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Analysis of Immunomodulatory Properties of Spheroids from Adipose-derived Stem Cells

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Abstract

Purpose: Adipose Stem Cells (ASCs) represent promising cell therapies for regenerative medicine and immunomodulation. The recently isolated spheroids from adipose-derived stem cells (S-ASCs) have shown being superior stemness and enhanced regenerative capability than the classical ASCs. The aims of this study were (1) to investigate the effects of adipose tissue donor-site, harvesting technique and patients' characteristics on the yield of S-ASCs and (2) to investigate the immunomodulatory properties of S-ASCs comparing them with the adherent ASCs.

Material and Methods: The study was divided into two phases. *Phase I* (prospective clinical study) between July 2016 and August 2019 was conducted to evaluate the effects of adipose tissue donor-site, harvesting technique and patients' characteristics on the yield of S-ASCs. Seventy-six subcutaneous fat tissues samples from flanks (n=13), abdomen (=30), thigh (n=21) and breast (n=12), were harvested from healthy patients using syringe-assisted Coleman technique with (n=34) or without centrifugation (n=42). Differences in S-ASC yields were assessed with one-way analysis of variance.

Phase II (Immunological *in vitro* study) was designed to assess and to compare the immunomodulatory properties of human S-ASCs and ASCs toward γδ T cells (subpopulations of PBMCs) using a CFSE-mediated Mixed lymphocyte reaction (MLR) assay and their cytokine secretion profile by Cytofluorimetric analysis.

Results: In *Phase I*, the total number of S-ASCs in fat harvested from the thigh was

significantly higher than flanks or breast ($P < 0.05$). Comparing the technique used for fat harvesting, samples from thigh and abdomen with centrifugation yielded significantly more S-ASCs thank those without centrifugation.

In *Phase II*, both ASCs and S-ASCs effectively suppressed mitogen-induced γδ T cells proliferation. Levels of interferon (IFN)- γ secreted from activated γδ T cells increased over time, but these levels were significantly reduced when co-cultured with both type of ASCs. A statistical significant stronger immunosuppressive effect of S-ASCs was observed in the downregulation of the pro-inflammatory cytokines and in upregulation of the anti-inflammatory cytokines ($P<0.05$ for both). The suppressive activity of S-ASCs regarding the proliferation of γδ T cells was shown to be cell-to-cell contact dependent and independent.

Conclusions: Adipose tissue harvested from the thigh through Coleman's technique with centrifugation was found to yield higher number of S-ASCs. The immunomodulatory findings support that S-ASCs share immunosuppressive properties with the classical adherent ASCs.

Summary

Mesenchymal stem cells (MSCs), and in particular Adipose-derived Stem Cells (ASCs), have emerged as promising cell therapies for regenerative medicine and immunomodulation and are currently being tested in preclinical and clinical settings. However, the biological mechanisms of ASCs by which this treatment modality exerts its therapeutic effects remain unclear. The recent isolation of Spheroids from Adipose-derived Stem Cells (S-ASCs) from adipose tissue has provided new perspectives on the biological and immunological potentials. Three-dimensional (3D) S-ASCs have shown superior stemness and enhanced regenerative capability than the classical ASCs. There is controversy regarding the most favorable method of tissue-harvesting and donor-site to obtain the most yield and viability of ASCs and no studies have investigated if exists significant heterogeneity in terms of S-ASCs yield. Indeed, unlike of adherent ASCs, the immunomodulatory effects of S-ASCs have not been investigated yet. Therefore, in this research study we will investigate the effects of adipose tissue donor-site, harvesting technique and patients' characteristics on the yield of S-ASCs. Furthermore, we will compare the immunomodulatory properties of S-ASCs and ASCs analysing the microenvironmental molecules produced by these stem cells.

CHAPTER 1

Introduction

The so-called Adipose-derived stem cells (ASCs) are a variety of mesenchymal stem cells (MSCs) identified for the first time in 2001 in the adipose tissue.[1,2] In the past decade, successful isolation of multipotent ASCs, and their ability to preferentially home to damaged tissue, made ASCs a promising tool in regenerative medicine.[3] Indeed, ASCs has been used as biological vehicles to deliver therapeutic molecules *in vivo,* [4,5] to support hematopoietic reconstitution in lethally irradiated mice and to provide immunosuppressive capabilities *in vitro* and *in vivo* experiments.[6] Their clinical appeal is due to an easy harvest in large quantities through a non-invasive and well-accepted approach with an extremely low risk of complications.[7,8]

Cells can be easily separated from subcutaneous fat deposits by different stages of washing, digestion and centrifugation, followed by in vitro growth under adherence or suspension conditions. It has been described that stem and progenitor cells in the stromal vascular fraction (SVF) usually amount to up to 3%, and this is 2.500 times more than the frequency of MSCs in the bone marrow (up to 0.002%).

In recent years, the scientific community has considered two-dimensional (2D) cell cultures being artificial. Instead, cells express their own tissue-specific characteristics when they reside within a three-dimension (3D) micro-environment called “niche”. It has been shown that

cells lose tissue-specificity when they are separated from the niche.[9] In the attempt to restore the tissue-specificity, several culture techniques have been attempted. Hence, 3D cultures have been performed in which cell-cell contacts are re-established. In this contest, cells give rise to suspension aggregates with a spheroidal shape, called Spheroids of Adipose-derived stem cells (S-ASCs).[10]

Donor-dependent differences exist in the yield of ASCs, in the proliferation and capacity of differentiation, that could be determined also by the age[11,12] and body mass index (BMI).[13,14] Indeed, controversy among the authors are still present if fat-harvesting technique and donor site, could influence the yield of SVF cells and ASCs, from adipose tissues.[15,16] However, so far, no studies have investigated if these factors could influence the yield of S-ASCs.

The use of novel cell-based therapies, that combine the benefits of immunoregulation with neuroregeneration, have proven to have a great potential in improving functional outcomes and the quality of life in vascularized composite allotransplant (VCA) patients.[17] Nonetheless, their use is strongly limited due to the need of systemic immunosuppression. However, such regimens utilized to prevent rejection lead to severe, life-disrupting adverse effects.

It is known that mesenchymal stem cells (MSCs) are "immune-privileged" due to their low expression of human leukocyte antigen (HLA) and co-stimulatory molecules. Furthermore, they exert potent immunosuppressive actions.[18] These desirable properties make MSCs a new therapeutic option; MSCs have emerged as a promising cell-based therapy for immunomodulation, and are currently being examined in preclinical and clinical settings as therapeutic solutions for autoimmune disorders or transplant rejection.[19-23]

To date, bone marrow-derived mesenchymal stem cells (BM-MSCs) are considered the standard cell type used in the fields of stem cell biology and clinical application. However, ASCs, abundant in the human body, could be harvested with minimal invasiveness and are becoming increasingly widespread in treating various diseases.[24] Many studies have aimed to investigate the phenotypic and intrinsic biological differences between traditional adherent and non-adherent

ASCs.[25,26] The recent isolation of a non-adherent ASCs population paved the way to the possibility that this niche could include cells at a less advanced stage of differentiation, and therefore more upstream in the stem cell hierarchy. Despite scientists have began using spheroids from ASCs in a number of laboratories worldwide, their identity as a separate stem cell niche with different phenotype, biological behavior and differentiation ability, has never been fully defined.

1.1 Immunomodulatory role of ASCs

The so-called Adipose-derived stem cells (ASCs) are a variety of MSCs extracted from adipose tissue and also defined as fibroblast-like, adherent and multipotent cells. Indeed, ASCs are able to differentiate along multiple lineages including adipocytes, osteoblasts and chondrocytes, as well as other cell types including hepatocytes, myocytes and neuronal-like cells.[3,27] The successful isolation of multipotent ASCs achieved in the past decade, and their ability to preferentially home to damaged tissue, make ASCs a promising tool in regenerative medicine.[27-29] Indeed, ASCs can be engineered and used as biological vehicles for vector-based genes to convey therapeutic molecules *in vivo*.[4,5] Furthermore, previous studies suggested that ASCs can support hematopoietic reconstitution in lethally irradiated mice.[6] Additionally, ASCs have proved to inhibit the production of inflammatory cytokines and stimulate the production of anti-inflammatory cytokines and antigen-specific Tregs.[30]

The immunomodulatory potential of MSCs was supported by robust *in vitro* and *in vivo* evidence. Consequently, a significant interest developed around the immunomodulatory properties of ASCs.[31]

1.2 ASC-Mediated Immunosuppression *in Vitro*

Due to their strong immunosuppressive properties, *in vitro* experiments have proven that both ASCs and BM-MSCs can suppress T-cell proliferation induced by alloantigens, mitogens

and soluble antigens (Figure 1A).[31,32] Interestingly, this inhibition appears not to be MHC restricted, given that both autologous and allogeneic ASC are able to determine this suppression.[4,33] For the first time, Puissant et al. investigated the immunosuppressive properties of ASCs *in vitro* demonstrating not only that ASCs do not elicit response of allogeneic lymphocytes, but they also repress allogenic peripheral blood mononuclear cells (PBMCs) from inhibiting lymphocyte proliferation.[34] This inhibition persisted when ASCs were separated from lymphocytes through a permeable membrane, suggesting a secretory, rather than contact-mediated, activity.

Further studies showed that ASCs inhibited the production of pro-inflammatory cytokines when co-cultured with activated leukocytes *in vitro* (TNF-α, IFN-γ and IL-12), and simultaneously increased the mRNA expression of immunosuppressive mediators (TGF-β, HGF, IDO, and COX-2). In particular, TNF-α increases the immunosuppressive effect of ASCs by enhancing the expression of prostaglandin E2 (PGE2).[31] Indeed, the implication of PGE2 was proved essential in the MSC-mediated immunosuppression, inhibiting dendritic cells (DCs) maturation, T lymphocytes proliferation and proinflammatory cytokines production.[35] Moreover, IFN-γ, a potent proinflammatory cytokine, proved to induce the production of IDO that, in turn, inhibits T-cell proliferation.[18] Additional immunosuppressive effects mediated by MSCs on T-cells include decreased T-$CD8^+$-mediated cytotoxicity and an increased level of Tregs.[30] In this regard, recent studies have shown that TGF-β play a significant role in T-cell regulation. The disruption of TGF-β signaling in T-cells would result in the down-regulation of Tregs and the up-regulation of activated effector T-cells.[36] Indeed, levels of TGF-β and IL-10 significantly increased while the IFN-γ was suppressed in ASCs co-cultures compared to controls, confirming the ability of ASCs to modulate T-cells while inducing immune tolerance by modulating the levels of the principle immune-mediators.[37] Recently, proteome studies have demonstrated that haptoglobin expression correlates with the modulation of allogenic lymphocytes and immune tolerance induction through ASCs.[38] Following co-culture with activated T cells, the fluorescence intensity

of heme oxygenase-1 expression in the ASCs was increased compared to controls.[37] Moreover, the analysis of serum proteins in immunotolerant animals showed that the increase of VCA survival induced by MSC was associated with the downregulation of haptoglobulin and the upregulation of vitamin D-binding protein, β2-glycoprotein, α1-macroglobulin and rat albumin.[38]

1.3 ASC-Mediated Immunosuppression *in Vivo*: Preclinical Studies in *VCA*

A number of *in vivo* animal studies also demonstrated the immunomodulatory properties of ASCs. These animal models were investigated with respect to alloreactive immunity, autoimmunity, GVHD, anti-tumor immunity, spinal cord injury and neurodegenerative diseases,[39] resulting in successful preclinical applications for SOT and VCA.

Previous studies successfully used BM-MSCs in combination with total body irradiation (TBI), transient immunosuppressant treatment with or without donor bone-marrow transplantation (BMT) in swine,[40-42] and rodent[43] VCA animal models (Table 1).

Following these encouraging results, more recent studies began investigating the effects of ASCs on prolonged VCA survival. Multiple injections of allogeneic ASCs, combined with antilymphocyte serum (ALS) and a transient immunosuppressant regimen, enabled long-term survival of rat hind-limb VCA model.[4] Prolonged survival of allografts with ASCs injection was deemed to be mediated by the blockade of inflammatory cytokines and the regulation of T-cell function. An increased level of $CD4^+CD25^+FoxP3^+$ Tregs population was detected in the blood of recipients treated with donor ASCs in combination with ALS and CsA at six weeks postoperatively. However, such level rapidly dropped to naive levels by week 12. On the other hand, peripheral concentrations of both TGF-β and IL-10, not IFN-γ, increased in the blood of ASC/ALS/CsA-treated recipients at 4 weeks and 21 weeks post-operation.[4] A later study observed a substantially delayed allograft rejection, even without immunosuppressants, using donor-derived ASCs.[44] Subsequently, Cheng et al. evaluated the effects of syngeneic ASCs in a rat hind-limb VCA model.[33] A single dose was able to induce donor-specific tolerance in 66% of

VCA recipients. Indeed, in the long-term, VCA-tolerant animals presented high levels of peripheral $CD4^+CD25^+$FoxP3 Tregs. Comparing the same animal model and immunosuppression therapy, a single dose of syngeneic ASCs proved to have greater efficacy in terms of tolerance induction compared to multiple donor ASCs infusions (66 vs. 33%).[33] Finally, Plock et al. investigated the influence of timing and frequency of donor ASCs administrations on VCA survival. The timing of ASCs administration resulted crucial for VCA survival and peripheral chimerism development. Repeated administrations revealed improved outcomes after VCA.[45]

1.4 ASCs: Immunosuppressive or immunostimulatory?

Recent studies revealed that ASCs could generate both immunosuppressive or immunostimulatory effects. Allogenic MSCs proved to induce alloantibodies and CD8-mediated cytotoxicity. They also activate complement pathways and contribute to tissue rejection.[46,47] By contrast, autologous MSCs are non-immunogenic. Some groups have proposed that donor characteristics, tissue sources and culture conditions could greatly influence the immunomodulatory features of BM-MSCs and ASCs. Immunostimulation by ASCs could be influenced by number of passages during culture: early passage of ASCs promote allogenic responder T-cells in MLRs, however, subsequent passages would lead to progressive cellular senescence, decreased proliferation rate and, indeed, a tendency to lose their immunomodulatory properties.[25,48,49] Recently, Wang et al. showed that in human ASCs, the levels of IL-10 and HGF decreased with the number of passage, while that of IFN- γ increased if co-cultured with PBMCs.[49] Therefore, while an increased number of passages has minor effects on cell phenotype, it appears to impair the immunomodulatory properties of ASCs. Nonetheless, the mechanism through which the ASCs regulate allogenic responder T-cells remains to be investigated. Previous studies suggested that, with increased passages, the reduction of immunostimulation of ASCs could be partially explained due to the absence of markers associated with antigen-presenting cells (APCs) such as MHC-II, CD45, CD80 and CD86, which seem to be correlated with their

capacity of acting as stimulator cells and triggering the immune response.[32,50] Another hypothesis involves the role of soluble factors, such as PGE2, IDO and HGF, as well as the leukemia inhibitory factor that seems to mitigate an active T cell response.[51] Indeed, the inhibition of each of these soluble factors eliminated the immunosuppressive effects of ASCs**.** Others cytokines such as IL-6 proved to have a role in the ASC-mediated immunosuppression mechanism. IL-6 is able to downregulate MHC-II, CD40, and CD86 on DCs and inhibit their differentiation, affecting antigen presentation and/or the costimulatory signaling of APCs.[52] Interestingly, the requirement of cell-cell contact proved to be essential in order to regulate the immunosuppressive capabilities of ASCs. However, the data available is still unclear and, while some studies have demonstrated that such contact is necessary for ASCs to exert an immunosuppressive effect,[34] other studies suggest that the cell-cell contact is not essentially required.[53] While, on the one hand, the recruitment of inflammatory cells promoted by MSCs can be considered a proinflammatory effect,[54] on the other, proinflammatory cytokines such as IFN-γ, TNF-α, IL-1, seem to be essential for MSC-mediated immunosuppressive effects.[55]

1.5 2D adherent vs. 3D spheroids from ASCs (S-ASCs)

Two-dimensional (2D) cell culture has been employed as a standard culture condition for *in vitro* expansion of ASCs.[56] However, previous studies demonstrated that stem cells (both naïve and cancerous) from different organs could grow in suspension if set in a serum-free culture condition. These cells present a phenotype characterized by round cells and do not adhere to plastic. Instead, they aggregate in clusters: spheroids (multiclonal) and spheres (monoclonal).[57,58] Comparing traditional 2D adherent ASCs and 3D non-adherent spheroids, the latter are closer to the physiologic state.[59] 3D cell aggregates are spared of the artificial state of cell adhesion to plastic, which alters phenotype as well as surface molecule expression. Although spheroids from ASCs began to be used in a number of laboratories worldwide, their qualification as a separate stem cell niche with different phenotype, biological behavior and differentiation ability have

never been fully explained.

Spheroid proved to feature several characteristics in both BM-MSCs and ASCs (Figure 1B). In a study carried out by Bartosh et al, BM-MSC spheroids were shown to enhance anti-inflammatory effects in both *in vitro* (up-regulating TNFAIP6/TSG6 and reducing macrophage activation) and *in vivo* in a mouse model of peritonitis.[60] Furthermore, BM-MSC spheroids may have an anti-tumor effect. Frith et al. demonstrated that prostate cancer cells growing in medium conditioned by dynamic 3D-MSCs would be selectively inhibited, possibly through up-regulation of IL-24.[61] In another study, 3D-MSCs spheroids from umbilical cords showed increased levels of HGF and TGF-β3, which may enhance the ability to regenerate or repair tissues.[62] Bhang et al. showed that the transplantation of spheroids from human cord blood MSCs into mouse ischemic hind-limbs could improve the survival rate of transplanted MSCs.[63] This may be related to the suppression of apoptotic signaling while simultaneously activating anti-apoptotic signaling.[63] Indeed, Laschke et al. have reported that spheroids consisting of murine ASCs could enhance angiogenesis.[64] Interestingly, Cheng et al. reported that ASCs spheroids prolonged replicative lifespan or delayed cell senescence of ASCs *in vitro*.[65]

1.1 Rationale and Objectives

For the past 5 years, our group has been focusing on the isolation and characterization of these spheroids from ASCs, which we now refer to as Spheroids from Adipose-derived Stem Cells (S-ASCs).[26,59,66] Studies from our group have demonstrated an enhanced ability of S-ASCs to differentiate *in vitro* into distinctive end-stage cell types such as osteoblast, chondrocyte and adipocyte.[26] Furthermore, gene expression profile analysis indicated that S-ASCs are endowed with stem cell potential that is gradually lost following adherence and after multi-lineage specific differentiation. S-ASCs possess a pronounced and unique *in vivo* ability to regenerate bone injury when seeded in a dermal matrix such as Integra®. In these settings, S-ASCs showed increased ability to facilitate bone vertebral body repair and regeneration after laminectomy in a mouse

model when compared to adherent ASCs. Indeed, S-ASCs have provided new perspectives on the biological and immunological potentials that such upstream cell niche may have in comparison with ASCs, which may by their downstream counterparts. The ability of these upstream progenitors to grow in suspension as spheres in a 3D-trend may potentially overcome the important limitations of autologous, allogenic, and xeno sera necessary for the growth of the adherent 2D monolayer ASCs.

There is controversy regarding the most favorable method of tissue-harvesting and donor-site to obtain the most yield and viability of ASCs and no studies have investigated if exists significant heterogeneity in terms of S-ASCs yield. Indeed, unlike of adherent ASCs, the immunomodulatory effects of S-ASCs have not been investigated yet.

The objectives of this study were as follow:

Specific Aim 1:

To investigate the effects of adipose tissue donor-site, harvesting technique and patients' characteristics on the yield of S-ASCs.

Specific Aim 2:

To assess the *in vitro* immunomodulatory properties of autologous S-ASCs through the analysis of pattern of cytokines produced by them and toward γδ T cells using a CFSE-mediated MLR assay and to compare them to commercially available ASCs.

CHAPTER 2

Materials and Methods

2.1 Phase I: Clinical Prospective Study

2.1.1 Adipose Tissue Patients

Between January 2016 and May 2018, adipose tissue from healthy individuals was withdrawn from 76 healthy donors (12 men, 64 women) at the Department of Plastic and Reconstructive Surgery of the University Hospital of Palermo. The average age of the donors was 47 years old (range 16-82 years) with mean BMI of 26 (range18.9-36.5). Inclusion criteria were healthy patients without any co-morbidity who underwent fat harvesting as a secondary revision following reconstructive procedures or for cosmetic purpose. Patients were excluded if they received previous radiotherapy. The hospital ethical committee approved the study, and from each patient an informed consent was collected. Tissues were obtained through liposuction from different areas, such as abdomen, thighs, flanks, and breast.

Patients were divided in 3 groups related to the age: 1) 16-45 years; 2) 46-55 years and 3) 56-82 years old. Then, the study population was then divided into three categories of BMI (normal, overweight and obesity) to evaluate a possible correlation between the average number of S-ASCs and body weight. We identified 3 groups related to the BMI: 1) 18.5-24.9; 2) 25-29.9;

3) >30.

2.1.2 Harvesting method of Adipose Tissue

Lipoaspirates were harvested by Coleman's technique using a 3-mm blunt cannula and a 10-mL syringe following infiltration of Klein solution (NaCl 0.9% 1 L, 2% Lidocaine 25 ml, Adrenaline 1: 1000 1ml, NaHCaO3 8.45% 12.5 ml). Fat-harvesting sites examined were as follow: thigh, flanks, breast and abdomen. Following the fat tissue harvesting, the samples were handled by centrifugation at 1500 rpm for 3 minutes or by sedimentation (decantation in syringe). In a double-blind study, lipoaspirates were then sent to the laboratory for digestion, cultured in suspension and extraction of adipose stem cells.

2.1.3 Cell isolation and culture

Lipoaspirates were subjected to a process of enzymatic and mechanical digestion in order to obtain a specific cell population. The samples were digested with collagenase (1.5 mg/ml, GIBCO) through mechanical action for 30 minutes at 37°C. Then, the digested samples were centrifuged at 1200 rcf for 5 min at 4 ° C and separated into 3 phases (from top to bottom):

- Upper phase: consisting of the oily content released by the digestion of adipocytes, with a characteristic golden yellow color.
- Central phase: consisting of digested adipocytes and connective tissue, presenting a whitish color and a soft consistency.
- Lower phase (pellet): consisting of blood and the vascular stromal portion (SVF) including: adipose stem cells, fibroblasts, smooth muscle vascular cells, erythrocytes and leukocytes.

The upper and central phases were removed while the lower phase (SVF portion) was cultured with the addition of growth factors such as bFGF (10 ng/ml; Sigma, St. Louis, MO) and

epidermal growth factor (EGF; 20ng/ml; Sigma) and antibiotic /antimycotic. Then it was plated on specific ultra-low culture flasks (Corning, NY) and placed at 37°C in a 5% CO_2 humidified incubator.[26] In these conditions, cells grew as floating spheroids and called Spheroids of Adipose derived Stem Cells (S-ASCs). Under the microscope, the S-ASCs, after a period of about 3-4 days, are easily identifiable both as single units and as spheroidal cellular aggregates.

2.2 Phase 2: Immunological in vitro study

2.2.1 Isolation of peripheral γδ T cells

S-ASCs were used for immunological experiments, in order to evaluate their immunomodulatory properties. These analyzes were carried out between distinct cell populations, γδ T cells (a subpopulation of PBMCs) together with ASCs or S-ASCs. γδ T cells are a population of T lymphocytes that combine innate and adaptive immune characteristics playing an important role in the host's primary response to infections and tumors. These cells have been preferred in this study due to non-recognition of the different major histocompatibility complex (MHC) class I, as different donors could be used for the fat and cell samples of the immune response. To obtain peripheral blood mononuclear cells (PBMCs), blood was stratified on a density gradient (Ficoll-Hypaque, Pharmacia Biotech, Uppsala; Sweden) following the following protocol:

1. Blood is diluted 1:1 with incomplete RPMI (RPMI 1640 medium, Hepes Buffer, 100U/ml penicillin, 0.1 mg / ml streptomycin) inside a sterile Falcon;
2. Diluted blood is slowly stratified on a volume of Ficoll equal to the initial volume of blood, before being diluted;

3. Stratified blood is centrifuged at a speed of 2000 rcp for 20 minutes, after that stratification of the various blood components is obtained
4. Then, the plasma is discarded and, using a sterile Pasteur, the Buffy Coat is taken;
5. PBMCs collected are placed inside a sterile Falcon and subjected to two washes (centrifugation at 1500 rcp for 5 minutes) with incomplete RPMI to eliminate any traces of plasma and Ficoll;
6. The PBMCs obtained are placed in complete RPMI, containing 10% of bovine Fetal Serum and 1X of glutamine;

In the immunology laboratory, human peripheral γδ T cells were isolated from the PBMCs of healthy donors by magnetic antibody cell sorting (MACS) technology using the TCR- γδ + T Cell Isolation kit (Miltenyi Biotec). Informed consent was obtained from each blood donor. The PBMCs were incubated for 15 min at 4° with PE-labelled anti-γδ TCR mAb (clone GL3; BD Biosciences). After washing twice with MACS buffer [0.5% bovine serum albumin, 2 mM ethylenediaminetetraacetic acid in phosphate-buffered saline (PBS)] anti-PE MicroBeads (Miltenyi Biotec) were diluited (1:5 dilution) and γδ T cells were enriched. Purity of γδ T cells was determined by flow cytometry and only cells with > 98% purity were used. For the culture of primary lines of γδ T cells obtained from PBMCs, a specific stimulation was used with Zoledronic acid (2.94 uM/ml). The culture is maintained for a maximum of 14 days, with a change of *land* every 3-4 days. Stimulation is carried out only on the first day while the addition of IL2 is performed every 3 days.

For to the cytofluorimetric analysis evaluation that allows to understand the percentage of γδ and the starting phenotype, cells are labeled with specific antibodies. The expression of surface markers is evaluated by cytofluorimetry (FACS Canto II) and the analysis carried out using the FlowJo software (BD Biosciences). Once the cells cultivated inside the flask have been counted, around 100,000 cells for each sample are distributed in the cytofluorimetric tubes, and are labeled with monoclonal antibodies conjugated to

fluorochromes. The fluorescences used are Fluorescein isothiocyanate (FITC), Ficoeritrina (PE), Alloficocianina (APC), Ficoeritrina-Cy7 (PECy7) and Alloficocianina-Cy7 (APC-Cy7), Peridinin chlorphyll protein (PerCP).

The following monoclonal antibodies conjugated with fluorochromes have been used to study the phenotype of T γδ lymphocytes: anti-CD45RA (PE), anti-CD27 (APC), anti-CD45 (APC-Cy7), anti-CD3 (PerCP), anti-Vδ2 (FITC).

2.2.2 Cell Cultures

S-ASCs were cultured as previously described. On the other hand, adherent 2D ASCs were the commercially available STEMPRO® human adipose-derived stem cells, plated as recommended by the manufacturer (Invitrogen, Carlsbad, CA).

T cells were cultured in the presence of phytohaemagglutinin (PHA, 1 mg/ml, Sigma, St Louis, Missouri, USA) and with various numbers of human ASCs or S-ASCs in flat-bottomed 96-well plates at different cell ratio ASC/SASCs: γδ T: 1:1, 1:5, 1:10; 5:1 and 10:1. In some experiments, γδ T cells were cultured with the presence of only the supernatant of S-ASCs. The supernatant was collected daily for 5 days from S-ASCs and then cultured with γδ T cells.

2.2.3 Flow cytometry

The cellular gating strategy used to identify the γδ T cell population and the expression of its surface markers was as follow: lymphocyte separation by FSC/SSC (physical parameters), single cell selection by FSC-A (amplitude) FSC-H (height), selection of live leukocytes by LiveDead (zombie /CD45, selection of T lymphocytes (FSC-A/CD3+), selection of T lymphocytes γδ (Vδ2), study of the phenotype (CD27 / CD45RA).

2.2.4 Cytokine determination

To evaluate the intracellular production of IFN-γ, IL-17, IL10 and IL-9 by γδ T cells obtained from peripheral blood of healthy subjects, the circulating cells have been seeded in wells of a culture plate and have been stimulated with Ionomycin (BD Biosciences, 10 μg / ml) and PMA (BD Biosciences, 15 μg / ml) for 4 hours at 37 °C at 5% CO2. After the first hour of incubation, Monensin (Sigma, 2 μl / ml) was added. Monesin is an inhibitor of intracellular protein transport to the Golgi complex, whose presence determines the accumulation of proteins at the level of the endoplasmic reticulum and favors the evaluation of intracellular cytokines by flow cytometric analysis. Then, a surface antibody fixation was performed for 30 minutes at 4 °C using Fixation/Permeabilization Solution (BD Cytofix / CytopermTM kit), containing 4% paraformaldehyde. Next, the cells were washed in 500 μl of FACS Buffer by centrifugation at 1500 rpm for 5 minutes and, subsequently, with a Permeabilization Buffer (BD Perm/WashTM Buffer). The monoclonal antibodies used for intracytoplasmic labeling were the following: FITC-labeled anti-IFN-γ (clone 4S.B3, Cat 502506 Biolegend), APC-labeled anti-IL17A (clone CZ8-23G1, Cat 130-096-748 Miltenyi), PeVio770 anti-TNF-α (clone cA2, Cat 130-096-755 Miltenyi). Coculture conditions of 1:5 and 1:10 and vice versa were used between ASCs/S-ASCs and γδ T cells in complete RPMI soil.

2.2.5 S-ASCs suppressive activity determination

The proliferating γδ T cells were recovered, counted and the phenotype evaluated. Part of these cells have been labeled with CSFE. CSFE is a dye that spontaneously enters the cells and at the cytoplasmic level is modified through an esterification preventing its release. The dye during the mitosis processes will be divided equally between the daughter cells, allowing control, starting from time 0, of the number of mitotic divisions performed. Proliferation-enriched γδ T lymphocytes were co-cultured with non-autologous ASCs or S-ASCs in order to evaluate the immunoregulatory capacity of these cells on γδ T cells. Positive

controls were created with γδ T cells cultured alone, stimulated with PHA and negative (without stimulation) with γδ T cells lymphocytes not labeled with CFSE. Other γδ T lymphocytes were co-cultured with ASCs or S-ASCs at different ratios (1:5; 1:10; 5:1 and 10:1).

CFSE-stained γδ T cells have been stimulated by the mitogen phytohaemagglutinin (PHA) and ASC or S-ASCs at different concentrations. Each sample has been plated in quintuplicates per each experiment. Five different sets of experiments were performed for each group to achieve statistical significance. At each cell division, cells will lose CFSE cellular expression. Cell proliferation has been assessed using flow cytometry analysis by the reduction of CFSE intensity. Immunomodulation of γδ T cells by ASCs and S-ASCs has been assessed by lack of CFSE stain reduction.

2.3 Statistical analysis

Statistical analysis was performed using SPSS software package (SPSS Inc, Chicago, USA). Descriptive data were presented as mean plus or minus SD. Comparison between different conditions was performed using the paired samples t test. Results were considered significant if p value was lower than 0.05.

CHAPTER 3

Results

3.1 Phase I: Clinical prospective study

Sixty-four participants were females and 12 were males with a mean age of 47 years old (range 16-82 years) and BMI between 18.9 and 36.5. Lipoaspirates samples were harvested from the following donor sites: abdomen (n = 30), flanks (n=13), thigh (n=21) and breast (n =12). After fat tissue was harvested, the samples were handled by centrifugation at 1500 rpm for 3 minutes (n = 33) or sedimentation (decantation in syringe) (n = 43).

3.1.1 Correlation between S-ASCs and patients' characteristics

Thirty patients were in the first group (18-45 years), 31 in the second group (46-55 years) and 15 in the third group (56-82 years). In the first group, there was an average of 1.682.000 S-ASCs, in the second group 1.118.000 S-ASCs and in the third group 695.000 S-ASCs. There was a statistically age-related difference regarding the average of S-ASCs among the first two groups and the latter group (P=0.03) (Table 2).

Regarding the BMI, 27 Patients were included in normal weight category, 35 were overweight and 14 obese (Table 3). A significant difference was found between the overweight

category, which presented the highest number of S-ASCs (average 1.493.000), and the other two categories obesity and normal weight, who presented respectively 900.000 and 344.000 average of S-ASCs respectively (P=0.03).

3.1.2 Correlation between S-ASCs yield and harvesting donor-site

The analysis investigating the percentage of the extracted S-ASCs for each sample in each different donor-site location showed 92% isolation capacity of S-ASCs in the flanks samples. Instead, the percentage of S-ASCs isolated from thigh region and breast was 86% and 75% respectively. Abdomen presented the lowest yield with 63% isolation capacity of S-ASCs (Figure 2).

The average number of S-ASCs extracted from the various harvesting sites partially reflected their percentage of isolation capacity. Hence, flanks exhibited the highest yield of S-ASCs (2.393.846) followed by thigh (1.953.190), breast (1.834.417) and abdomen (585.833).

However, considering the S-ASCs yield in relation to the volume of adipose tissue harvested (S-ASCs/ml), it was found that the best fat-harvesting site was the thigh region showing the highest yield of S-ASCs (87.724), followed by abdomen (64.710), flanks (39.297) and breast (12.946). A significant difference was observed regarding the S-ASCs yield among thigh/abdomen donor sites and flanks/breast regarding S-ASCs yield (P=0.03). (Figure 3).

3.1.3 Correlation between S-ASCs yield and Harvesting Method (Centrifugation vs. Sedimentation)

A comparison of the S-ASCs yield using Coleman's technique with or without centrifugation is illustrated in Figure 4. An average of 1.216.200 and 1.422.619 S-ASCs was extracted respectively with the centrifugation and sedimentation technique. One-way analysis of variance showed that there was not a significant difference between the two techniques used in the operating room regarding the S-ASCs yielded (P > 0.08) However, by analyzing the two

different methods in the individual harvesting donor-sites, S-ASCs yield in the abdomen and thigh region the centrifugation technique was clearly superior to the sedimentation technique. However, in the breast the S-ASCs yield has been substantially superior with the sedimentation technique than centrifugation. (Figure 4)

3.2 Phase II*:* Immunological *In vitro* study

3.2.1 ASCs vs S-ASCs: Cytokine determination

When γδ T cells were co-cultured with classic adherent 2D ASCs, a reduction in expression levels of the proinflammatory cytokine IL-17 was observed after stimulation by PHA. The reduction of IL-17 was 75% and 85% respectively in 1:5 and 1:10 ratios. However, IFN-γ increased of 10% in the 1:5 ratio and partially decreased of 7.5% in 1:10 ratio. Regarding the role of ASCs in the expression of the anti-iinflammatory cytokines, an evident upregulation of 67% of Il-10 was observed only in the 1:5 ratio. The expression of IL-9 did not show any upregulation but was reduced of 64% in the 1:5 ratio (Figure 5).

In the co-culture of γδ T cells with 3D S-ASCs, an evident reduction of the proinflammatory cytokines and an increase of anti-inflammatory cytokines expressed in γδ were observed. IFN-γ reduction was observed in the percentage of 47% and 30% respectively in the 1: 1 and 1:10 ratios. A reduction of 90% and 100% regarding IL-17 was observed respectively in 1:5 and 1:10 ratios. Inverting the stoichiometry ratios between γδ T cells and S-ASCs, using 5:1 & 10:1 ratios, we observed a stronger down-regulation of IFN-γ and IL-17. A drastic reduction of the proinflammatory cytokine IFN-γ of 98% and 60% was observed respectively in the 5:1 and 10: 1 ratios. IL-17 also showed a reduction of 80% and 90% respectively in both 5:1 and 10:1 ratios (Figure 6). Regarding the effect of S-ASCs in the expression anti-inflammatory cytokines by γδ T cells, it was shown an upregulation of IL-10 of 330% and 25% respectively in the ratio 1:1 and 10:1. IL-9 showed an increase of 40% and

200% in the ratios 1:5 and 1:10 respectively. Comparing the effect of ASCs and S-ASCs on the expression of pro-inflammatory and anti-inflammatory cytokines produced by γδ T cells, we observed a statistical significant stronger immunosuppressive effect of S-ASCs in the downregulation of the pro-inflammatory cytokines and in upregulation of the anti-inflammatory cytokines ($P<0.05$ for both) (Figure 7).

The analysis of cytokine expression following the co-culture of γδ T cells in the presence of only the conditioned medium produced by S-ASCs did not show any significant change of cytokines concentration. These data highlighted how a direct co-culture of S-ASCs and γδ T immune cells is essential to obtain an evident anti-inflammatory effect. On the other hand, the use of only the conditioned medium produced by S-ASCs causes a pro-inflammatory effect not detected in the cell-cell contact (Figure 8).

3.2.2 ASCs and S-ASCs both inhibit the proliferative capacity of γδ T cells

Using the Mixed Lymphocyte Reaction (MLR) test mediated by CFSE (a marker capable of entering cells), the proliferative capacity of γδ T cells was evaluated in the presence of the conditioned medium produced by ASCs and S-ASCs or in cell-to-cell co-culture of ASCs and S-ASCs.

An evident suppression of γδ T cell proliferation has been evaluated when co-cultured with ASCs from 75% to 15% due to cell-cell contact in a 1:10 ratio. In contrast, in presence of only the conditioned medium produced by the ASCs an inhibitory effect of the γδ T cells was not detected (Figure 9). While, in the co-culture of the γδ T cells with S-ASCs, there was a reduction of the γδ proliferation from 75% to 65-55% in the ratios 1:5 and 1:10. However, a strong suppression of the proliferation of γδ T cells from 75% to 3.5% was noted in presence of only the S-ASCs conditioned medium (Figure 10).

CHAPTER 4

Discussion

In recent years research has focused on the study of stem cells due to their strong proliferative and differentiating capacity that can be exploited to many applications of regenerative medicine and immunology. Nowadays, adipose tissue is considered the most suitable source of stem cells, due to its abundance and easy access and an excellent yield of ASCs.[2,67] Adipose tissue is frequently used in both cosmetic and reconstructive surgery, and many researches have been conducted in the last years with a progressive increasing of their application. Hence, ASCs are commonly used in the treatment of burn scars, skin dystrophies, ischemic revascularization, tissue regeneration and immunomodulation.

VCA is an emerging therapeutic option for patients who have exhausted alternative reconstructive solutions. Since their first description, over 100 human hand and 37 face VCAs have been reported in the past 18 years,[68-71] proving not only their technical feasibility, but also the superior outcomes compared to conventional reconstruction in selected patients. It represents the very top of modern reconstructive ladders with more and more cases reported every year worldwide. Nonetheless, VCA is not routinely performed due to the condition of lifelong

immunosuppression and toxicity profiles.[69] The major complications resulting from immunosuppressive therapies include increased propensity to develop infections, organ toxicity (i.e., nephrotoxicity) and malignancy.[72] Furthermore, unlike solid organ transplantation (SOT), VCAs are life-*enhancing* rather than life-*saving*, leading to many ethical and medical issues involving individual surgeons as well as professional societies.[68]

Legal and ethical issues have been raised with regard to performing VCA. Considering that VCAs are life-enhancing and not life-saving, their use has been criticized due to the possible serious complications of lifelong immunosuppression. In the past decade, new research has been proposed in the field of reconstructive transplantation with cell-based protocols able to induce tolerance in VCAs. Hence, the possibility to decrease, or even totally eliminate, the administration of immunosuppressive drugs could definitively favor the development and extend the indications of VCA. Moreover, there are no general guidelines on the use of adult stem cells as cell-based therapy in VCA recipients. Due to several controversies, VCAs are not yet a routine procedure.

With the aim to reduce ethical concerns, clinical research is currently focusing on designing protocols that could allow indefinite graft survival that do not require life-long immunosuppression, a condition referred to as tolerance. To date, most VCA programs still use conventional immunosuppressive regimens consisting of induction (anti-thymocyte globulin) and long-term immunosuppression with triple therapy (tacrolimus, mycophenolate mofetil, and corticosteroids).[73] However, there is still a high rate of acute rejection episodes despite immunosuppression as well as adverse toxic effects.[74] The "Pittsburgh Protocol" is the first cell therapy available in clinical VCAs and has been applied in five hand transplant cases. Following an inductive drug treatment, donor bone marrow is infused intravenously to improve the efficacy of tacrolimus monotherapy.[17] Despite tolerance has not been clinically seen to date, the Pittsburgh Protocol, now called the "Hopkins-Pittsburgh Protocol", has allowed tremendous steps forward

by shifting the paradigm of immunosuppression from a triple immunosuppressant regimen to a single agent (tacrolimus). Furthermore, this protocol allowed a radical reduction of immunosuppression agent doses in order to maintain constant minimal levels and reduce tacrolimus nephrotoxicity.

Limited clinical evidence for a possible role of stem cells in immunomodulation after VCAs comes from a case report by Del Bene et al. who proposed autologous BM-MSCs as a new immunosuppressant strategy in a double hand allotransplantation that enabled minimal maintenance levels of immunosuppressants without acute rejection 20 months after transplantation.[75]

There are various approaches to improve the efficacy of ASC-based therapeutics.

Traditionally, 2D cell culture has been utilized as a standard culture condition for *in vitro* expansion of ASCs.[56] However, previous studies, including some of our own, have shown that stem cells (both naïve and cancerous) from different organs could be obtained directly from lipoaspirate samples, and grow in suspension when the SVF portion is cultured in a serum-free culture condition. These cells display a phenotype characterized by round cells and do not adhere to plastic.[57,58]

Comparing the traditional 2D adherent ASCs and 3D non-adherent spheroids, the latter are closer to the physiologic state. 3D cell aggregates are spared of the artificial state of cell adhesion to plastic which alters phenotype as well as surface molecules expression. It is our opinion that the new culturing techniques that allow isolation of S-ASCs yield the true stem cells fraction from fat, and that this cell population may provide new perspectives on the biological potentials of stem cells from fat.

Although spheroids of adipose stem cells began being used in a number of laboratories worldwide, their dignity as a separate stem cell niche with different phenotype, biological

behavior and differentiation ability have never been fully delineated.

In this study, we provided more insights regarding this new population of S-ASCs. We have analyzed the yield of S-ASCs by correlating them with different harvesting sampling donor-sites and manipulation techniques. Indeed, we investigated for the first time the immunomodulatory effects of S-ASCs *in vitro*.

Previously, several authors have suggested that a number of factors, including the fat-harvesting site, fat collection technique, patient's age and BMI, could affect yield and the quality of ASCs. However, results from different studies are inconsistent and did not take into consideration the yield of 3D S-ASCs. With regards to the individual differences between the patients, we have taken into account both age and BMI. Similarly, to previous studies, we have found that in younger patients (under 55 years old), the number of S-ASCs obtained is almost double compared to the older population. Regarding the BMI, patients in the overweight category showed the highest average yield of S-ASCs with a statistically significant difference compared to the other two weight categories. In our study, flanks, abdomen, thigh, and breast were investigated as fat-harvesting donor-site and correlated with S-ASCs yield. Jurgens et al.[15] found that the number of ASCs extracted is greater in the abdomen than hips. Likewise, Iyyanki and Hubenak showed a greater yield of total SVFs in the abdomen than axilla or hips;[76] however, no difference between the fat depots was found regarding the number of ASCs. In contrast, Tsekouras and Mantas revealed that the external thigh is an optimal donor-site with higher yield of ASCs than abdomen, waist and knee.[77] Finally, Oedayrajsingh-Varma et al.[78] and Rohrich et al.[79] did not found significant differences between different fat-harvesting donor-sites.

In our study population, the thigh presented a clear advantage in S-ASCs yield. Not only we found presence of S-ASCs in 86% samples analyzed, but also the average number of cells obtained was the second highest (1.953.190) in terms of total average of S-ASCs yield followed

by flanks (2.393.846). Indeed, in this donor-site a proportional correlation has been found between the number of S-ASCs extracted and the amount of adipose tissue (ml) harvested (87.724 S-ASCs/ml). In contrast to other studies that have shown the abdomen as the best donor-site for the ASCs yield, in our study, the yield of S-ASCs from the abdomen revealed that this donor-site is one of the less favorable both on average and per volume of tissue.

With respect to the fat-harvesting technique, previous studies suggested a greater yield of ASCs using the sedimentation compared to the centrifugation technique, due to the lower cells manipulation and less damage. In our study, however, there was not a significant difference between the two techniques. Indeed, analyzing the fat-harvesting method with the number of S-ASCs per volume (ml) in individual donor-sites, it seems that in the thigh and abdomen the centrifugation technique provides higher results.

The S-ASCs are known in literature for their stem properties and for their marked regenerative capacity demonstrated on an *in vivo* model of bone lesion, as a result of their intrinsic therapeutic properties. These cells therefore provide a hopeful strategy in the field of regenerative medicine for the treatment of a wide range of diseases and injuries, therefore, it is essential to determine the best sampling site in order to standardize the technique. The data reported by us could support the choice of a specific site from which to obtain a better cell yield in a regenerative medicine context, although it will be necessary to expand the patient cohort to verify our results.

The role of mesenchyme-like stem cells in VCA remains unclear and is currently being investigated. Previous *in vitro* and *in vivo* researches suggest that ASCs could play a valuable role in tolerance induction and may one day eliminate the need for lifelong immunosuppression, which is currently the main hindrance to the widespread application of VCA.

In this study, we compared the immunomodulatory effect of adherent ASCs and non-adherent S-ASCs *in vitro* with regards to inhibit the proliferation of γδ T cells isolated from

healthy donors. ASCs significantly inhibited the secretion of IFN-γ and the proliferation of γδ T cells stimulated with PHA, a polyclonal mitogen. On the other hand, 3D S-ASCs showed a statistically significant stronger immunosuppressive effect in the downregulation of the pro-inflammatory cytokines and in upregulation of the anti-inflammatory cytokines. Importantly, S-ASCs dose-dependently decreased the proliferative response of γδ T cells isolated from healthy patients. However, we did not observe any statistical difference regarding the ability to suppress the proliferation of γδ T cells using the MLR assay. Interestingly, the suppressive activity of S-ASCs seems not dependent to the cell-to-cell contact. These findings support that S-ASCs share immunosuppressive properties with the classical adherent ASCs. Therefore, S-ASCs cell-based reconstructive therapy due to their immunosuppressive properties, could be an alternative source to ASCs to treat VCA patients.

In this regard, researches in small and large animal models prior to clinical trials, applying cell-based approaches for establishing tolerance in VCA, are mandatory before their clinical application as a cell-based therapy. Our group is planning to evaluate *in vivo* if S-ASCs administration can induce and eventually maintain clinical tolerance toward the allograft in a heterotopic rat hind-limb VCA model. This VCA model, used to assess tolerance to the various components of limb transplants, has demonstrated advantages causing minimal recipient morbidity/mortality; and indeed, its long bone components act as a source of vascularized bone marrow.

With a new cell therapy, such as ASCs injection, its translation to clinical use requires, indeed, detailed understanding of the fate of the transplanted cells *in vitro*. In the planned experiments, *in vivo* tracking of S-ASCs with bioluminescence will be performed to characterize their fate, destinations, and longevity in this surgical VCA model. The *in vivo* tracking of these cells with long-term follow up could help defining their migration, homing and behaviour, give

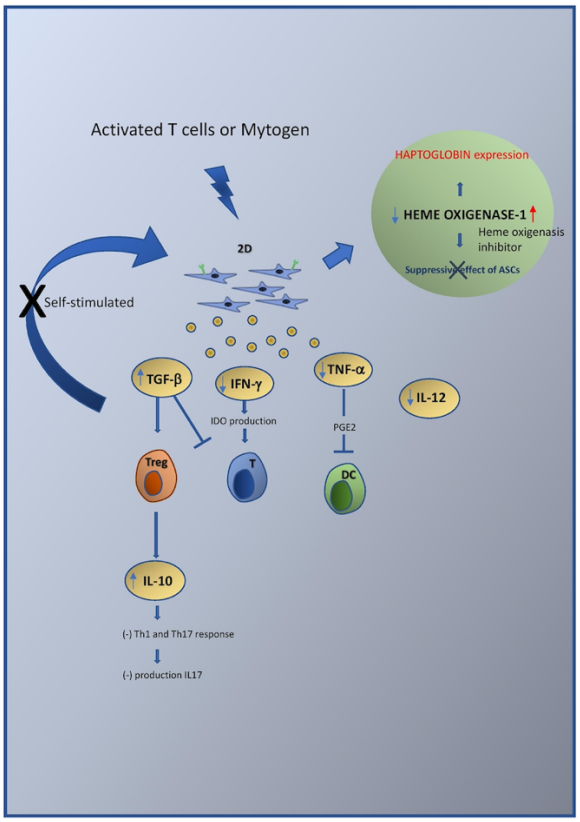
information on regulatory mechanisms and, finally correlate ASCs presence in the tissues with clinical outcomes.

Validation of stem cell-mediated immunomodulatory treatment could contribute to tolerance induction, or prolongation of graft survival, and reduce immunosuppressant dosage, still having a potential impact on VCA. Preclinical animal studies and clinical trials are therefore desirable to address the hypothesis and open issues presented in this study.

In conclusion, the present study showed that adipose tissue harvested from the thigh through Coleman's technique with centrifugation was found to yield higher number of S-ASCs. Indeed, S-ASCs also exhibit similar *in vitro* immunosuppressive properties to the adherent ASCs. This reinforces the therapeutic interest in S-ASCs although further studies are required to fully characterize the immunosuppressive properties of S-ASCs and to evaluate their *in vivo* properties in animal models.

CHAPTER 5

Tables and Figures



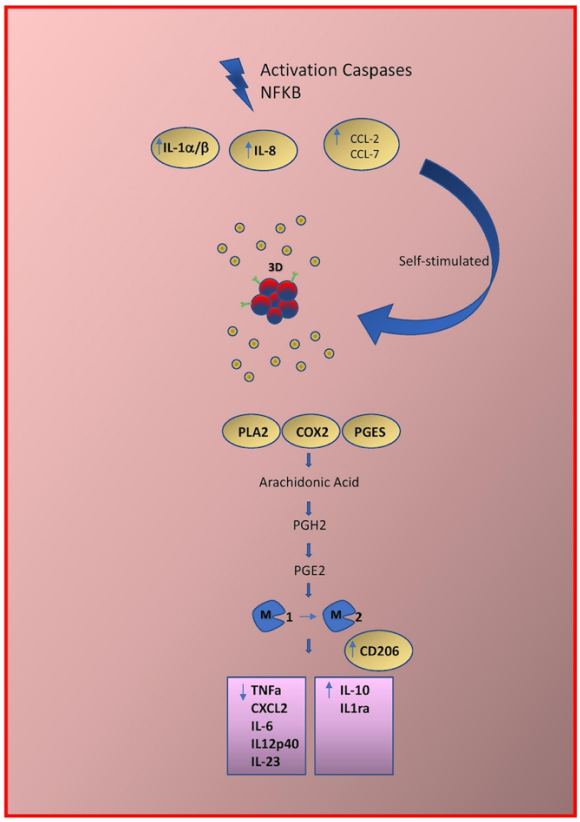


Figure 1. A shows the interplay between ASCs and T cells mediated by cytokines. Classic adherent ASCs require to be primed by proinflammatory cytokines (TNF-α, IFN-γ) produced by activated T cells to acquire immunosuppressive effects. In turn, ASCs suppress T-cell proliferation down-regulating TNF-α and IFN-γ. Tumor necrosis factor α significantly enhances the production of PGE_2, and IFN-γ induces the induction of IDO. Concurrently, high levels of TGF-β and IL-10 significantly increase the secretion of Tregs, suppressing the autoreactivity of T cells. Proteome study showed a high expression of haptoglobin. Heme oxygenase 1 is involved in the suppressive effect of ASCs and abrogated following pretreatment with heme oxygenase 1 inhibitor. B shows the immunomodulatory properties of spheroids of MSCs (S-MSCs). Conversely, to the adherent ASCs, S-MSCs need to be self-stimulated by autocrine proinflammatory signaling (increased IL-1α/β, IL-8, CCL2, and CCL7) to produce immune-suppressive effects. Phospholipase A2 (PLA2), COX-2, and PGE synthase (PGES) are strongly up-regulated by spheroids of MSCs. Further passages consist in the production of arachidonic acid from PLA2 that is subsequently processed by COX-2 to PGH_2 that produces PGE_2. Up-regulation of PGE_2 skews macrophages differentiation from M1 to M2 with increased M2 markers (CD206). M2 macrophages produce anti-inflammatory cytokines, such as IL-10 and IL-1ra, and reduce proinflammatory cytokines including TNF-α, CXCL2, IL6, IL12p40, and IL23.

Table 1: Pre-clinical VCA studies in swine and rodent using MSCs or ASCs

Authors	Animal Type	Donor	Recipient	VCA model	MSC source	MSC cells dose (n injections)	TBI	BMT	Combined immunosuppression	VCA Survival	Chimerism	Tregs	TGF-β1/IL-10	Tollerance rate (%)
Kuo et al. 2009 [33]	Swine	Lan-Yu	Hwa-Ban	Hind-limb	Allogeneic BM-MSCs	$1x10^7$ (5)	+	+	CsA	↑	-	↑	N/A	0
Pan et al 2010 [36]	Rat	BN	LEW	Hind-limb	Allogeneic BM-MSCs	$10x10^6$ (1)	+	+	RPM+ALG	↑	+	↑	N/A	0
Kuo et al 2011 [34]	Swine	Lan-Yu	Hwa-Ban	Hind-limb	Allogeneic BM-MSCs	$1x10^7$ (5)	+	-	CsA	↑	-	↑	N/A	67
Kuo et al 2011 [20]	Rat	BN	LEW	Hind-limb	Allogeneic ASCs	$2x10^6$ (3)	-	-	ALS+CsA	↑	+	↑[a]	↑	33
Kuo et al 2012 [35]	Swine	Lan-Yu	Hwa-Ban	Haemifacial	Allogeneic BM-MSCs	$2.5x10^7$ (6)	-	-	CsA	↑	-	↑	↑	0
Jeong et al 2014 [37]	Rat	SD	Wistar	Hind-limb	Allogeneic ASCs	$1x10^5$ (4)	-	-	No immunosuppression	↑	-	↑	N/A	0
Cheng et al. 2014 [26]	Rat	BN	LEW	Hind-limb	Syngeneic ASCs	$2x10^6$ (1)	-	-	AlG+CsA	↑	N/A	↑	N/A	66
Plock et al. 2015 [3]	Rat	BN	LEW	Hind-limb	Allogeneic BM-MSCs/ ASCs	$1x10^6$ / $5x10^6$ (3)	-	-	ALS, FK-506	↑	+[d]	↑	N/A	47
Plock et al. 2017 [38]	Rat	BN	LEW	Hind-limb	Allogeneic ASCs	$1x10^6$ (1 or 3)	-	-	ALS	↑[b]	+[d]	↑[c]	N/A	12.5

Table1. Preclinical VCA Studies in Swine and Rodent Using MSCs or ASCs

Samples number	Age Range	S-ASCs Average
30	18-45	1.682.000
31	46-55	1.118.000
15	56- 82	695.000

Table 2. Correlation between S-ASCs and patients' age

Samples number	BMI (kg/m2)	S-ASCs Average
27	Normal (18.50-24.99)	344.000
35	Overweight (25 – 29,9)	1.493.000
14	Obesity (>30)	900.000

Table 3. Correlation between S-ASCs and patients' BMI

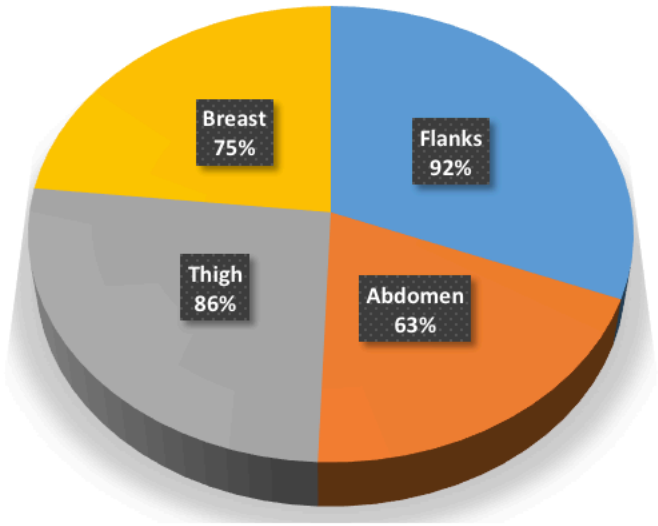
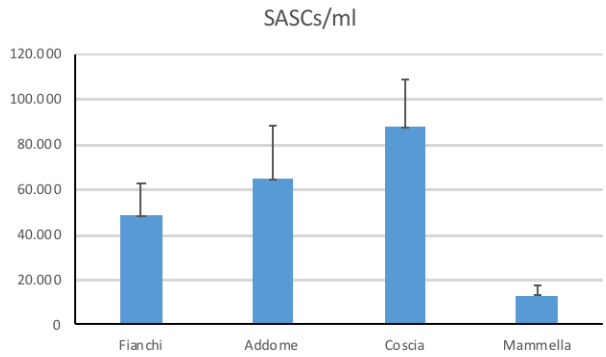
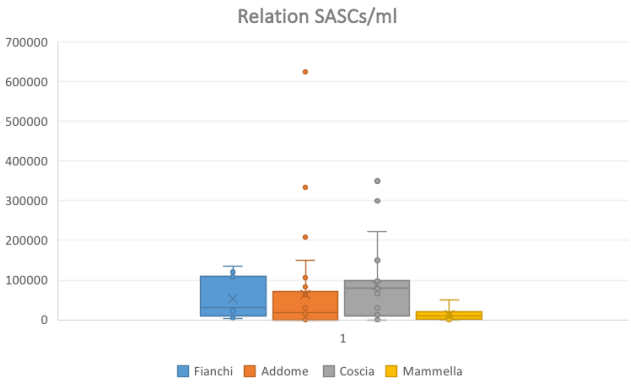


Figure 2. S-ASCs extracted for each sample in each different donor-site





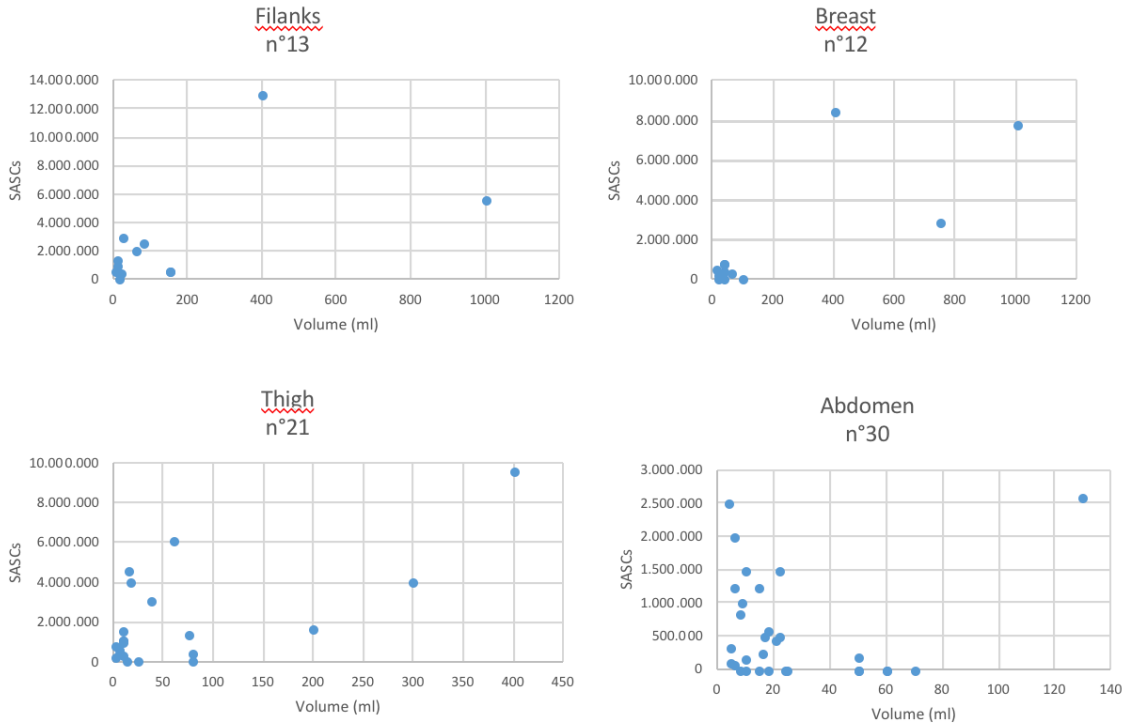


Figure 3. Relation between S-ASCs to the volume of adipose tissue harvested(S-ASCs/ml)

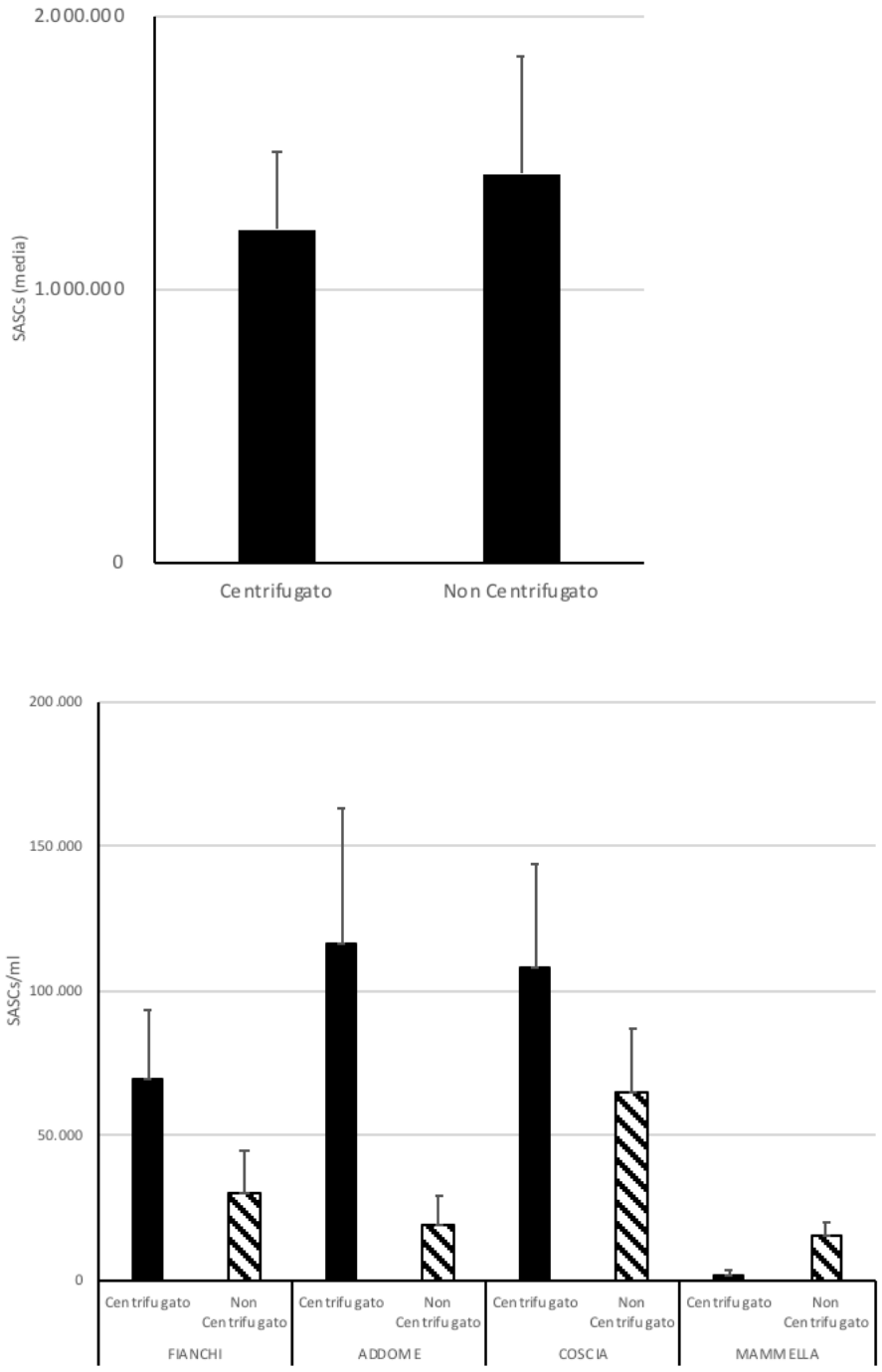


Figure 4. Correlation between S-ASCs yield and Harvesting Method (Centrifugation vs. Sedimentation)

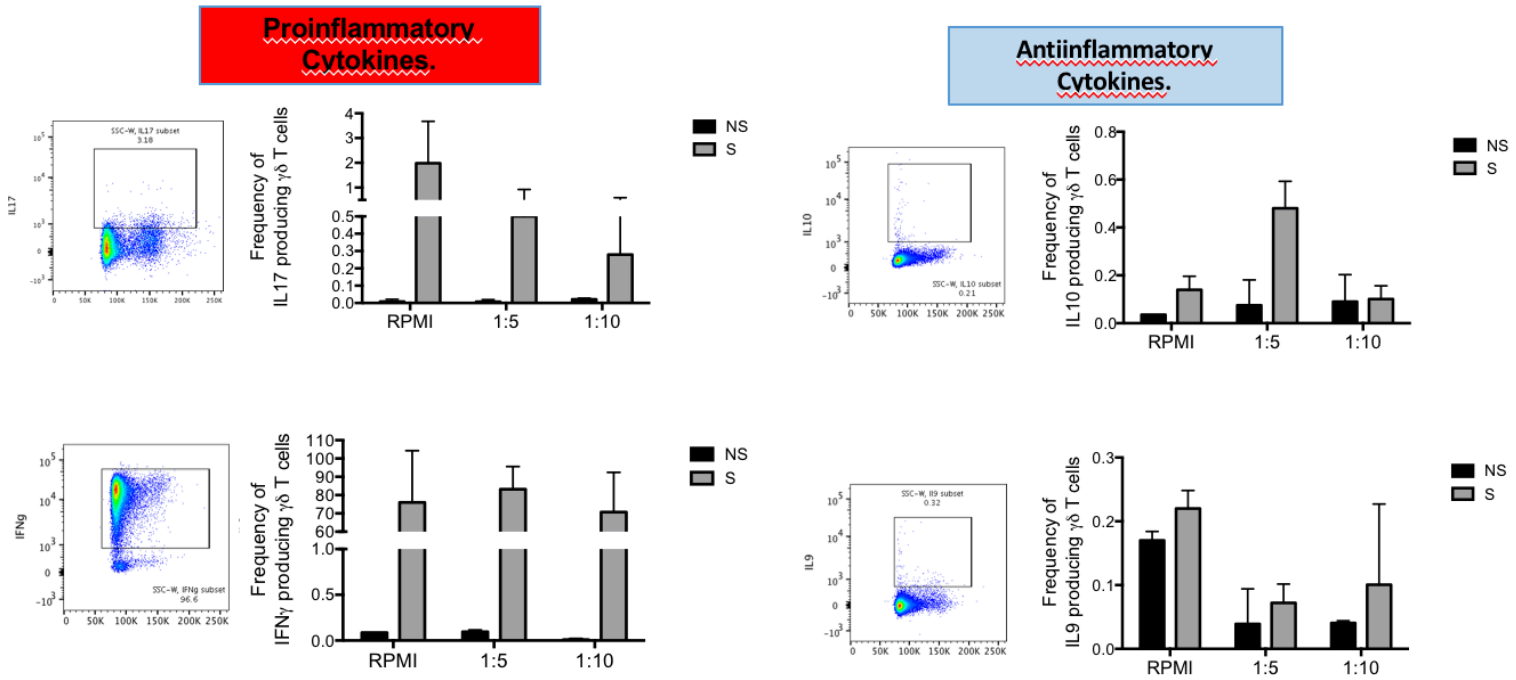


Figure 5. Analysis of the Cytokines produced by γδ T cells when co-coltured with ASCs

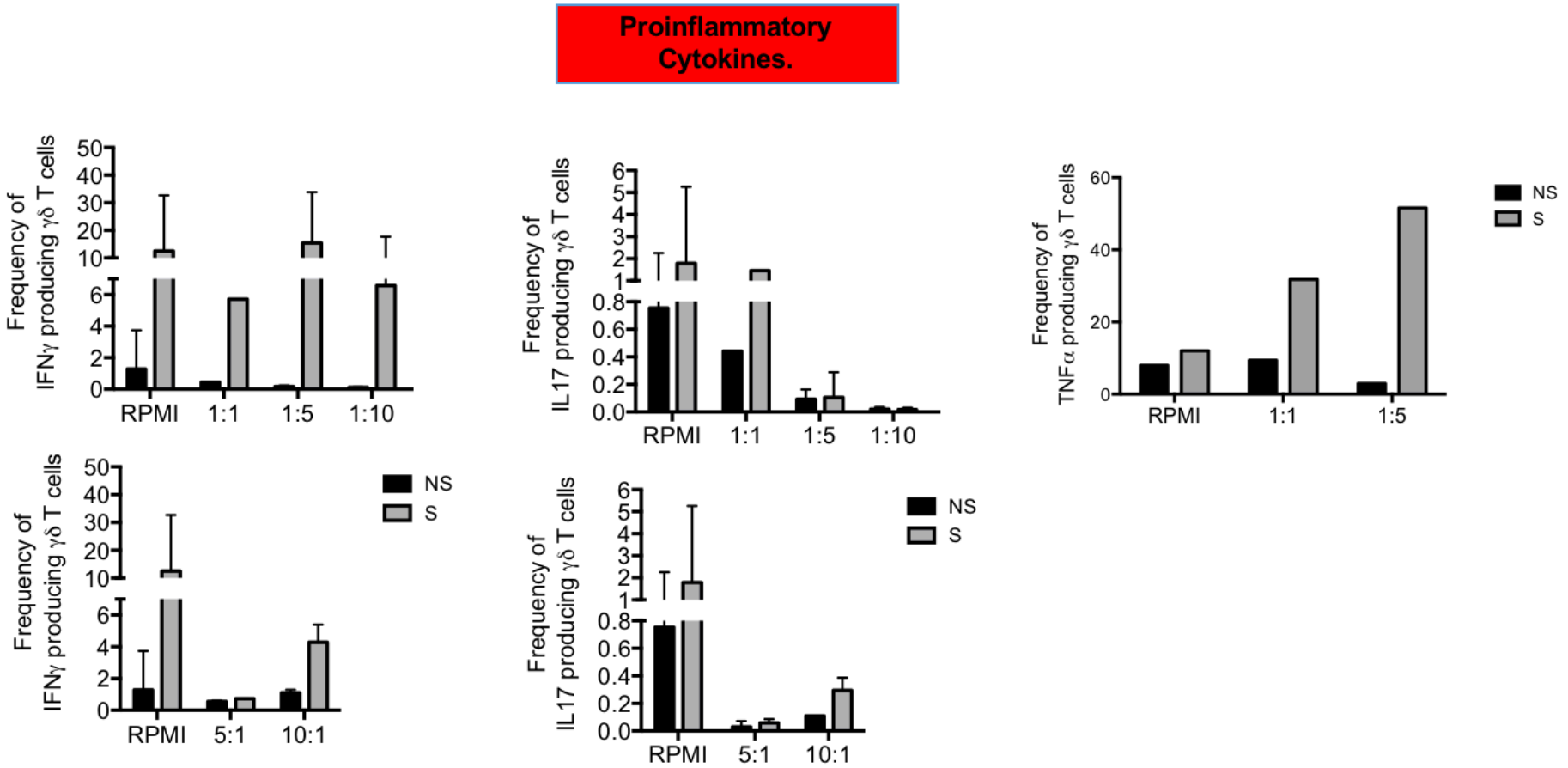


Figure 6. Analysis of the Proinflammatory Cytokines produced by γδ T cells when co-coltured with S-ASCs

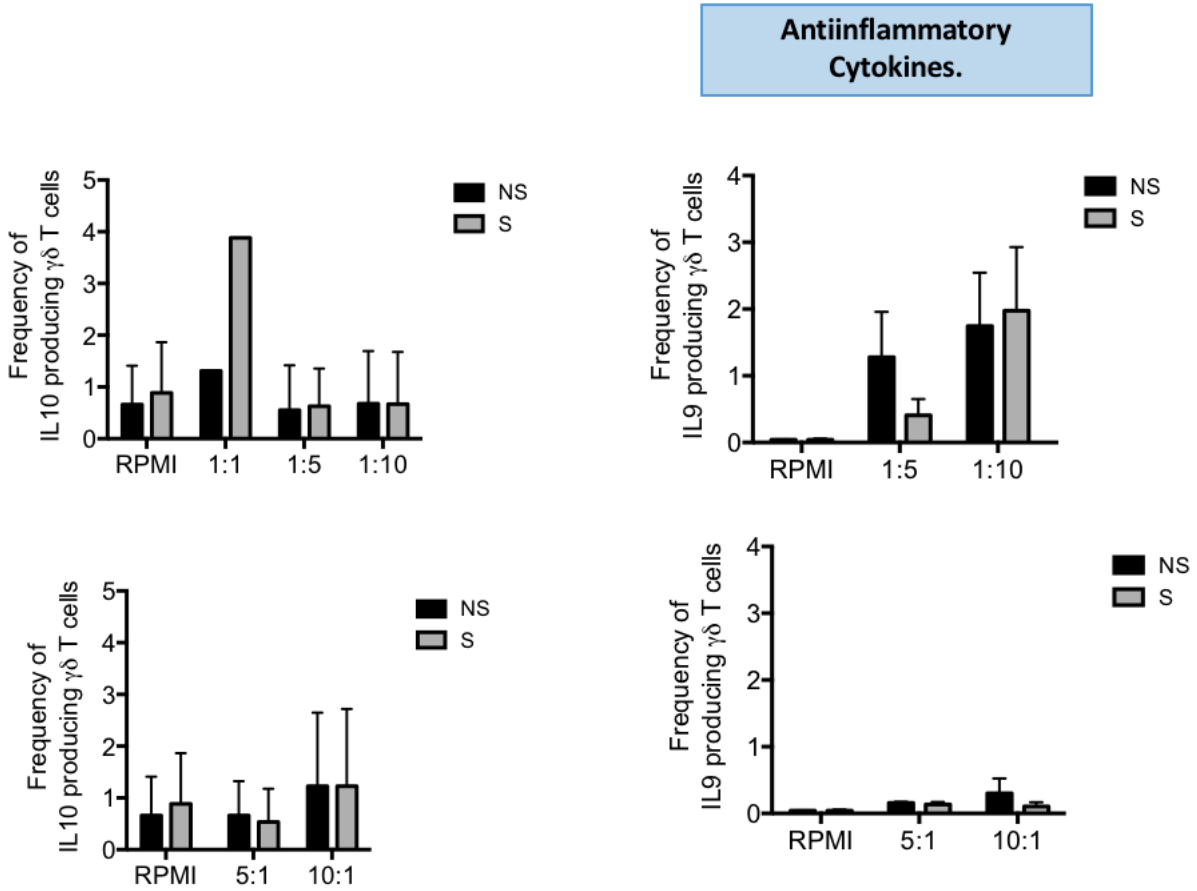


Figure 7. Analysis of the Anti-inflammatory Cytokines produced by γδ T cells when co-coltured with S-ASCs

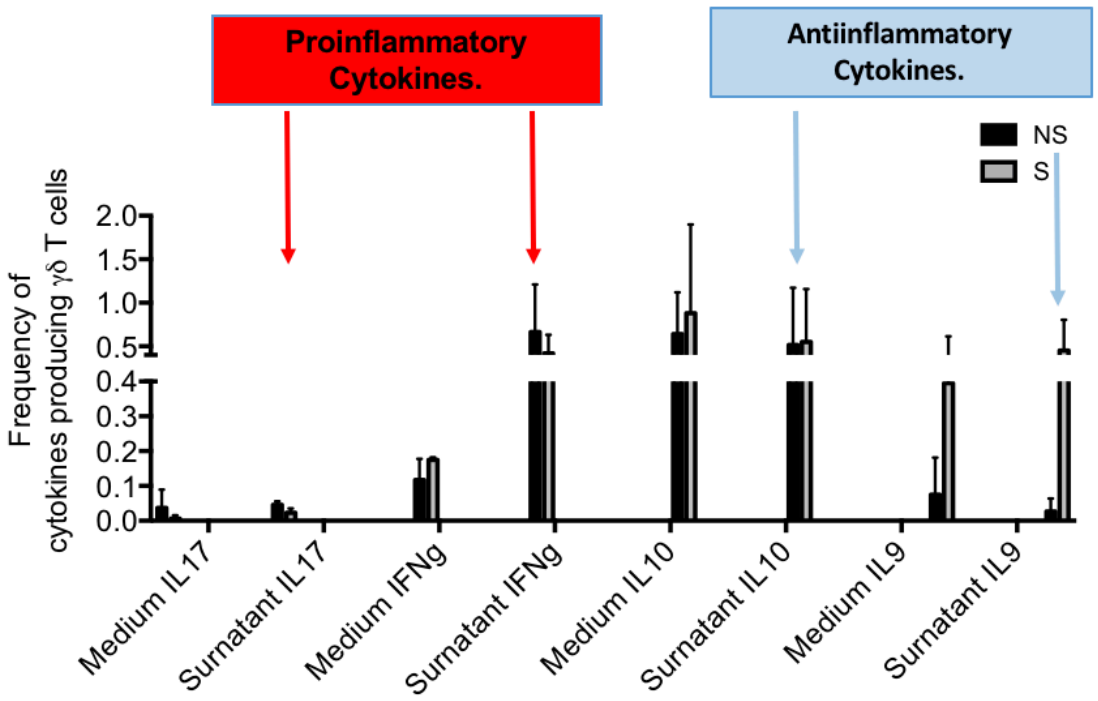


Figure 8. Analysis of the Cytokines produced by γδ T cells when co-coltured with the conditioned medium produced by S-ASCs

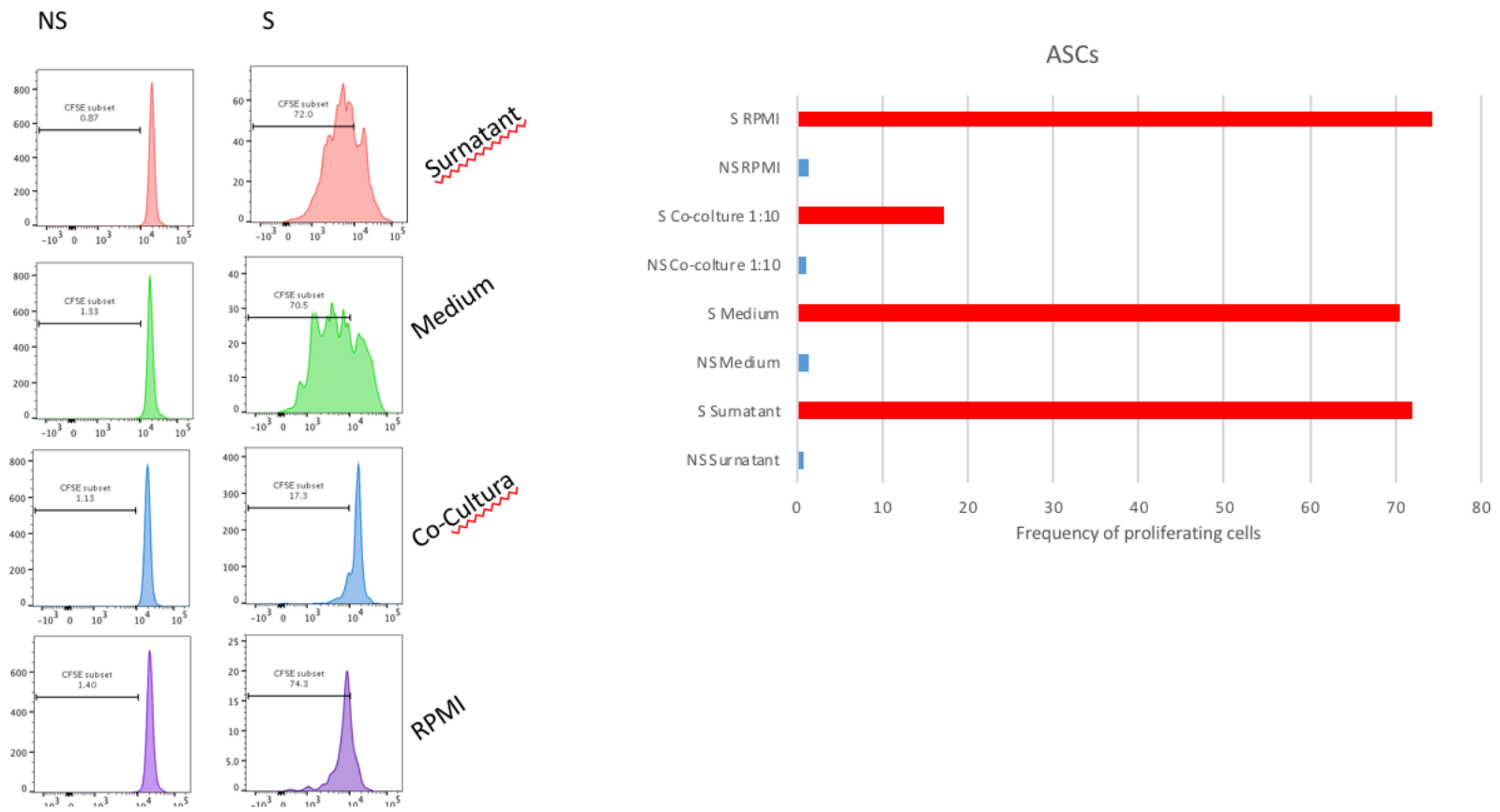


Figure 9. Analysis of the proliferative capacity of γδ T cells with ASCs

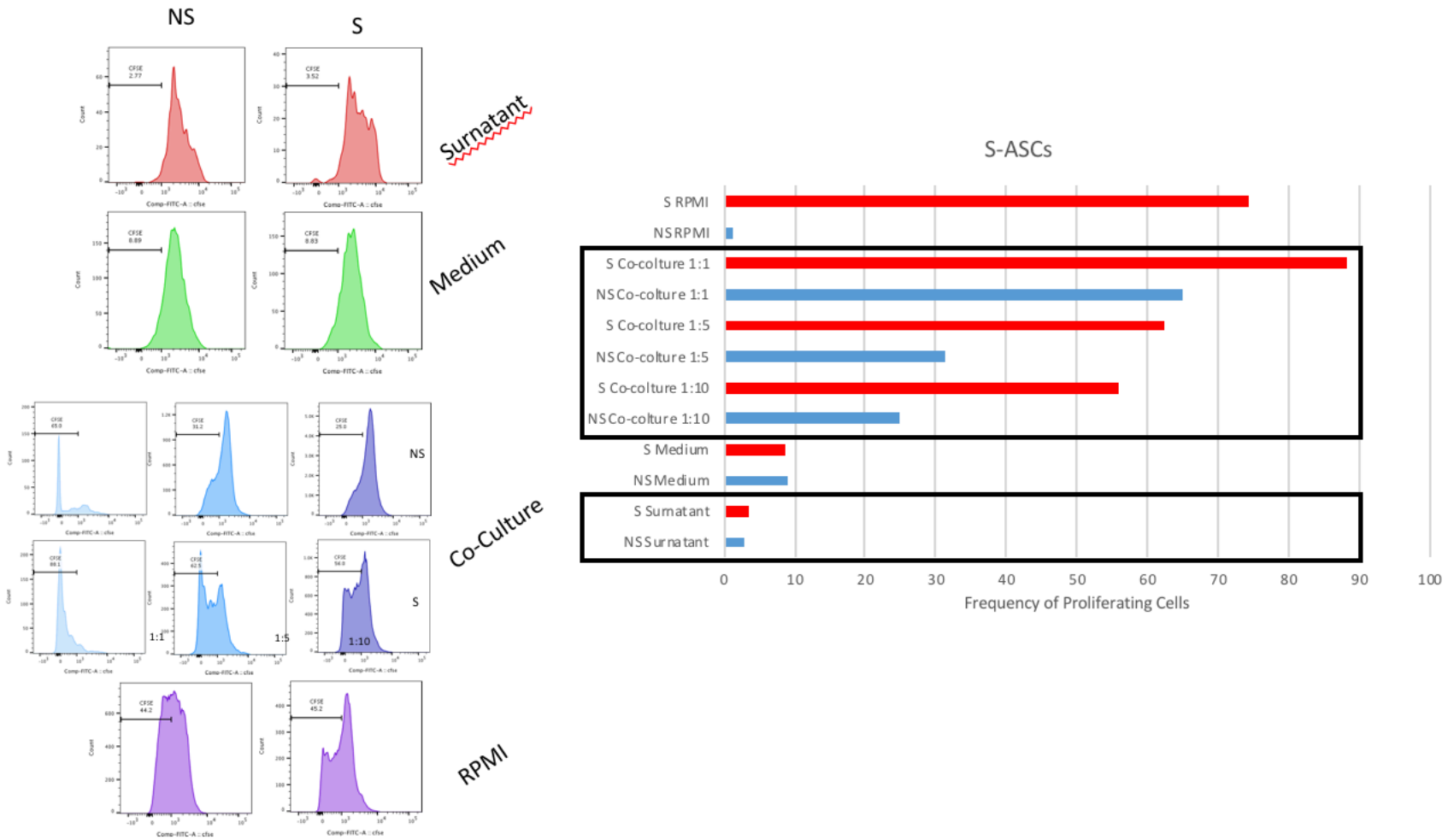


Figure 10. Analysis of the proliferative capacity of γδ T cells with S-ASCs

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Scientific Production – NON ISI

- Diploma della Clinical and Research Microsurgery Fellowship at the Department of Plastic and Reconstructive Microsurgery, Center of Vascularized Composite Allotrasplantation of Chang Gung Memorial Hospital Linkou, Taiwan.
- One book Chapter in press, Lymphoscintigraphy Interpretation, Staging and Lymphedema Grading, "Cheng: Principles and Practice of Lymphedema Surgery, 2e" Elsevier.
- One book Chapter in press, Lymphedema: Surgical Management after Pelvic/Perineal Resections, “Perineal Reconstruction - Principles and Practice”. Springer