phosphorylation of NF-κB and Smad1/5 were determined only from direct MLR assays with two ASC donors (ASC 3/14 and ASC 4/14). Phosphorylation of NF-κB was decreased when ASCs were co-cultured with both MLR reactions. The phosphorylation of NF-κB was higher in PBMCs compared to ASC controls or ASCs with MLR reaction (Figure 13). To summarize, NF-κB phosphorylation was significantly higher in PBMCs compared to ASCs and co-culturing decreased the NF-κB levels in PBMCs .

Smad1/5 phosphorylation was increased in direct co-culture with ASC 3/14 donor, whereas the phosphorylation was inhibited with ASC 4/14 donor in direct co-culture. According to western blot images, Smad1/5 was more phosphorylated in ASCs than PBMCs (MLR controls) (Figure 14). The Smad1/5 phosphorylation was higher in PBMCs alone compared to direct co-culture of PBMCs and ASCs. The phosphorylation of Smad1/5 was also ASC donor-dependent.

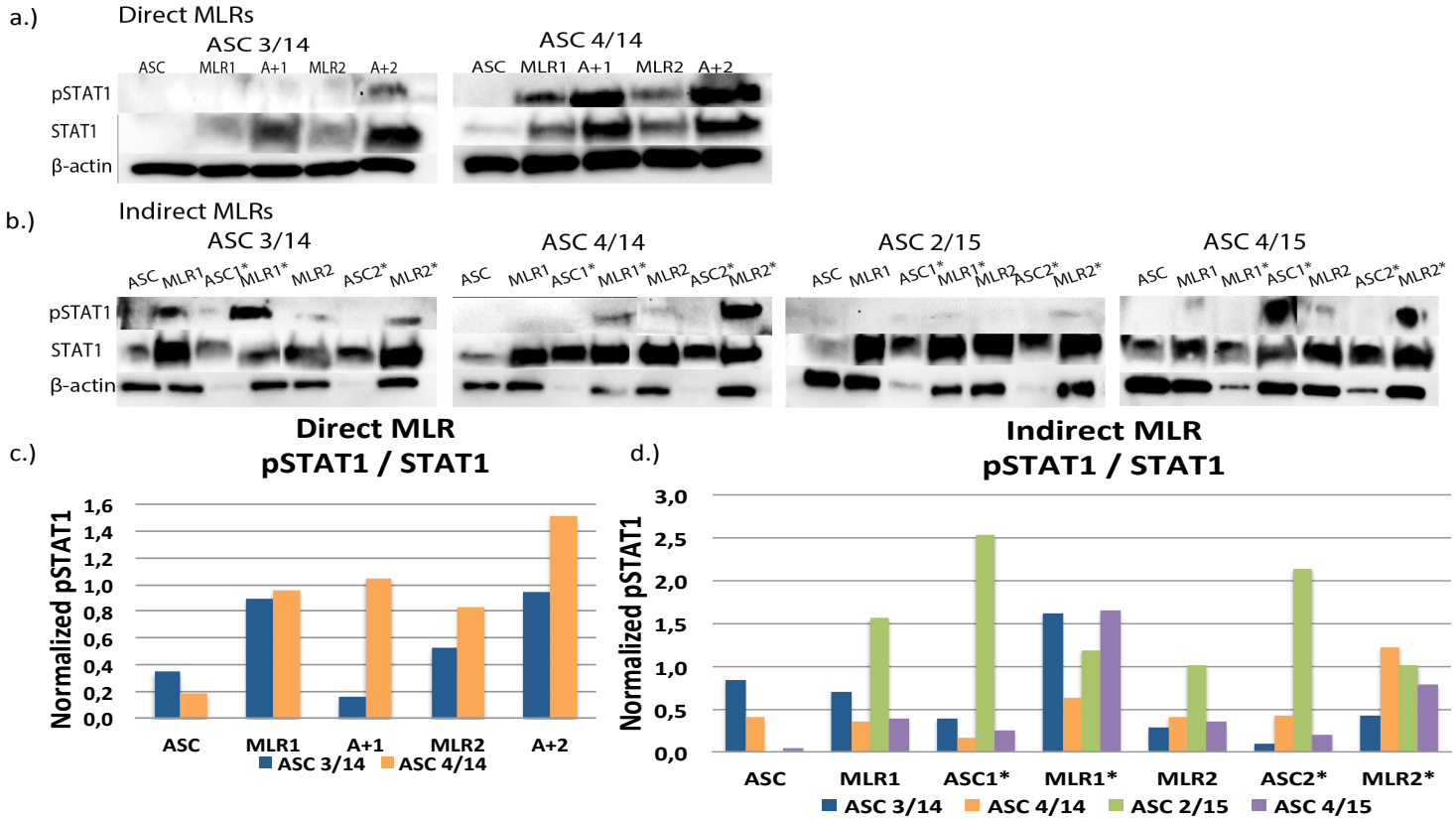

Figure 11. Phosphorylation of STAT1 in direct and in indirect MLR assays. a) Representative images of Western Blot analysis of STAT1 phosphorylation, basal STAT1 and β-actin in direct MLR assays with two ASC donors (ASC 3/14 and 4/14). b) Representative images of Western Blot analysis of STAT1 phosphorylation, basal STAT1 and β-actin in indirect MLR assays with cells of four ASC donors (ASC 3/14, 4/14, 2/15 and 4/15). c), d) Phosphorylated STAT1 levels were quantified by normalizing them with STAT1 basal protein levels by using the ImageJ analysis tool. Abbreviations: ASC = Adipose stem cells, MLR1 = Lymphocytes from donors 1 and 2, MLR2 = Lymphocytes from donors 1 and 3, A+1 or 2 = ASCs and MLR1 or 2 in *direct co-culture,* ASC* = ASCs from *indirect co-culture*, MLR* = Lymphocytes from *indirect co-culture* (*means co-culture where the ASCs are in insert and lymphocytes in the bottom of the well).

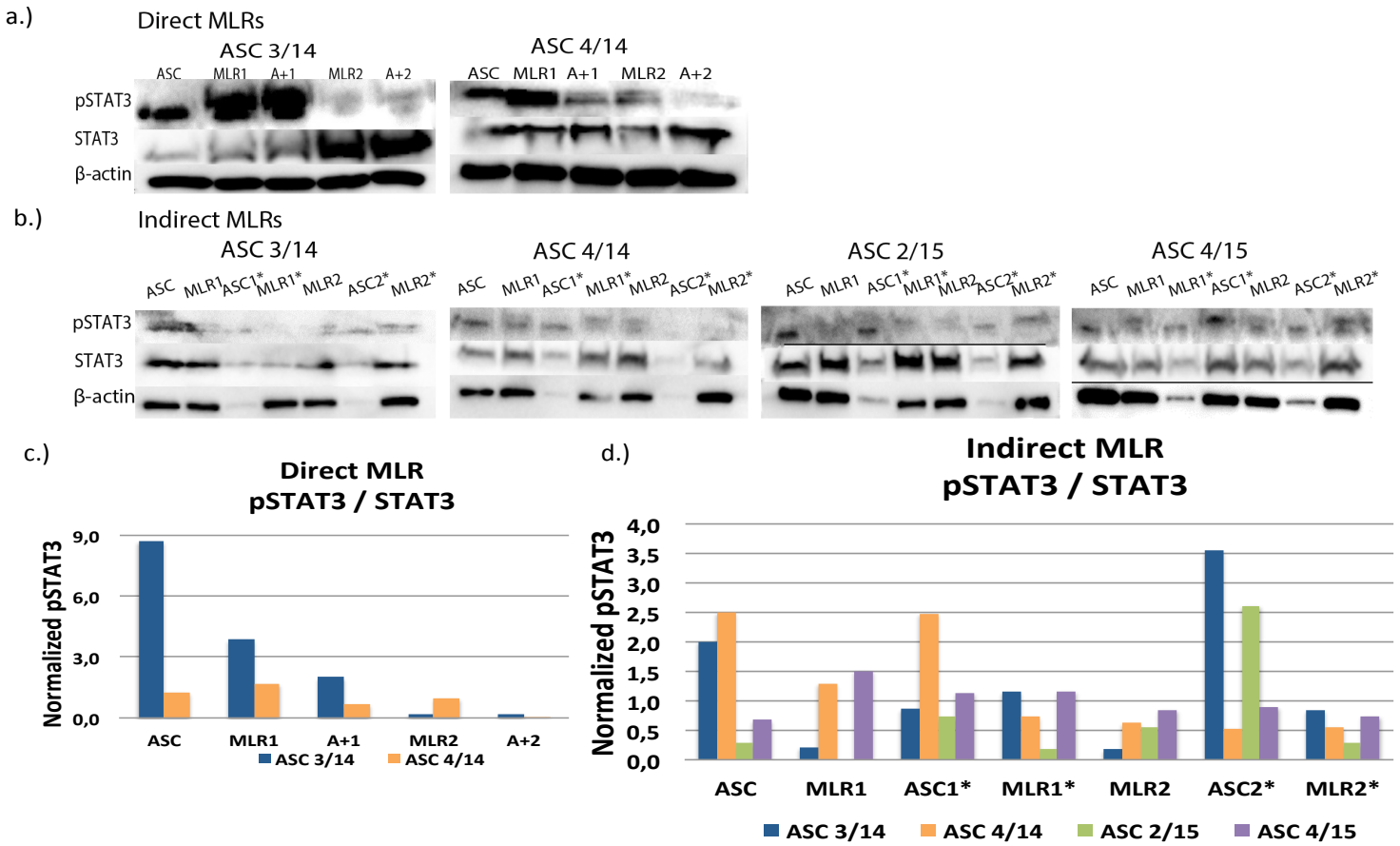

Figure 12. Phosphorylation of STAT3 in direct and in indirect MLR assays. a) Representative images of Western Blot analysis of STAT3 phosphorylation, basal STAT3 and β-actin in direct MLR assays with two ASC donors (ASC 3/14 and 4/14). b) Representative images of Western Blot analysis of STAT3 phosphorylation, basal STAT3 and β-actin in indirect MLR assays with cells of four ASC donors (ASC 3/14, 4/14, 2/15 and 4/15). c), d) Phosphorylated STAT3 levels were quantified by normalizing them with STAT3 basal protein levels by using the ImageJ analysis tool. Abbreviations: ASC = Adipose stem cells, MLR1 = Lymphocytes from donors 1 and 2, MLR2 = Lymphocytes from donors 1 and 3, A+1 or 2 = ASCs and MLR1 or 2 in *direct co-culture,* ASC* = ASCs from *indirect co-culture*, MLR* = Lymphocytes from *indirect co-culture* (*means co-culture where the ASCs are in insert and lymphocytes in the bottom of the well).

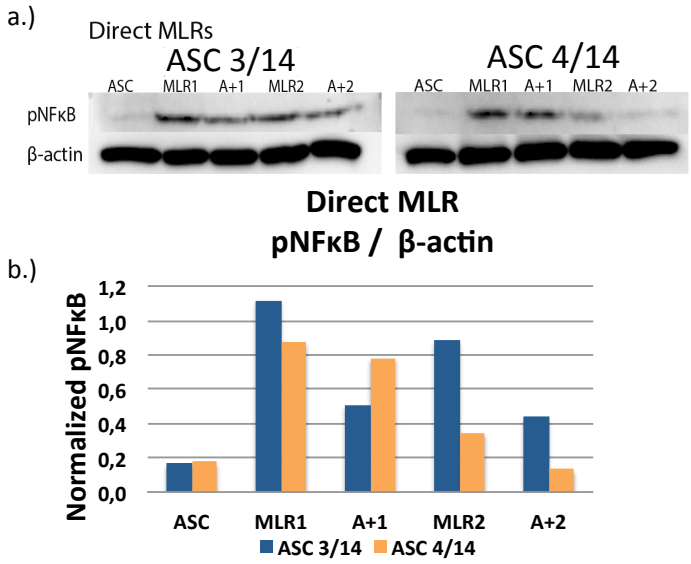

Figure 13. Phosphorylation of NF-κB in direct MLR assays. a) Representative images of Western Blot analysis of NF-κB phosphorylation and β-actin in direct MLR assays with cells of two ASC donors (ASC 3/14 and 4/14). b) Phosphorylated NF-κB levels were quantified by normalizing them with β-actin protein levels by using the ImageJ analysis tool. Abbreviations: ASC = Adipose stem cells, MLR1 = Lymphocytes from donors 1 and 2, MLR2 = Lymphocytes from donors 1 and 3, A+1 or 2 = ASCs and MLR1 or 2 in *direct co-culture*

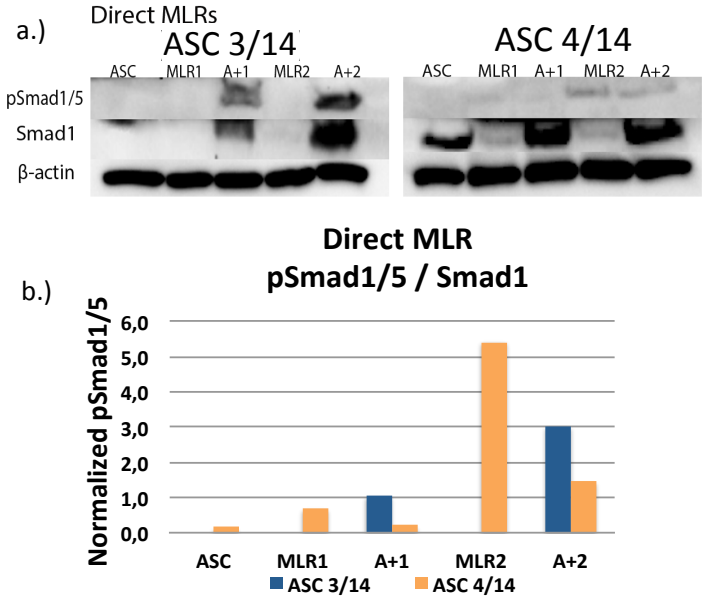

Figure 14. Phosphorylation of Smad1/5 in direct MLR assays. a) Representative images of Western Blot analysis of Smad1/5 phosphorylation, basal Smad1 and β-actin in direct MLR assays with cells of two ASC donors (ASC 3/14 and 4/14). b) Phosphorylated Smad1/5 levels were quantified by normalizing them with basal Smad1 protein levels by using the ImageJ analysis tool. Abbreviations: ASC = Adipose stem cells, MLR1 = Lymphocytes from donors 1 and 2, MLR2 = Lymphocytes from donors 1 and 3, A+1 or 2 = ASCs and MLR1 or 2 *in direct co-culture.*

6 DISCUSSION

This study analyzed the suppressive potential of ASCs in direct versus indirect co-cultures and the activation of intracellular cytokine signaling pathways during ASC-mediated immunosuppression. Patrikoski *et al.* has previously studied the cytokine secretion during ASC-mediated immunosuppression (Patrikoski, et al. 2014). Based on these results, the current study investigates the activation of intracellular signaling pathways STAT1, STAT3, NF-κB and Smad1/5.

Immunosuppressive capacity of ASCs has been studied worldwide and also in our laboratory (Atoui and Chiu. 2012, Baer. 2014, McIntosh, et al. 2006, Patrikoski, et al. 2014). The cytokines that are involved in ASC-mediated immunosuppression are described in previous publication (Patrikoski, et al. 2014). Nevertheless, there are very few studies on the activation of intracellular signaling pathways in ASC– or MSC–mediated immunosuppression and therefore it is difficult to compare the signaling results with previous studies that discuss the signaling from a different viewpoint. However, certain speculations and hypotheses on the results are described below.

6.1 Characteristics of adipose stem cells

6.1.1 Proliferation of ASCs

Multiple factors have an influence on ASCs characteristics such as age, body mass index, gender, ethnicity and medical history including preexisting diseases, smoking or alcohol abuse (Baer and Geiger. 2012). In this study, ASCs from four different female donors by ages 28, 31, 34 and 52 were used. Gender and age are the only known information on cell donors, which makes it difficult to estimate how much other factors affect to cell viability and proliferation. Based on the observations during cell expansion, viability and proliferation rate of three ASC donors were typical but one donor showed decreased proliferation rates. It is known that the age of donor affects the viability and proliferation rate of cells and it has been shown that cells from younger donors (<40 years) have better viability and proliferation capacity (Choudhery, et al. 2014). However, the donor age does not explain the lower proliferation rate because the ASC 2/15 donor was the youngest (28 years) of all donors. Also

the site of harvest has been shown to affect the proliferation capacity of ASCs (Baglioni, et al. 2012). It is known that ASCs collected from subcutaneous fat samples have better proliferation capacity compared with other ASC sources (Baglioni, et al. 2012). In this study, all of the used ASCs were harvested from subcutaneous fat samples, and thus, the site of harvest did not explain the difference in the proliferation rate between different donors. However, ASCs 2/15 was isolated from liposuction sample, whereas the other three cell lines were harvested from adipose tissue. This should not explain the difference in cell proliferation as it was shown previously that no difference in cell viability or proliferation was observed between cells harvested from liposuction or adipose tissue samples (Oedayrajsingh-Varma, et al. 2006). Although the proliferation capacity was decreased with ASC 2/15 donor, the immunosuppression and cell signaling results were not significantly different from the results of other donors.

6.1.2 Surface marker expression

The IFATS and ISCT have established minimal criteria for phenotypic identification of MSCs and ASCs that was first published in 2006 by Dominici *et al.* (Dominici, et al. 2006). In 2013, Bourin *et al.* published the update for the minimal criteria (Bourin, et al. 2013). Flow cytometry results of this study were typical to MSCs and ASCs and the surface marker expression demonstrated the profile defined by Dominici *et al*. and Bourin *et al*. (Bourin, et al. 2013, Dominici, et al. 2006). The ASCs expressed stromal markers CD73, CD90 and CD105 and ASCs lacked the expression of CD3, CD11a, CD14, CD19, CD45, CD80, CD86 and HLA-DR.

The results of surface marker expression are combined result of four ASC donors. Three ASC donors (ASC 3/14, 2/15 and 4/15) had low CD34 expression (2-10%) but ASC 4/14 showed higher CD34 expression (66,7 %). The CD34 expression was decreased during further passaging and on passage 5 the expression was decreased to the same level than expression of other three ASC donors. Compared to BM-MSCs that are considered as CD34 negative cells, it is shown that freshly harvested ASCs contain CD34 positive cell population, which will disappear during passaging (Baer and Geiger. 2012, Baer. 2014, Maumus, et al. 2011, Tsuji, et al. 2014). Maumus *et al.* has reported that during 3 days culture the proportion of CD34 positive cells will increase, but during longer culture the CD34 positive cells decrease as the total cell number starts to increase (Maumus, et al. 2011). Therefore the variation in CD34 expression may also depend on the culture period. Nevertheless, the higher CD34 expression

that was observed for ASC 4/14 cells did not influence the immunosuppression results or cell signaling results, which were performed in passage 2.

6.2 Immunosuppressive capacity of adipose stem cells

6.2.1 Pre-test to evaluate the strongest mixed lymphocyte reaction combinations

Pre-test was performed to evaluate the strongest MLR combinations for immunosuppressive analyses. Four PBMC donors were used to form six different MLR combinations. For functional MLR assay, the proliferation of PBMC should be strong in MLRs in order to efficiently detect the immunosuppressive capacity of ASC. Thus, two strongest reactions were chosen, containing PBMCs from three different donors.

6.2.2 Adipose stem cells possess immunosuppressive capacity

The study demonstrated that ASCs have immunosuppressive capacity, as previously demonstrated (Atoui and Chiu. 2012, Baer. 2014, McIntosh, et al. 2006, Patrikoski, et al. 2014). Immunosuppression was studied with four ASC donors and two different MLR combinations (MLR1 and MLR2). Every ASC donor was co-cultured with MLR1 and MLR2 combinations in indirect cultures. To evaluate difference between strength of immunosuppression in direct and indirect reactions, two ASC donors (ASC 3/14 and 4/14) were co-cultured with MLR1 and MLR2 combinations in direct MLR. In indirect co-cultures, the ASCs and PBMCs were separated by a semipermeable membrane to block the effects of cell-cell contacts.

Immunosuppression was observed with three ASC donors (ASC 3/14, 4/13 and 2/15) with both MLR combinations in both direct and indirect co-cultures. When ASC 4/15 cells were co-cultured in direct contact with MLR2, they showed immunosuppressive capacity, which was absent in co-culture with MLR1 and indirect co-culture with MLR2. Lack of immunosuppression in co-cultures with MLR1 combination may be explained with the lower proliferation of PBMCs in MLR1. Thus, it may be speculated that with stronger initial PBMC response, also ASC-mediated suppression may have been observed. In general, the suppression in direct co-cultures was stronger compared to indirect reactions because cell-cell contacts seem to affect to the strength of immunosuppression (Ankrum, et al. 2014, Di Nicola, et al. 2002, Leto Barone, et al. 2013, Yoo, et al. 2009). This may explain why the

immunosuppression was observed in direct co-culture of ASC 4/15 and MLR2 but not in indirect co-culture.

When immunosuppression of each ASC donor was combined, statistically significant immunosuppression was observed only in reactions containing MLR2. This may be explained by a stronger initial proliferation of MLR2 compared with MLR1, and thus, the suppressive potential of ASCs appears more clearly. The HLA type of each PBMC donor also affects the strength of the PBMC proliferation in MLR and dissimilarity between two donors provokes the activation of T-cells (Nicolaidou, et al. 2015). Although the PBMC reactivity was tested before starting the co-cultures, it is likely that the donors in MLR1 were not dissimilar enough regarding the HLA type. In this study, the HLA type of PBMC donors was not available, and the effects of HLA-profiles and other factors affecting the strength of the proliferation were only evaluated in pre-tests. There may be more variation in HLA-profiles of PBMCs used in MLR2 compared to PBMCs used in MLR1.

When the immunosuppression results of four ASC donorss and two MLR combination were combined, a stronger immunosuppression was observed in direct reactions (17.3% suppression) compared to indirect reactions (11.8% suppression). However, the immunosuppression was statistically significant in both reactions. As earlier mentioned, there are published data about the changes in immunosuppressive capacity between direct and indirect co-cultures (Ankrum, et al. 2014, Di Nicola, et al. 2002, Leto Barone, et al. 2013, Yoo, et al. 2009).

6.3 Signaling pathway analysis

Activation of intracellular signaling pathways in ASC-mediated immunosuppression has not been widely studied and there are only few published studies on this subject carried out using ASCs. A small number of published results on this subject makes it difficult to compare the results of this study to previous studies. Immunomodulation of ASCs includes several signaling pathways but only few of them are studied in this thesis. Cell signaling is complex and there are multiple pathways for each cytokine, but in this thesis only some of the main pathways (STAT1, STAT3, NF-κB and Smad1/5) are studied. The pathways were selected based on previous studies, in which the secretion of cytokines (IFN-γ, IL-6, TNF-α and TGF-β) during ASC-mediated immunosuppression was analyzed (Patrikoski, et al. 2014). These

signaling pathways have multiple functions in ASC– and MSC–mediated immunosuppression as shown in Figure 15.

In discussion of ASC–mediated immunosuppression it was mentioned that ratio between PBMCs and ASCs have an effect on the strength of observed immunosuppression in MLR assay. If the immunosuppression would have been stronger, it is likely that the activation of signal proteins would have been higher. In this study the cytokine profile of MLR cultures were not analyzed. Thus, the differences in levels of signaling protein phosphorylation may be due to the different cytokine profiles between reactions. Analyzing the cytokine profiles and comparing them to signal protein activation, it would be easier to estimate the activation of signaling pathways.

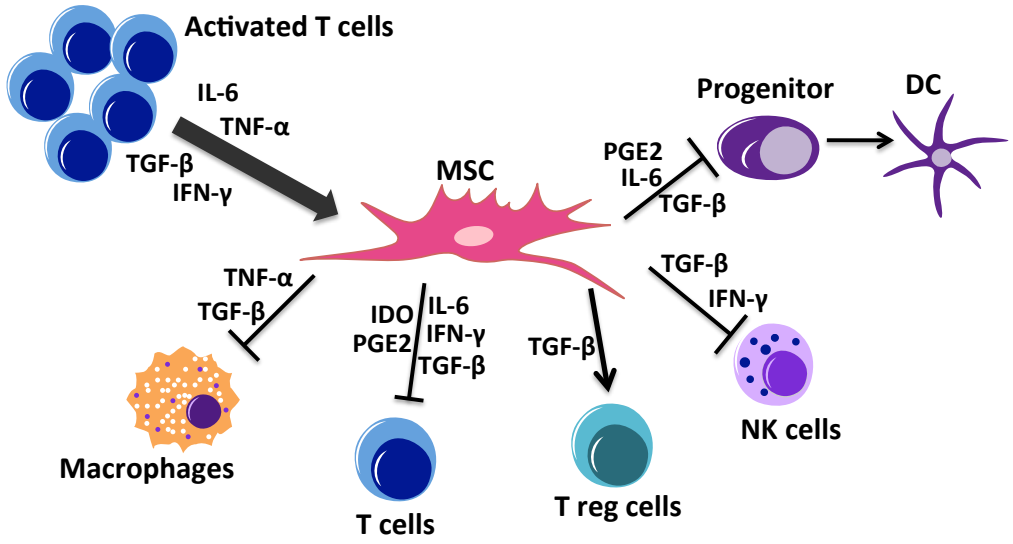

Figure 15. Schematic presentation of the effects of immunosuppressive factors. Activated T-cells secrete cytokines that activates MSCs to secrete soluble mediators such as idoleamine 2,3-dioxygenase (IDO), prostaglandin E2 (PGE2) and interleukin 6 (IL-6). These mediators regulates the proliferation and functions of immune cells with different products of signaling pathways triggered by IL-6, tumor necrosis factor α (TNF-α), transforming growth factor β (TGF-β) and interferon γ (IFN-γ). Abbreviations: T regulatory cell (T reg), natural killer (NK) and dendritic cell (DC).

6.3.1 IFN-γ activates STAT1 in both ASCs and PBMCs

IFN-γ is a pro-inflammatory cytokine secreted by activated T-cells and it activates the immunomodulative functions in MSCs (Yagi, et al. 2010). In this thesis, the IFN-γ mediated activation of STAT1 was investigated. Activation of STAT1 was studied from direct and

indirect co-cultures using Western blot analysis. Phosphorylated protein levels were normalized to total basal protein levels to observe the level of activation. In direct reactions STAT1 was phosphorylated both in PBMCs and ASCs. In indirect reactions STAT1 was phosphorylated in PBMCs with three ASCs donors (ASCs 3/14, 4/14 and 4/15) and in two ASC donors (ASCs 2/15 and 4/15) with both MLR combinations. IFN-γ should activate the STAT1 pathway in MSCs and inhibit the proliferation and differentiation of T-cells, as shown in Figure 4 (Liu, et al. 2015, Yagi, et al. 2010). In light of this theory, the activation of STAT1 in ASCs during the immunosuppression is rational. Additionally, it has been shown that IFN-γ is able to phosphorylate STAT1 via multiple pathways in T-cells and B-cells, which are the cell types of PBMCs (Girdlestone and Wing. 1996, Sakatsume and Finbloom. 1996, Shao, et al. 2015a). This explains the STAT1 activation during immunosuppression in both ASCs and PBMCs.

The phosphorylation of STAT1 was exceptional high in ASC donor 2/15 in indirect co-culture compared to other ASC donors. Additionally, ASC 2/15 was the only donor that had stronger capacity for immunosuppression in indirect reactions compared to direct reactions. That may explain why the activation of STAT1 was higher in ASCs donor 2/15. The stronger the immunosuppression is, the higher the activation of intracellular signaling protein should be. Interestingly, the results showed that in indirect reactions STAT1 was activated only in ASCs, which were isolated in 2015 (ASCs 2/15 and 4/15) compared to ASCs isolated in 2014 (ASCs 3/14 and 4/14), in which the activation did not occur. In indirect reactions, the cell-cell contacts were lacking and the immunosuppression was generally lower compared to direct reactions, which may explain why the activation occurred in direct reactions with ASCs 3/14 and 4/14 (Ankrum, et al. 2014, Di Nicola, et al. 2002, Leto Barone, et al. 2013, Yoo, et al. 2009). There is no literature on how the cryopreservation time affects the immunosuppressive functions of ASCs, but it may be speculated to have some effects. The current results suggest that the older cell linages do not activate STAT1 as effectively as newer cell lines in indirect reactions. Generally, ASCs have been found to maintain the capability to proliferate and differentiate efficiently after cryopreservation (Shu, et al. 2015).

6.3.2 IL-6 have pro-inflammatory effects in direct MLRs and anti-inflammatory effect in indirect MLRs

During inflammation, IL-6 is secreted by several cell types and it has both pro- and anti-inflammatory functions depending on the context (Schaper and Rose-John. 2015, Scheller, et

al. 2011). Anti-inflammatory functions of IL-6 are mediated trough the activation of classical signaling pathway (Figure 2) and pro-inflammatory functions trough the trans-signaling pathway. Classical IL-6 signaling pathway is triggered when IL-6 binds to the IL-6R that is expressed only on a limited number of cell types, such as immune cells and hepatocytes, leading to the immunosuppressive functions via activation STAT3 and STAT3-mediated signaling. Most cell types cannot express IL-6R on the cell surface. In these cells IL-6 signaling occurs via trans-signaling pathway. It is triggered when IL-6 bind to the sIL-6R and results the pro-inflammatory functions via activation of STAT3 and STAT3-mediated signaling (Liu, et al. 2015, Scheller, et al. 2011, Wolf, et al. 2014).

Compared to other studied signaling proteins, the production of STAT3 was the highest. Multiple cell types secrete IL-6, and thus, the amount of IL-6 may be higher compared to other cytokines, affecting the protein production (Schaper and Rose-John. 2015). In this study, it was observed that in direct reactions STAT3 phosphorylation was inhibited and in indirect reactions STAT3 was mainly activated.

It could be speculated that the differences between IL-6 functions between direct and indirect reactions may be in the concentration of IL-6. It is likely that the level of IL-6 concentration may affect the final functions of IL-6; however, this is only a speculation because there are no published data about concentration-dependent signaling regarding IL-6. Kondo *et al.* have reported that undifferentiated MSCs do not express membrane bounded IL-6 receptors (IL-6R) as much as soluble IL-6 receptors (sIL-6R) (Kondo, et al. 2015). However, anti-inflammatory effects of IL-6 are mediated trough classical IL-6 signaling, where the IL-6 binds to the IL-6R (Schaper and Rose-John. 2015, Scheller, et al. 2011). This may explain the balance between pro- and anti-inflammatory effects of IL-6, but it does not explain the difference between direct and indirect reactions because the ASCs are undifferentiated in both co-cultures. However, undifferentiated MSCs may activate STAT3 via trans-signaling or using some other pathway, which may explain the phosphorylated STAT3 levels in ASCs in this study. Alternatively, it may be speculated that PBMCs and ASCs may form cell-cell contacts in direct reactions that may lead to changes in STAT3 phosphorylation activating the IL-6 signal. However, the reason for differences is not the strength of immunosuppression because the inhibition of STAT3 occurred in direct reactions, which generally had stronger immunosuppressive capacity (Ankrum, et al. 2014, Di Nicola, et al. 2002, Leto Barone, et al. 2013, Yoo, et al. 2009).

In addition to IL-6 and IFN-γ, TGF-β have the ability to inhibit pro-inflammatory effects of IL-6 by inhibiting the STAT3 production produced by IL-6 trans-signaling (Liu, et al. 2015, Massague, et al. 2005, Shao, et al. 2015b). If this inhibition is stronger in indirect reaction compared to direct reactions, it may explain the difference between the results.

6.3.3 ASCs inhibit the phosphorylation of NF-κB in PBMCs in direct co-culture

TNF-α is a cytokine that is secreted from activated T-cells and it has immunosuppressive functions activating the NF-κB pathway (Dorronsoro, et al. 2014, Prasanna, et al. 2010). Phosphorylation of NF-κB was determined only from direct MLR assays. In direct reaction the phosphorylation of NF-κB was higher in PBMCs compared to ASCs. Because the phosphorylation of MLR controls without ASCs where higher compare to direct reactions containing ASCs and PBMCS, ASCs may decrease the NF-κB levels of PBMCs.

The NF-κB pathway was activated only in direct reactions, which suggested that TNF-α – mediated activation of NF-κB needs cell-cell contacts. The activation of NF-κB pathway was also studied in indirect co-cultures of ASCs and PBMCs in pre-tests. However, due to lack of NF-κB phosphorylation in pre-tests, the NF-κB pathway during indirect co-cultures were not studied in actual experiments. In addition to TNF-α, IFN-γ is also able to activate NF-κB pathway (Xiao, et al. 2015, Yagi, et al. 2010). Besides the controlling of immune cells, NF-κB phosphorylation inhibits the T-cells to produce TNF-α through feed-back-loop mechanism. TNF-α mainly activates the NF-κB pathway and if the secretion of TNF-α is decreased it may not effectively activate the signaling pathway, which leads to inhibition of NF-κB phosphorylation (Dorronsoro, et al. 2014). Phosphorylated NF-κB inhibits also the proliferation and activation of T-cells, which decrease of TNF-α production that is secreted by activated T-cells (Dorronsoro, et al. 2014, Yagi, et al. 2010). If the TNF-α level is decreased during co-culture in our study, it would explain the inhibition of NF-κB. However, the cytokine levels have not measured in this study. Phosphorylated NF-κB levels were normalized by using β-actin instead of the basal NF-κB antibody. Since the β-actin level was high it may skew the results of normalized NF-κB levels. In fact, the results of NF-κB activation may be different if the normalization was performed using the basal protein levels.

6.3.4 Activation of Smad1/5 signaling pathway was ASC donor-dependent

TGF-β is secreted by ASCs and activated in T-cells (Ock, et al. 2016, Patrikoski, et al. 2014, Rodriguez, et al. 2015). In this thesis, the activation of Smad1/5 was studied based on TGF-β secretion during ASC –mediated immunosuppression (Patrikoski, et al. 2014). In the current study, TGF-β was considered to activate Smad1/5 pathway during ASC-mediated immunosuppression, although TGF-β is also able to activate other signaling pathways (Massague, et al. 2005, Miyazono, et al. 2010). Smad1/5 activation was studied only in direct cultures because there were no signs of the activation of Smad1/5 in indirect reactions on pre-tests thus, this pathway may also need the cell-cell contacts for the activation. Smad1/5 signal pathway activation was donor dependent because Smad1/5 was phosphorylated only in reactions with ASC 3/14, whereas in reaction with ASC 4/14 the phosphorylation of Smad1/4 was inhibited.

Principally, BMPs activate Smad1/5 and TGF-β activates Smad2/3 but it is reported that TGF-β is also able to activated Smad1/5 (Massague, et al. 2005, Nurgazieva, et al. 2015). It may be possible that Smad1/5 can be activated indirectly by other Smad pathways or through BMP-2 mediated activation. TGF-β may activate Smad2/3, which together with Smad pathways is able to activate the Smad1/5 pathway. Smad2/3 activation by TGF-β may also stimulate the production of BMP-2 that is the activator of Smad1/5 pathway. However, this is only a speculation because there are no published data on this subject. TGF-β –mediated Smad2/3 signaling in immunosuppression has been more reported (Wrzesinski, et al. 2007, Yoshimura, et al. 2010), but publications carried out with ASCs were not found. In future, it may be interesting to study activation of Smad2/3 signal pathway triggered by TGF-β during immunosuppression.

6.4 Methodological consideration

The used ASCs were harvested from adipose tissue samples from four different female donors. Because all donors were female, this study is not able to compare the immunosuppressive effect between ASCs from males and females. Still, there is evidence that ASCs from male and female donors may have different characteristics, e.g., the differentiation capacity (Aksu, et al. 2008) and it may be possible that there is also difference in immunomodulatory functions. The used PBMCs were isolated from three donors but no further details are known

on these donors. The results of immunosuppression analyses and activation of cell signaling pathways show that there is variation between different donors. Variable results of signaling pathway activation may partly depend on variation between cell donors. It has been previously reported that characteristics of ASCs are affected by age, body mass index, gender, ethnicity and medical history including preexisting diseases and medication, smoking or alcohol abuse (Baer and Geiger. 2012). Since only age and gender of ASC donors are known, there are many unknown factors that may affect the signaling pathway results.

The isolation methods of ASCs and PBMCs are widely used in our laboratory. The isolation of ASCs was performed by using mechanical and enzymatic methods as described previously (Zuk, et al. 2001). PBMCs were isolated by method standardized by our laboratory. Therefore, the isolation procedures should not cause any bias for the results.

Many factors may affect the MLR assay results. There may be a higher rate of HLA similarity between PBMCs and ASCs although the possibility for that is not high. The HLA type of PBMC donors affects the strength of PBMC proliferation and dissimilarity between two PBMC donors provoke the activation of T-cells. The HLA-profile of used cells was not available, thus the pre-tests were performed to observe the strength of the PBMC proliferation in MLRs. The strongest MLR combinations were chosen according to the pre-tests, however it may be possible that proliferation of PBMCs was not strong enough that immunosuppressive effect of ASCs would have been seen properly.

Cell ratio between PBMCs and ASCs may also affect to the strength of immunosuppression. The used ratio between PBMCs and ASCs was 27:1. These cell numbers were used to prevent the overgrowth and detachment of cells during 5 days culture. However, the ratio should be further optimized and it could be smaller than the used 27 PBMCs for one ASC. Changing the PBMC-ASC ratio for immunosuppression analyses may strengthen the immunosuppression observed from MLR assay.

ASCs expanded prior to direct MLR assays in BM did not proliferate efficiently with certain cell lines, which may be due to used HS lot. The used serum lot was recently expired, which may have affected the cell proliferation, although the same serum lot was used successfully in the expansion of ASCs for indirect MLR assays. Therefore, ASCs were changed into medium containing fresh HS (Biowest), where they started to proliferate more efficiently and the morphology was normalized. Fresh HS lot was used only for direct MLR assays, and thus, it may have some effect on results but no major differences were observed.

7 CONCLUSION

In this study the immunosuppressive capacity of ASCs was analyzed as well as difference in immunosuppression between direct and indirect co-cultures of ASCs and PBMCs. Furthermore, the activation of intracellular signaling pathways (STAT1, STAT3, NF-κB and Smad1/5) in ASC –mediated immunosuppression was studied. The results showed that ASCs possess immunosuppressive capacity both in direct and in indirect MLR assays. Immunosuppression was stronger in direct assays, which was in line with previous studies. There was a lot of variation in the results of signaling pathway studies and the protein phosphorylations were ASC donor-dependent.

The major difference between direct and indirect co-cultures was the lack of activation in NF-κB and Smad1/5 pathways in indirect reactions. The other studied pathways (STAT3 and STAT1) were activated in both direct and indirect co-cultures. NF-κB phosphorylation was significantly higher in PBMCs compared to ASCs and co-culturing decreased the NF-κB levels in PBMCs. Smad1/5 activation was donor-specific; it was activated with other ASC donor and inhibited with the other. The STAT3 was phosphorylated in PBMCs and ASCs when cells were cultured alone, whereas in direct co-cultures STAT3 phosphorylation was inhibited and in indirect co-cultures STAT3 phosphorylation was donor-dependent. STAT1 was phosphorylated in PBMCs and to a lesser degree in ASCs in direct co-cultures, but in indirect co-culture, STAT1 phosphorylation was increased in PBMCs.

Cell signaling behind ASC -mediated immunosuppression is not much studied making the studied subject highly interesting. Results of this study produced more detailed information on the immunosuppressive capacity of ASCs and especially new data on the intracellular signaling behind the ASC –mediated immunosuppression. Further studies on cell signaling will be based on this basic characterization of the pathways. This study also obtained novel knowledge on how to technically perform the signaling pathway analyses from MLR assays.

New experiments with more ASC and PBMC donors are needed to obtain more specific data for the activation of intracellular signaling in ACS –mediated immunosuppression. Nevertheless, immunomodulative functions of ASCs make them attractive cells for clinical application. ASCs have already been used to treat some autoimmune diseases. More detailed information on the immunological properties of ASCs will enable the safe use of ASCs in clinical applications to treat immune diseases.

REFERENCES

1. Wankhade UD, Shen M, Kolhe R, Fulzele S. Advances in adipose-derived stem cells isolation, characterization, and application in regenerative tissue engineering. *Stem Cells Int*. 2016;2016: 3206807.

2. Kapur SK, Dos-Anjos Vilaboa S, Llull R, Katz AJ. Adipose tissue and stem/progenitor cells: Discovery and development. *Clin Plast Surg*. 2015;42: 155-167.

3. Katz AJ, Llull R, Hedrick MH, Futrell JW. Emerging approaches to the tissue engineering of fat. *Clin Plast Surg*. 1999;26: 587-603, viii.

4. Zuk PA, Zhu M, Mizuno H et al. Multilineage cells from human adipose tissue: Implications for cell-based therapies. *Tissue Eng*. 2001;7: 211-228.

5. Zuk PA, Zhu M, Ashjian P et al. Human adipose tissue is a source of multipotent stem cells. *Mol Biol Cell*. 2002;13: 4279-4295.

6. Tsuji W, Rubin JP, Marra KG. Adipose-derived stem cells: Implications in tissue regeneration. *World J Stem Cells*. 2014;6: 312-321.

7. Zuk PA. The adipose-derived stem cell: Looking back and looking ahead. *Mol Biol Cell*. 2010;21: 1783-1787.

8. McIntosh K, Zvonic S, Garrett S et al. The immunogenicity of human adipose-derived cells: Temporal changes in vitro. *Stem Cells*. 2006;24: 1246-1253.

9. Niemeyer P, Kornacker M, Mehlhorn A et al. Comparison of immunological properties of bone marrow stromal cells and adipose tissue-derived stem cells before and after osteogenic differentiation in vitro. *Tissue Eng*. 2007;13: 111-121.

10. Baer PC. Adipose-derived mesenchymal stromal/stem cells: An update on their phenotype in vivo and in vitro. *World J Stem Cells*. 2014;6: 256-265.

11. Atoui R, Chiu RC. Concise review: Immunomodulatory properties of mesenchymal stem cells in cellular transplantation: Update, controversies, and unknowns. *Stem Cells Transl Med*. 2012;1: 200-205.

12. Patrikoski M, Sivula J, Huhtala H et al. Different culture conditions modulate the immunological properties of adipose stem cells. *Stem Cells Transl Med*. 2014;3: 1220-1230.

13. Kuo YR, Chen CC, Goto S et al. Modulation of immune response and T-cell regulation by donor adipose-derived stem cells in a rodent hind-limb allotransplant model. *Plast Reconstr Surg*. 2011;128: 661e-72e.

14. Jeong SH, Ji YH, Yoon ES. Immunosuppressive activity of adipose tissue-derived mesenchymal stem cells in a rat model of hind limb allotransplantation. *Transplant Proc*. 2014;46: 1606-1614.

15. McIntosh KR, Frazier T, Rowan BG, Gimble JM. Evolution and future prospects of adipose-derived immunomodulatory cell therapeutics. *Expert Rev Clin Immunol*. 2013;9: 175-184.

16. Fang B, Song Y, Liao L, Zhang Y, Zhao RC. Favorable response to human adipose tissue-derived mesenchymal stem cells in steroid-refractory acute graft-versus-host disease. *Transplant Proc*. 2007a;39: 3358-3362.

17. Garcia-Olmo D, Herreros D, Pascual I et al. Expanded adipose-derived stem cells for the treatment of complex perianal fistula: A phase II clinical trial. *Dis Colon Rectum*. 2009;52: 79-86.

18. Choumerianou DM, Dimitriou H, Kalmanti M. Stem cells: Promises versus limitations. *Tissue Eng Part B Rev*. 2008;14: 53-60.

19. Wobus AM, Boheler KR. Embryonic stem cells: Prospects for developmental biology and cell therapy. *Physiol Rev*. 2005;85: 635-678.

20. Brignier AC, Gewirtz AM. Embryonic and adult stem cell therapy. *J Allergy Clin Immunol*. 2010;125: S336-44.

21. Rao MS, Mattson MP. Stem cells and aging: Expanding the possibilities. *Mech Ageing Dev*. 2001;122: 713-734.

22. Evans MJ, Kaufman MH. Establishment in culture of pluripotential cells from mouse embryos. *Nature*. 1981;292: 154-156.

23. Martin GR. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc Natl Acad Sci U S A*. 1981;78: 7634-7638.

24. Thomson JA, Itskovitz-Eldor J, Shapiro SS et al. Embryonic stem cell lines derived from human blastocysts. *Science*. 1998;282: 1145-1147.

25. Mallon BS, Park KY, Chen KG, Hamilton RS, McKay RD. Toward xeno-free culture of human embryonic stem cells. *Int J Biochem Cell Biol*. 2006;38: 1063-1075.

26. Jensen J, Hyllner J, Bjorquist P. Human embryonic stem cell technologies and drug discovery. *J Cell Physiol*. 2009;219: 513-519.

27. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell*. 2006;126: 663-676.

28. Takahashi K, Tanabe K, Ohnuki M et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell*. 2007;131: 861-872.

29. Fong CY, Gauthaman K, Bongso A. Teratomas from pluripotent stem cells: A clinical hurdle. *J Cell Biochem*. 2010;111: 769-781.

30. Till JE, McCulloch EA. A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. 1961. *Radiat Res*. 2012;178: AV3-7.

31. BECKER AJ, McCULLOCH EA, TILL JE. Cytological demonstration of the clonal nature of spleen colonies derived from transplanted mouse marrow cells. *Nature*. 1963;197: 452-454.

32. Friedenstein AJ, Petrakova KV, Kurolesova AI, Frolova GP. Heterotopic of bone marrow. analysis of precursor cells for osteogenic and hematopoietic tissues. *Transplantation*. 1968;6: 230-247.

33. Friedenstein AJ, Chailakhyan RK, Latsinik NV, Panasyuk AF, Keiliss-Borok IV. Stromal cells responsible for transferring the microenvironment of the hemopoietic tissues. cloning in vitro and retransplantation in vivo. *Transplantation*. 1974;17: 331-340.

34. Dominici M, Le Blanc K, Mueller I et al. Minimal criteria for defining multipotent mesenchymal stromal cells. the international society for cellular therapy position statement. *Cytotherapy*. 2006;8: 315-317.

35. Tatullo M, Marrelli M, Paduano F. The regenerative medicine in oral and maxillofacial surgery: The most important innovations in the clinical application of mesenchymal stem cells. *Int J Med Sci*. 2015;12: 72-77.

36. Eggenhofer E, Luk F, Dahlke MH, Hoogduijn MJ. The life and fate of mesenchymal stem cells. *Front Immunol*. 2014;5: 148.

37. Caplan AI. Adult mesenchymal stem cells: When, where, and how. *Stem Cells Int*. 2015;2015: 628767.

38. Hanley PJ. Therapeutic mesenchymal stromal cells: Where we are headed. *Methods Mol Biol*. 2015;1283: 1-11.

39. Najar M, Raicevic G, Boufker HI et al. Mesenchymal stromal cells use PGE2 to modulate activation and proliferation of lymphocyte subsets: Combined comparison of adipose tissue, wharton's jelly and bone marrow sources. *Cell Immunol*. 2010;264: 171-179.

40. Lotfy A, Salama M, Zahran F, Jones E, Badawy A, Sobh M. Characterization of mesenchymal stem cells derived from rat bone marrow and adipose tissue: A comparative study. *Int J Stem Cells*. 2014;7: 135-142.

41. Caplan AI. Mesenchymal stem cells. *J Orthop Res*. 1991;9: 641-650.

42. Baer PC, Geiger H. Adipose-derived mesenchymal stromal/stem cells: Tissue localization, characterization, and heterogeneity. *Stem Cells Int*. 2012;2012: 812693.

43. Bourin P, Bunnell BA, Casteilla L et al. Stromal cells from the adipose tissue-derived stromal vascular fraction and culture expanded adipose tissue-derived stromal/stem cells: A joint statement of the international federation for adipose therapeutics and science (IFATS) and the international society for cellular therapy (ISCT). *Cytotherapy*. 2013;15: 641-648.

44. Choudhery MS, Badowski M, Muise A, Pierce J, Harris DT. Donor age negatively impacts adipose tissue-derived mesenchymal stem cell expansion and differentiation. *J Transl Med*. 2014;12: 8-5876-12-8.

45. Aksu AE, Rubin JP, Dudas JR, Marra KG. Role of gender and anatomical region on induction of osteogenic differentiation of human adipose-derived stem cells. *Ann Plast Surg*. 2008;60: 306-322.

46. Yang HJ, Kim KJ, Kim MK et al. The stem cell potential and multipotency of human adipose tissue-derived stem cells vary by cell donor and are different from those of other types of stem cells. *Cells Tissues Organs*. 2014;199: 373-383.

47. Baglioni S, Cantini G, Poli G et al. Functional differences in visceral and subcutaneous fat pads originate from differences in the adipose stem cell. *PLoS One*. 2012;7: e36569.

48. Huff NK, Spencer ND, Gimble JM, Bagby GJ, Nelson S, Lopez MJ. Impaired expansion and multipotentiality of adult stromal cells in a rat chronic alcohol abuse model. *Alcohol*. 2011;45: 393-402.

49. Garcia-Olmo D, Guadalajara H, Rubio-Perez I, Herreros MD, de-la-Quintana P, Garcia-Arranz M. Recurrent anal fistulae: Limited surgery supported by stem cells. *World J Gastroenterol*. 2015;21: 3330-3336.

50. Fang B, Song Y, Lin Q et al. Human adipose tissue-derived mesenchymal stromal cells as salvage therapy for treatment of severe refractory acute graft-vs.-host disease in two children. *Pediatr Transplant*. 2007b;11: 814-817.

51. Fang B, Song Y, Zhao RC, Han Q, Lin Q. Using human adipose tissue-derived mesenchymal stem cells as salvage therapy for hepatic graft-versus-host disease resembling acute hepatitis. *Transplant Proc*. 2007c;39: 1710-1713.

52. Lin CS, Lin G, Lue TF. Allogeneic and xenogeneic transplantation of adipose-derived stem cells in immunocompetent recipients without immunosuppressants. *Stem Cells Dev*. 2012;21: 2770-2778.

53. Riordan NH, Ichim TE, Min WP et al. Non-expanded adipose stromal vascular fraction cell therapy for multiple sclerosis. *J Transl Med*. 2009;7: 29-5876-7-29.

54. Ankrum JA, Ong JF, Karp JM. Mesenchymal stem cells: Immune evasive, not immune privileged. *Nat Biotechnol*. 2014;32: 252-260.

55. Leto Barone AA, Khalifian S, Lee WP, Brandacher G. Immunomodulatory effects of adipose-derived stem cells: Fact or fiction? *Biomed Res Int*. 2013;2013: 383685.

56. Puissant B, Barreau C, Bourin P et al. Immunomodulatory effect of human adipose tissue-derived adult stem cells: Comparison with bone marrow mesenchymal stem cells. *Br J Haematol*. 2005;129: 118-129.

57. Wang X, Liu C, Li S et al. Effects of continuous passage on immunomodulatory properties of human adipose-derived stem cells. *Cell Tissue Bank*. 2015;16: 143-150.

58. Kim I, Bang SI, Lee SK, Park SY, Kim M, Ha H. Clinical implication of allogenic implantation of adipogenic differentiated adipose-derived stem cells. *Stem Cells Transl Med*. 2014;3: 1312-1321.

59. Crop MJ, Baan CC, Korevaar SS et al. Inflammatory conditions affect gene expression and function of human adipose tissue-derived mesenchymal stem cells. *Clin Exp Immunol*. 2010;162: 474-486.

60. Ghannam S, Bouffi C, Djouad F, Jorgensen C, Noel D. Immunosuppression by mesenchymal stem cells: Mechanisms and clinical applications. *Stem Cell Res Ther*. 2010;1: 2.

61. Yoshimura A, Wakabayashi Y, Mori T. Cellular and molecular basis for the regulation of inflammation by TGF-beta. *J Biochem*. 2010;147: 781-792.

62. Melief SM, Zwaginga JJ, Fibbe WE, Roelofs H. Adipose tissue-derived multipotent stromal cells have a higher immunomodulatory capacity than their bone marrow-derived counterparts. *Stem Cells Transl Med*. 2013;2: 455-463.

63. Scheller J, Chalaris A, Schmidt-Arras D, Rose-John S. The pro- and anti-inflammatory properties of the cytokine interleukin-6. *Biochim Biophys Acta*. 2011;1813: 878-888.

64. Di Nicola M, Carlo-Stella C, Magni M et al. Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. *Blood*. 2002;99: 3838-3843.

65. Yoo KH, Jang IK, Lee MW et al. Comparison of immunomodulatory properties of mesenchymal stem cells derived from adult human tissues. *Cell Immunol*. 2009;259: 150-156.

66. Haddad R, Saldanha-Araujo F. Mechanisms of T-cell immunosuppression by mesenchymal stromal cells: What do we know so far? *Biomed Res Int*. 2014;2014: 216806.

67. Quaedackers ME, Baan CC, Weimar W, Hoogduijn MJ. Cell contact interaction between adipose-derived stromal cells and allo-activated T lymphocytes. *Eur J Immunol*. 2009;39: 3436-3446.

68. Schaper F, Rose-John S. Interleukin-6: Biology, signaling and strategies of blockade. *Cytokine Growth Factor Rev*. 2015;26: 475-487.

69. Wolf J, Rose-John S, Garbers C. Interleukin-6 and its receptors: A highly regulated and dynamic system. *Cytokine*. 2014;70: 11-20.

70. Liu X, Ren S, Qu X, Ge C, Cheng K, Zhao RC. Mesenchymal stem cells inhibit Th17 cells differentiation via IFN-gamma-mediated SOCS3 activation. *Immunol Res*. 2015;61: 219-229.

71. Luig M, Kluger MA, Goerke B et al. Inflammation-induced IL-6 functions as a natural brake on macrophages and limits GN. *J Am Soc Nephrol*. 2015;26: 1597-1607.

72. Najar M, Rouas R, Raicevic G et al. Mesenchymal stromal cells promote or suppress the proliferation of T lymphocytes from cord blood and peripheral blood: The importance of low cell ratio and role of interleukin-6. *Cytotherapy*. 2009;11: 570-583.

73. Park SJ, Nakagawa T, Kitamura H et al. IL-6 regulates in vivo dendritic cell differentiation through STAT3 activation. *J Immunol*. 2004;173: 3844-3854.

74. Pistoia V, Raffaghello L. Unveiling the role of TNF-alpha in mesenchymal stromal cell-mediated immunosuppression. *Eur J Immunol*. 2014;44: 352-356.

75. Prasanna SJ, Gopalakrishnan D, Shankar SR, Vasandan AB. Pro-inflammatory cytokines, IFNgamma and TNFalpha, influence immune properties of human bone marrow and wharton jelly mesenchymal stem cells differentially. *PLoS One*. 2010;5: e9016.

76. Dorronsoro A, Ferrin I, Salcedo JM et al. Human mesenchymal stromal cells modulate T-cell responses through TNF-alpha-mediated activation of NF-kappaB. *Eur J Immunol*. 2014;44: 480-488.

77. Yagi H, Soto-Gutierrez A, Parekkadan B et al. Mesenchymal stem cells: Mechanisms of immunomodulation and homing. *Cell Transplant*. 2010;19: 667-679.

78. Xiao J, Yang R, Biswas S, Qin X, Zhang M, Deng W. Mesenchymal stem cells and induced pluripotent stem cells as therapies for multiple sclerosis. *Int J Mol Sci*. 2015;16: 9283-9302.

79. Rodriguez TM, Saldias A, Irigo M, Zamora JV, Perone MJ, Dewey RA. Effect of TGF-beta1 stimulation on the secretome of human adipose-derived mesenchymal stromal cells. *Stem Cells Transl Med*. 2015;4: 894-898.

80. Ock SA, Baregundi Subbarao R, Lee YM et al. Comparison of immunomodulation properties of porcine mesenchymal stromal/stem cells derived from the bone marrow, adipose tissue, and dermal skin tissue. *Stem Cells Int*. 2016;2016: 9581350.

81. Wrzesinski SH, Wan YY, Flavell RA. Transforming growth factor-beta and the immune response: Implications for anticancer therapy. *Clin Cancer Res*. 2007;13: 5262-5270.

82. Taylor AW. Review of the activation of TGF-beta in immunity. *J Leukoc Biol*. 2009;85: 29-33.

83. Gu AD, Wang Y, Lin L, Zhang SS, Wan YY. Requirements of transcription factor smad-dependent and -independent TGF-beta signaling to control discrete T-cell functions. *Proc Natl Acad Sci U S A*. 2012;109: 905-910.

84. Massague J, Seoane J, Wotton D. Smad transcription factors. *Genes Dev*. 2005;19: 2783-2810.

85. Nurgazieva D, Mickley A, Moganti K et al. TGF-beta1, but not bone morphogenetic proteins, activates Smad1/5 pathway in primary human macrophages and induces expression of proatherogenic genes. *J Immunol*. 2015;194: 709-718.

86. Vanhatupa S, Ojansivu M, Autio R, Juntunen M, Miettinen S. Bone morphogenetic protein-2 induces donor-dependent osteogenic and adipogenic differentiation in human adipose stem cells. *Stem Cells Transl Med*. 2015;4: 1391-1402.

87. Miyazono K, Kamiya Y, Morikawa M. Bone morphogenetic protein receptors and signal transduction. *J Biochem*. 2010;147: 35-51.

88. Jaroszeski MJ, Radcliff G. Fundamentals of flow cytometry. *Mol Biotechnol*. 1999;11: 37-53.

89. Monici M. Cell and tissue autofluorescence research and diagnostic applications. *Biotechnol Annu Rev*. 2005;11: 227-256.

90. McIntosh KR. Evaluation of cellular and humoral immune responses to allogeneic adipose-derived stem/stromal cells. *Methods Mol Biol*. 2011;702: 133-150.

91. Oedayrajsingh-Varma MJ, van Ham SM, Knippenberg M et al. Adipose tissue-derived mesenchymal stem cell yield and growth characteristics are affected by the tissue-harvesting procedure. *Cytotherapy*. 2006;8: 166-177.

92. Maumus M, Peyrafitte JA, D'Angelo R et al. Native human adipose stromal cells: Localization, morphology and phenotype. *Int J Obes (Lond)*. 2011;35: 1141-1153.

93. Nicolaidou V, Stylianou C, Koumas L, Vassiliou GS, Bodman-Smith KB, Costeas P. Gene expression changes in HLA mismatched mixed lymphocyte cultures reveal genes associated with allorecognition. *Tissue Antigens*. 2015;85: 267-277.

94. Girdlestone J, Wing M. Autocrine activation by interferon-gamma of STAT factors following T cell activation. *Eur J Immunol*. 1996;26: 704-709.

95. Sakatsume M, Finbloom DS. Modulation of the expression of the IFN-gamma receptor beta-chain controls responsiveness to IFN-gamma in human peripheral blood T cells. *J Immunol*. 1996;156: 4160-4166.

96. Shao WH, Gamero AM, Zhen Y et al. Stat1 regulates lupus-like chronic graft-versus-host disease severity via interactions with Stat3. *J Immunol*. 2015a;195: 4136-4143.

97. Shu Z, Gao D, Pu LL. Update on cryopreservation of adipose tissue and adipose-derived stem cells. *Clin Plast Surg*. 2015;42: 209-218.

98. Kondo M, Yamaoka K, Sakata K et al. Contribution of the interleukin-6/STAT-3 signaling pathway to chondrogenic differentiation of human mesenchymal stem cells. *Arthritis Rheumatol*. 2015;67: 1250-1260.

99. Shao WH, Gamero AM, Zhen Y et al. Stat1 regulates lupus-like chronic graft-versus-host disease severity via interactions with Stat3. *J Immunol*. 2015b;195: 4136-4143.