IMMUNOLOGICAL PROFILE OF ADULT HUMAN MESENCHYMAL STEM CELLS DERIVED FROM VARIOUS SOURCES – BONE MARROW, ADIPOSE TISSUE, AND UMBILICAL CORD MATRIX / BLOOD

A THESIS

Submitted by

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in fulfilment for the award of the degree

of

DOCTOR OF PHILOSOPHY

Under the Supervision of

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LIST OF SYMBOLS AND ABBREVIATIONS

Symbols

α	-	Alpha
β	-	Beta
ϒ	-	Gamma
°	-	Degree

Abbreviations

ECM	-	Extra Cellular Matrix
iPSCs	-	induced pluripotent stem cells
SSEA	-	stage specific embryonic antigen
SCF	-	Stem cell factor
hMSCs	-	human Mesenchymal Stromal Cells
ISCT	-	International Society for Cellular Therapy
MSCs	-	Mesenchymal Stromal Cells
ESCs	-	Embryonic stem cells
TGF-β	-	Transforming growth factor
IDO	-	Indolamine 2, 3 dioxygenase
HLA-G	-	Human Leukocyte Antigen G
ROS	-	Reactive oxygen species
EGF	-	Epithelial growth factor
VEGF	-	Vascular endothelial growth factor
FGF	-	Fibroblast growth factor
KGF	-	Keratinocyte growth factor
PDGF	-	Platelet-derived growth factor
HGF	-	Hepatocyte growth factor

IGF-1	-	Insulin growth factor
Ang-1	-	Angiopoietin-1
SDF-1	-	stromal derived factor
NK	-	Natural killer cells
SCI	-	Spinal cord injury
ILD	-	Interstitial lung disease
PBMCs	-	Peripheral blood mononuclear cells
MNCs	-	Mononuclear cells
MDS	-	Myeloid dysplastic syndromes
IBD	-	Inflammatory bowel disease
MS	-	Multiple Sclerosis
GVHD	-	Graft versus host diseases
AT	-	Adipose tissue
BM	-	Bone marrow
WJ	-	Wharton’s Jelly
UCB	-	Umbilical cord blood
ACD	-	Acid citrate dextrose
PBS	-	Phosphate buffered saline
PHA	-	Phytohemagglutitin
FBS	-	Fetal bovine serum
PTGS-2	-	Prostaglandin-endoperoxide synthase 2
IC-SCR	-	Institutional Committee for Stem Cell Research
IC	-	Informed consent
SVF	-	Stromal vascular fraction
TMB	-	3,3’,5,5’-Tetramethylbenzidine

PDT	-	population doubling time -
SMA	-	smooth muscle actin
FSC	-	Forward scatter
SSC	-	Side scatter
ELISA	-	Enzyme Linked Immunosorbent Assay
COX-2	-	Cyclooxygenase
GAPDH	-	Glyceraldehyde 3-phosphate
CFSE	-	Carboxyfluorescein Succinimidyl Ester
PCR	-	Polymerase chain reaction
IFN-ϒ	-	Interferon gamma
IL	-	Interleukin
TNF-α	-	Tumour Necrosis factor
iNOS	-	inducible Nitric oxide synthase
ANGPT	-	Angiopoietin
DMEM	-	Dulbecco's Modified Eagle Medium
EDTA	-	Ethylene diamine tetra acetic acid
DPBS	-	Dulbecco’s Phosphate buffered saline
FACS	-	Fluorescence-activated cell sorting
qPCR	-	quantitative Polymerase chain reaction
FOXP3	-	forkhead boxP3
Tregs	-	T regulatory cells
LPS	-	Lipopolysaccharides
TLR	-	Toll like receptors
SHH	-	Sonic Hedgehog
BrdU	-	5-bromo-2'-deoxyurid

ABSTRACT

Mesenchymal Stromal Cells (MSCs) are very advantageous in the field of regenerative medicine due to their immunomodulatory properties. However, reports show that these properties vary from source to source. Hence, understanding the source-dependent specificity of MSCs in their immunomodulatory abilities will enable optimal use of MSCs in cell-based therapies. This study investigated the human MSCs from three different sources: adipose tissue (AT), bone marrow (BM) and Wharton's jelly (WJ) with respect to phenotypic responses of human peripheral blood mononuclear immune cells (MNCs) and the concurrent changes in cytokine expressions in MSCs, under a mitogen stimulated co-culture condition. The study employed cytometric analysis for studying the immunoregulatory properties of MSCs and cytokine profiling using a customized PCR array and solid-phase sandwich Enzyme-Linked Immunosorbent Assay (ELISA). The results reveal differential modulation of both immune cells as well as MSCs upon activation by the mitogen PHA, independently and in co-culture. Notably, we observed source-specific MSC-cytokine-signatures under stimulated conditions. The study results show that AT-MSCs up-regulate VEGF, BM-MSCs up-regulate PTGS-2 and WJ-MSCs increase expression of IDO over several fold as compared to the controls. This remarkable increase in source specific cytokine expression was also validated at a functional level by quantitative protein expression studies. In our hands, even though MSCs from AT, BM and WJ sources exhibit characteristic immunomodulatory properties, study results highlight that MSCs sourced from different tissues may exhibit unique cytokine signatures and thus may be suitable for specific regenerative applications.

CHAPTER - 1
INTRODUCTION

1.1 Regenerative medicine

Regenerative medicine, the latest and evolving field of medicine that emphasizes on the functional restoration of damaged tissues or organs in patients with chronic diseases or injuries in which their own regenerative responses are insufficient (1). In the current scenario, tissue and organ transplantation do not meet the needs of aged and diseased populations due to several reasons such as shortage of organ donors, invasive procedure and economic reasons that driven the quest for alternatives. Cellular therapy using stem cells are considered as the right choice of alternative for various conditions due to its indefinite cell division and trans-differentiation potential (1). Stem cells provide the framework for the whole body's tissue and organ system and mediate various roles in disease progression, development, and tissue repair processes in the human body. The remarkable breakthrough in the area of Stem Cell Research has paved the way for cell based therapies for various diseased conditions that cannot be cured by conventional treatments.

1.2 Stem Cells

Stem cells are undifferentiated cells with unique characteristics. They are defined mainly by two characteristics

i) its ability to self- replicate through asymmetric cell division

ii) its capacity to differentiate into multiple cell types depending on the suitable signals received by them (2).

Several signalling pathways, such as Wnt, Hedgehog, and Notch signalling pathways are responsible for the ability of stem cells to self- renew by themselves.

These cells can be replicate through two types of cell division

i) Symmetric cell division ii) Asymmetric cell division (3).

1.2.1 Symmetric cell division

Symmetric divisions are defined as the development of daughter cells that are destined to attain the same fate. i.e. In symmetric self-renewal, both daughter cells are identical as the parent stem cells. It facilitates the repopulation of undifferentiated stem cell niches.

1.2.2 Asymmetric cell division

An asymmetric cell division gives rise to two daughter cells with different cellular fate. i.e. one of the two cells is original copy or identical to parent stem cells whereas the other one is programmed to differentiate into more specialized cell lineage. Asymmetric cell division facilitates stem cell pool retention and homeostasis maintenance. Stem cell descendants differentiated from the sub population is crucial for tissue repair and regeneration in the body. (3)

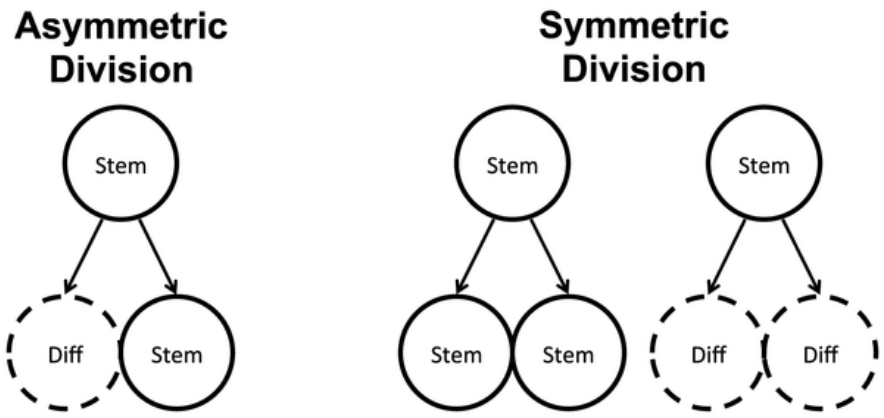


Figure 1. Types of cell division

Reference - Shahriyari L, Komarova NL. Symmetric vs. asymmetric stem cell divisions: an adaptation against cancer? PLoS One. 2013;

1.3 Stem cell niches

Stem Cells resides in a specialized microenvironment called niche which differs in their composition, nature and location depending on the source of the tissue type. In 1978, Schofield proposed the "niche" theory to define the physiological microenvironment that nourishes stem cells (4). In the past, the term “niche” has been used to describe the location

of the stem cells. Later, it was discovered that it also includes the cellular components of the microenvironment and the signals originating from the supporting cells. In 2006, Scadden gave complete definition for stem cell niches. According to him, niches are specific anatomic locations that regulate the participation of stem cells in tissue generation, maintenance and repair (5). It helps in protecting the host from over-exuberant stem-cell proliferation, also saves stem cells from depletion. It constitutes a fundamental unit of tissue physiology, integrating signals that facilitate the balanced response of stem cells to the needs of organisms. The interplay between stem cells and their niche creates the dynamic system necessary for sustaining tissues, and for the ultimate design of stem-cell therapeutics (5).

Therefore, the regenerative capacity of any stem cells can be retained only when they are in contact with the specialized microenvironment called stem cell niche to regulate the cell fate. It can be referred to either *in-vitro* or *in-vivo* stem cell microenvironment. Eg: During embryonic development various factors in the stem cell niche act on the embryonic stem cells are responsible for proliferation or differentiation which leads to the fetal development.

Stem-cell niches within the human body preserve adult stem cells in a quiet state but after any tissue injury, the underlying microenvironment signals to the stem cells to facilitate either self-renewal or differentiation into new tissues which helps in the regeneration. In order to regulate the stem cell attributes with in the microenvironment, several factors are important: Cell- cell interactions between Stem cells with

i) another stem cells

ii) neighbouring differentiated cells

iii) adhesion molecules

iv) Extra cellular matrix (ECM) components

v) Other factors such as oxygen tension, cytokines, chemokines, growth factors and physicochemical nature of the micro environment like pH, ionic strength (e.g. Ca2+ concentration) and metabolites, like ATP.

Functionally stem cell niches are classified as

1. Simple niche
2. Complex niche
3. Storage niche (6)

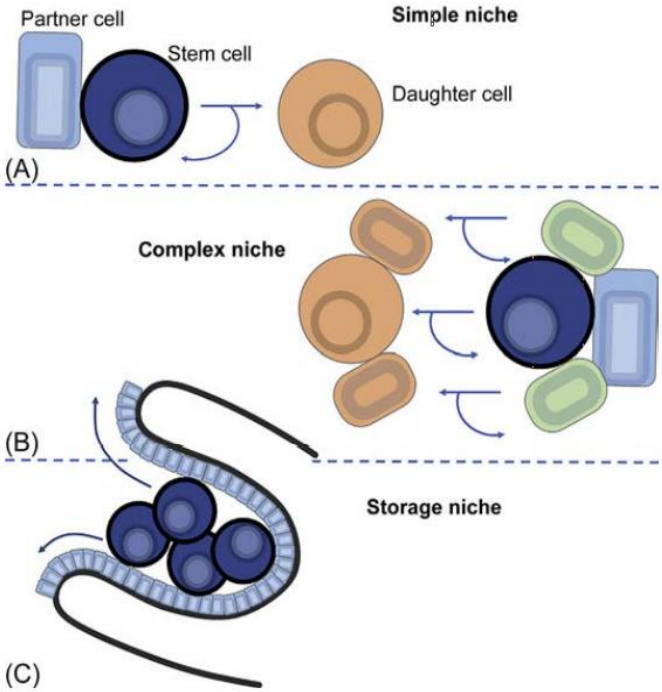


Figure 2. Classification of Stem cell niches

Reference - Ohlstein B, Kai T, Decotto E, Spradling A. The stem cell niche: Theme and variations. Curr Opin Cell Biol. 2004;16(6):693–9.

1.3.1 Simple niche

In Simple niche, stem cells bind to each other by unique adhesion molecules which lock stem cells in a position until they receive signals for proliferation. They are simple in structure and characteristic of epithelial, digestive tissues and gonads. (6, 7)

1.3.2 Complex niche

In Complex Niche, stem cells bind with other stem cells and differentiated partner cells, tend to form large cell complexes which provide better regulatory control mechanisms than in a simple niche, i.e. neural stem cells reside in a complex niche in the sub ventricular zone.

1.3.3 Storage niches

Storage niches are generally reservoirs of stem cells. Typically, these cells do not proliferate but they migrate from the niche after receiving the signals. E.g.: Hair follicles and niches storing melanoblast progenitors niches (7).

1.4 Classification and Sources of Stem Cells

Stem Cells are broadly classified into two types based on the potency and origin of the stem cells.

1.4.1 Stem Cells classification based on the potency

Based on the regeneration potential or plasticity, stem cells are classified as totipotent, pluripotent, multipotent, oligopotent, and unipotent.

1.4.1.1 Totipotent

Totipotent stem cells have the ability to divide and differentiate into all three germ layers: mesoderm, endoderm and ectoderm including both embryonic and extra embryonic structures. Totipotent cells have the maximum differentiation potential. E.g.: Zygote which is formed after the fertilization. Zygote have the ability to divide and differentiate into any germ layer cells or extra embryonic tissue like placenta. After 4 days, inner cell mass of blastocyst becomes pluripotent.

1.4.1.2 Pluripotent

These are stem cells which have the ability to differentiate into cells of all the germ layers except the extra embryonic tissue like placenta.

E.g.: 1. Embryonic stem cells derived from the inner cell mass of blastocyst and induced pluripotent stem cells (iPSCs). iPSCs are generated artificially from somatic cells by inducing some factors and it is functionally similar to pluripotent stem cells.

1.4.1.3 Multipotent

Multipotent stem cells have the ability to divide and differentiate into limited number of cells types i.e. into the cells originate from the same germ layer. E.g.: Adult haematopoietic stem cells which has the ability to differentiate into red and white blood cells or platelets. Another example is Adult Mesenchymal Stem Cells.

1.4.1.4 Oligopotent

Oligopotent stem cells have the ability to differentiate into only few types of stem cells. E.g.: Myeloid stem cells or lymphoid stem cells.

Myeloid stem cells can be differentiate into white blood cells but not red blood cells.

1.4.1.5 Unipotent

Unipotent have the ability to differentiate into only one type of cells which is their own.

E.g.: Adult muscle stem cells.

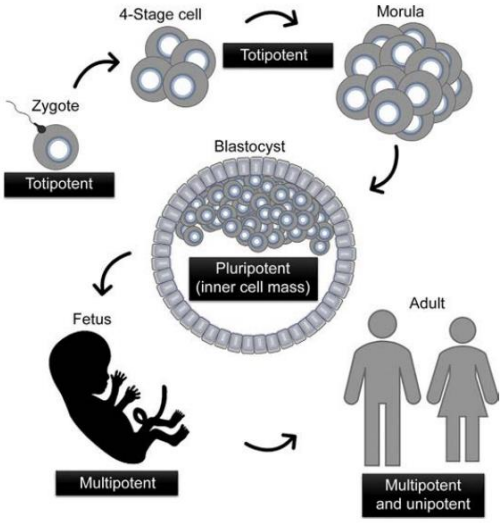


Figure 3. Classification of Stem cells based on the potency

Reference (7): Łos et al Chapter -2 "Stem cells"- -2018

1.4.2 Classification of Stem Cells based on the origin

Based on the origin, Stem cells are classified as

1. Embryonic stem cells
2. Adult stem cells and
3. Induced pluripotent stem cells (iPSCs).

1.4.2.1 Embryonic Stem Cells

Embryonic Stem Cells are pluripotent cells derived from the inner cell mass of the late blastocyst. Embryonic stem cells were isolated from mouse embryo in 1981 and from human in 1998. They have the ability to differentiate into cells of all the three germ layers but there are some limitations. They are:

- Ethical concern – Since the most of the isolation method requires the destruction of embryos.
- Culturing of ESCs *in-vitro* requires feeder layers in order to maintain it in undifferentiated state.
- Self-renewal ability and multi differentiation potential deteriorates in long term culture.
- Teratoma formation
- It is difficult to differentiate ESCs from cancer cells and other types of stem cells.

1.4.2.1.1 Markers of Embryonic Stem Cells

The most widely used cell surface markers for the identification of ESCs are Stage-specific embryonic antigens (SSEA-1, -3, -4), cluster of differentiation antigens (CD133, CD31, CD59, CD49f), TRA-1-60 and TRA-1-81, secreted stem cell factor (SCF), and terato-carcinoma-derived growth factor (7).

Among transcription factors, genes that are involved in regulating essential stem pathways, such as Oct-3/4, Sox2, KLF-4, and Nanog-4, are identified (7).

ESCs are crucial in the area of research such as human development, differentiation process of early embryonic stage, development of model of genetic disorders etc. Also ESCs could be used to regenerate cardiomyoctes, neuronal cells, pancreatic cells etc.

However, their use in clinical trial is hampered due to the teratoma formation and heterogeneous population of cells

1.4.2.2 Adult Stem Cells

Adult Stem Cells also known as somatic stem cells or tissue-specific stems are derived from the mature tissues like bone marrow, adipose tissue, liver, skin etc. They are multipotent cells and they play a vital role in tissue repair and regeneration.

E.g.: Hematopoietic Stem Cells and Mesenchymal Stem Cells.

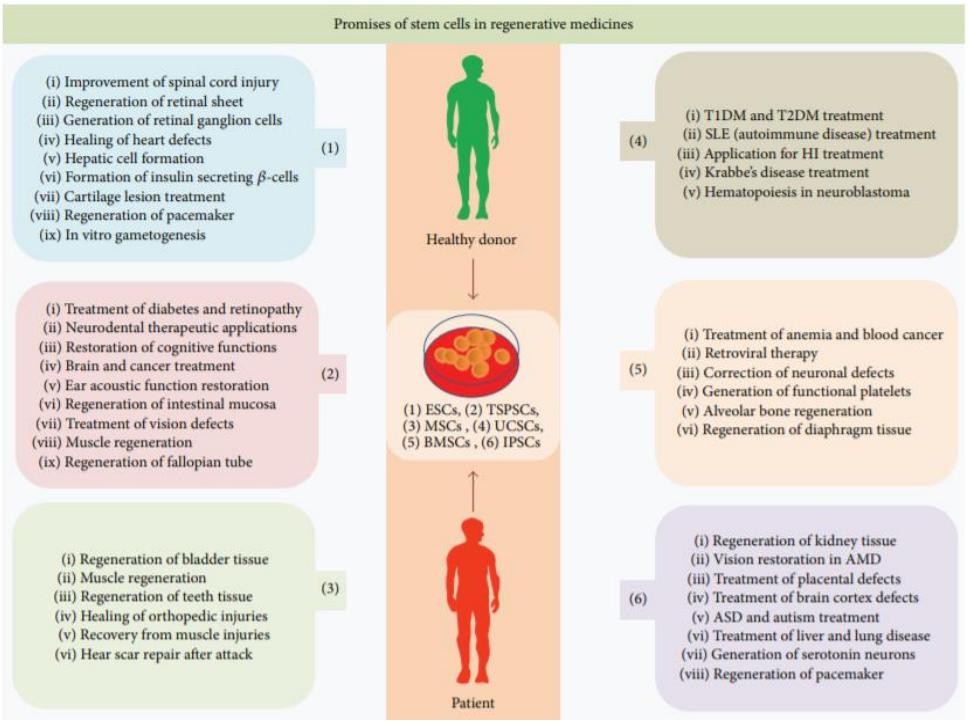


Figure 4. Application of stem cells in regenerative medicine

Promises of stem cells in regenerative medicine:

The six classes of stem cells, that is, embryonic stem cells (ESCs), tissue specific progenitor stem cells (TSPSCs), mesenchymal stem cells (MSCs), umbilical cord stem cells (UCSCs), bone marrow stem cells (BMSCs), and induced pluripotent stem cells (iPSCs), have many promises in regenerative medicine and disease therapeutics. Reference Ranjeeth Singh Mahla 2016 "Stem Cells Applications in Regenerative Medicine and Disease Therapeutics" Volume 2016, Article ID 6940283, 24 pages *"International Journal of Cell Biology"* https://doi.org/10.1155/2016/6940283

1.4.2.2.1 Hematopoietic Stem Cells

Hematopoietic stem cells are the multipotent cells having the ability to self-renew and differentiate into all the hematopoietic lineages. Hence, they are used for transplantation in haematological disorders such as leukaemia, lymphoma, severe aplastic anaemia, multiple myeloma and so on.

1.4.2.2.2 Mesenchymal Stem Cells

Human mesenchymal Stromal cells (hMSCs), are non-hematopoietic, multipotent, undifferentiated cells resides naturally in the human body. They are typically characterised as spindle shaped morphology of cells with an ability to adhere to the plastic surfaces under standard tissue culture conditions. The property of MSCs to proliferate extensively with ability to differentiate *in-vitro* into several lineages of cells such as osteoblasts, adipocytes, chondrocytes, myocytes, astrocytes etc. when received the suitable signals to differentiate defining their stem cell nature (8). Due to its enhanced regenerative potential, it has been considered as an emerging strategy for the treatment of various immune related disorders.

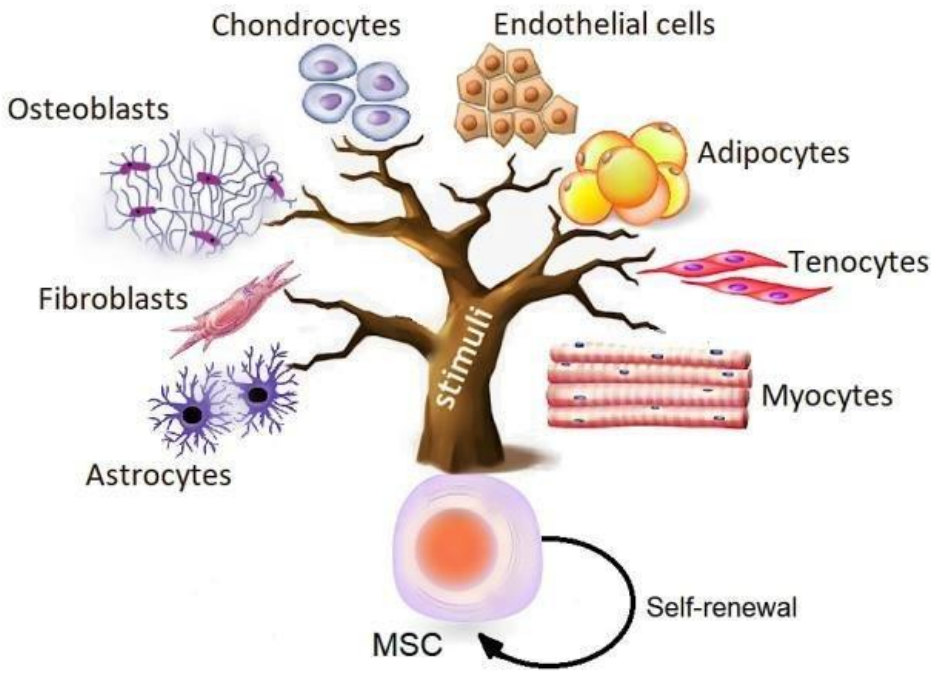


Figure 5. Self-renewal and differentiation of MSCs

1.4.2.3 Sources of Mesenchymal Stem Cells

Mesenchymal Stem cells were identified first in bone marrow by Friedenstein et al in 1991(9). Though MSCs were found initially in bone marrow, later on researchers from worldwide investigated and reported the existence of MSCs in various primitive as well as adult sources. Other sources of MSCs includes adipose tissue (10-12), amniotic fluid (13,14), amniotic membrane (15), dental tissues (16), endometrium (17), limb bud (18) menstrual blood (19), tonsil (20) peripheral blood (21) placenta and fetal membrane (22) salivary gland (23) skin and foreskin (24,25) sub-amniotic umbilical cord lining membrane (15) synovial fluid (26) and Wharton's jelly (10, 27-31).

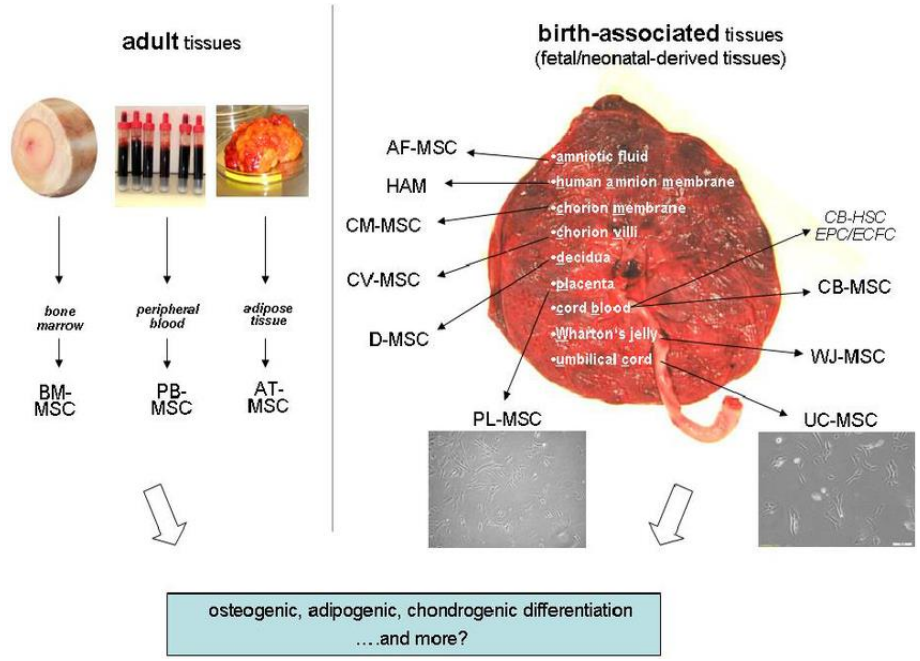


Figure 6. Fetal and adult sources of Mesenchymal stem cells

Reference -Hass et al "Different populations and sources of human mesenchymal stem cells (MSC)" : A comparison of adult and neonatal tissue-derived MSC. *Cell Communication and Signaling.* 2011.

MSCs are easy to culture and manipulate under the standard culture conditions. However, heterogeneous laboratory methods for isolation and cultivation of MSCs have prompted the Mesenchymal and Tissue Stem Cell Committee of International Society for Cell

Therapy (ISCT) to issue guidelines to define human MSCs. According to the ISCT, the MSCs must meet the following minimal requirements to define it as Mesenchymal Stem Cells.

1.4.2.4 Minimal criteria to define Mesenchymal Stem Cells as per ISCT

According to the minimal criteria proposed by International Society for Cellular Therapy (ISCT),

i) MSCs must be adherent to plastic surface when the cells are maintained in standard culture conditions.

ii) It must be positive and negative for certain cell surface markers (positive for CD73, CD90 and CD105; negative for CD45, CD34, CD14 or CD11b, CD79alpha or CD19, and HLADR).

iii) It must be able to differentiate into tri-lineage (Osteo, Adipo and Chondrogenic lineages) of the mesodermal origin (32).

In addition, they also differentiate into cell types of ecto- and endodermal lineages (31). In the recent few decades, the promising characteristics of mesenchymal stem cells (MSCs), including their regenerative properties and ability to differentiate into various cell lines, have created significant interest among researchers whose work has provided interesting perspectives on cell-based therapies for different diseases.

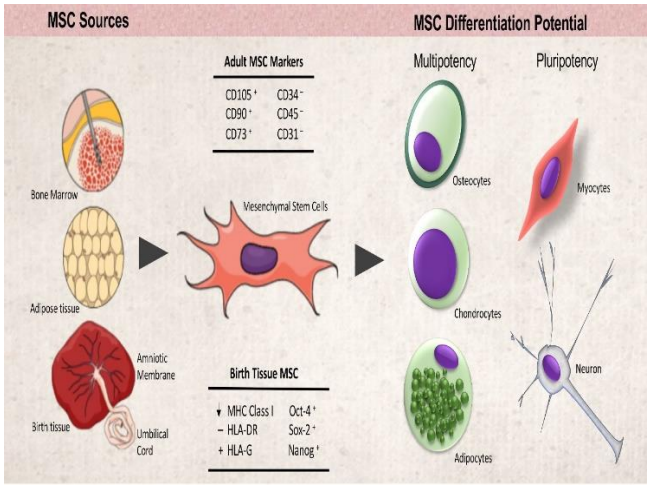


Figure 7. Minimal criteria of ISCT to define MSCs

Reference - Diller RB, et al. OBM Transplant. 2020

Though Embryonic Stem Cells (ESCs) or iPSCs are pluripotent, their genetic instability and teratoma forming potential has limited their use in human applications. Hence, there is a need to find an ideal alternative cellular candidate from an ideal source in regenerative medicine. Therefore, MSCs being prolific in their population are considered to be a suitable alternate candidate in cell based regenerative therapy.

1.4.2.5 Properties of Mesenchymal Stem Cells

The major properties of Mesenchymal Stem Cells that make them more attractive in the field of regenerative medicine are

- Easy availability in various tissue sources and possibility of autologous source.
- Easy to isolate and manipulate under the standard culture conditions.
- Multipotent differentiation potential under certain physiological and experimental conditions.
- Immune privileged or hypo immunogenic due to the low expression of MHC class I and absence of MHC class II molecules, absence of co-stimulatory molecules like CD28, CD40, CD 80, CD 86 etc.
- Immunosuppressive properties by inhibiting the proliferation of immune cells and modify them into tolerogenic phenotype along with secretion of immunosuppressive cytokines (IL-10, TGF-β, IDO, HLA-G).
- Ability to migrate and homing to the site of injury.
- Immunomodulatory properties depending upon the microenvironment of the tissue injury.
- Resistant to oxidative stress (ROS)
- Safety and efficacy was proven in many clinical trial involving MSCs in the treatment of immunological and auto immune diseases like GVHD, SLE, etc.

1.5 Understanding the Interaction between the MSCs and immune responses

1.5.1 Communication between MSCs and damaged tissue

MSCs play a crucial role in tissue repair due to their multipotent differentiation potential and immunomodulatory properties. During tissue injury, adaptive immune cells (CD 4^+ T cells, CD 8^+ T cells and B cells) and naive immune cells (macrophages, neutropihils etc.) are triggered by the secretory factors produced by necrotic cells, apoptotic cells, damaged microvasculature, and stroma.

Meanwhile, in response to injured cells and spilled cell contents, phagocytes immediately create an inflammatory microenvironment by producing inflammatory mediators such as TNF-α, IL-1β, free radicals, chemokines, and leukotrienes. As a result of the pro-inflammatory stimuli along with combined action of immune cells, endothelial cells and fibroblasts, changes in the microenvironment is orchestrated. The changes in the microenvironment leads to the migration and homing of MSCs at the site of injury and aids in the replacement of damaged tissue by differentiation and paracrine activity (33). Growth factors are produced by MSCs during tissue repair including transforming growth factor-β (TGF-β), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), keratinocyte growth factor (KGF), platelet-derived growth factor (PDGF), hepatocyte growth factor (HGF), insulin growth factor-1 (IGF-1), angiopoietin-1 (Ang-1), and stromal cell derived factor-1 (SDF-1) (34-36) . These growth factors, in turn, stimulate the proliferation of tissue progenitor cells, fibroblasts and endothelial cells which are responsible for tissue regeneration and repair. Hence, inflammation or pro-inflammatory microenvironment created during tissue injury is absolutely necessary for the stimulation of MSCs to take part in tissue repair and regeneration (37).

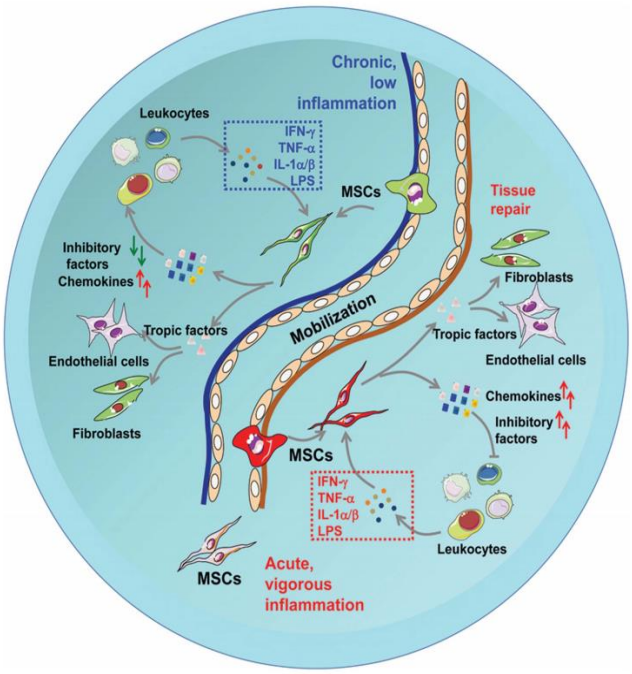


Figure 8. Interaction between MSCs and immune responses during tissue repair.

Pro-inflammatory stimulus in the tissue injury triggers the MSCs to secrete cytokines and chemokines responsible for tissue repair and regeneration.

Reference: Ma S, Xie N, Li W, Yuan B, Shi Y, Wang Y. Immunobiology of mesenchymal stem cells. Cell Death Differ [Internet]. 2014;21(2):216–25. Available from: http://dx.doi.org/10.1038/cdd.2013.158

1.5.2 Immunosuppressive properties of MSCs

In an inflammatory microenvironment, the pro-inflammatory stimulus such as IFN-ϒ, TNF-α, IL-1α, IL-1β stimulates the MSCs to produce high level of immunosuppressive cytokines, chemokines, cell adhesion molecules like CXCR3 ligands, CCR5 ligands, ICAM-1, VCAM (38). These molecules stimulate the immune cells to be in close association with MSCs to produce high concentration of immunosuppressive factors such as IL-10, IL-6, PGE-2, HO-, IDO, HLA-G which inhibits the proliferation and activation of immune cells (39). Of these, PTGS-2 plays a major role in immunosuppression. PTGS-2 produced by MSCs stimulates the macrophages to produce another immunosuppressive molecule IL-10 which inhibit the maturation of dendritic cells and shift the balance between Th1 and Th2 response

(40, 41). It has been reported that in the presence of PTGS-2, MSCs inhibit the proliferation of activated NK cells through the secretion of IDO, an another immunosuppressive molecule (42). Likewise, IL-10, was highly upregulated when MSCs are co-cultured with immune cells, splenolytes and also during *in-vivo* administration (43). Hence, the immunosuppressive property of MSCs exerted mainly through the concerted action of major immunosuppressive molecules which orchestrate the inflammatory niche during tissue injury.

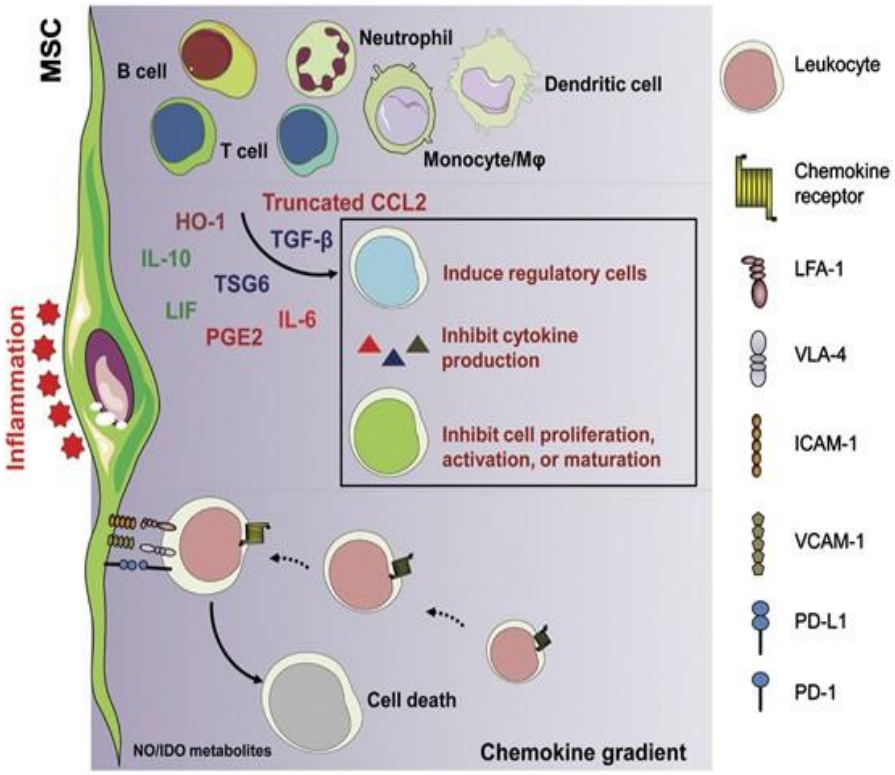


Figure 9. Immunosuppressive properties of MSCs.

Tissue injury associated with infiltration of immune cells and MSCs. Inflammatory stimulus triggers the production of chemokines and cell adhesion molecules in MSCs (CXCR3, CCR5 ligands, ICAM-1 and VCAM-1). These molecules stimulate the accumulation of immune cells in close association with MSCs, whereby high concentrations of immunosuppressive cytokines could affect immune cell activation, proliferation and functions

Reference: Ma S, Xie N, Li W, Yuan B, Shi Y, Wang Y. Immunobiology of mesenchymal stem cells. Cell

1.5.3 Immunomodulation of MSCs *in-vitro* and *in-vivo*

Immunomodulatory functions of MSCs could be due to both cell-cell interaction and via secretion of soluble factors such as cytokines and chemokines (44,45). MSCs are known to directly interact with immune cells such as T and B cells, dendritic cells (DC), natural killer cells (NK) etc…and alter their function: inhibit the proliferation of T and B lymphocytes, maturation of dendritic cells, polarization of macrophages from Pro-inflammatory (M1 phenotype) to anti-inflammatory (M2) or tolerogenic phenotype (42, 46). It is also known that they can exert immunomodulatory functions of mitogen-stimulated as well as mixed lymphocyte reactions *in-vitro* (43,47-49). It has been shown that their microenvironment plays a crucial role in their immunomodulatory function (50). MSCs respond positively to an inflammatory microenvironment *in-vivo* (51). Inflammatory conditions include injury to a specific location or a systemic trigger presented as chronic inflammation. MSCs, in general, can therefore respond to respective inflammatory stimulus and alleviate the burden. hMSCs were shown to reduce localized inflammation caused by spinal cord injury (SCI) in mice. The cellular mechanism in reducing the inflammatory response involved an increase in anti-inflammatory cytokine (IL-4) production in the neighbouring neuron-like cells and is believed to be effected cell-non-autonomously involving signalling from the MSCs (52). When BM-MSCs were co-cultured with T cells under inflammatory condition such as PHA stimulation, increased secretion of anti-inflammatory cytokines such as IL-10 and IL-11 have been reported (53). It is not uncommon for MSCs to adapt their cytokine profile based on the tissue of action (injury site). For e.g. kidney perivascular cells (MSCs) have been known to secrete Hepatocyte Growth Factor (HGF) to bring about organ homeostasis. Interestingly, a role for the microenvironment structure in influencing the MSC effector function has also been identified (50). On the other hand, growing evidence points to the long-range paracrine activities of MSCs. Paracrine effect of MSCs, in organ remodelling was observed in the case

of interstitial lung disease (ILD) in an experimental mice model, where systemic administration of human umbilical cord derived MSCs show suppression of inflammation and fibrosis of the lung tissue. The MSCs were shown to act via lung macrophages where the IL6-IL10-TGFβ axis is suppressed to reduce inflammation during ILD (54). An integral step in the systemic response of allogeneic MSCs is the encounter of blood-effector cells such as the Peripheral blood mononuclear cells (PBMCs)/ Mononuclear cells (MNCs). Recently, it has been shown that cytokine profiles of both MSCs and MNCs alter during *in vitro* co-culture conditions (55). MSCs, on their own, constitutively express IL-6 and TGF-β; upon inflammation, their expression pattern changes: IL-6 is up-regulated whereas TGF-β is down-regulated (56). Hence, such a see-saw expression of cytokines by MSCs and the effector cells will have to be tightly regulated and fine-tuned to bring about the desired biological outcome. The ability of MSCs to modulate their own immune responses is observed even in a systemic disease condition. For e.g. Cytokines such as VEGF, SCF, and ANGPT of BM- MSCs of patients of myeloid dysplastic syndromes (MDS) show a decrease in their gene expression as compared to the control MSCs (57). In fact, such change in response at the gene expression level happens as early as 6 hours upon exposure to soluble "injury" factors (58). Together, these studies point to the increasing need for a comprehensive understanding of MSC from various sources and their responses to a wide range of immune cells.

The use of MSCs are advantageous over other adult stem cells in regenerative therapy due to their strong immunosuppressive properties and lack of elicitation of host versus graft reaction that may occur in the recipient. In fact, it has also been reported that even the paracrine activity of MSCs are capable of modulating the host immune responses (33, 59). These characteristics of MSCs led researchers to further explore their immunomodulatory capacity in order to identify a source that would be more immune-tolerant in allogeneic models of cell transplantation. Therefore, their therapeutic effects have been investigated in

various immunological and inflammatory disorders such as inflammatory bowel disease (IBD), Multiple Sclerosis (MS) and Graft versus host reaction (GVHD) (60-64). Though their therapeutic effects are promising, MSCs have exhibited differences in their differentiation and immunomodulation abilities. These differences have been attributed to their source of origin and the quality of cells (early or late passages) (65,66). Therefore, the central need of cell-based regenerative therapy is to identify a source(s) which has high plasticity, enhanced differentiation potential and effective immunomodulatory functions for better clinical outcomes.

Here, MSCs derived from Adipose tissue(AT), Bone marrow(BM) and Wharton's Jelly (WJ) were co-cultured with immune cells (MNCs) in order to assess their immunomodulatory properties and cytokine profile to deepen the understanding of the role of MSCs under an inflammatory microenvironment. Conversely, understanding the responses of immune cells to MSCs will also add to the knowledge gap in this field. Most importantly, characterization of the immunomodulatory function of MSCs from different sources will aid in the identification of a better MSC source for various cell therapy approaches. Our present study therefore compares three different sources of MSCs: BM, AT and WJ and their respective cytokine profiles and immunoregulatory effects.

1.6. AIM & OBJECTIVE

1.6.1 Aim

To investigate the immunological Profiling of Adult Human Mesenchymal Stem Cells derived from various sources –Adipose tissue, Bone marrow, and Umbilical Cord Matrix /Blood

1.6.2 Objectives

1.6.2.1 Objective 1

To isolate, and expand the Mesenchymal Stem Cells derived from the various sources – Adipose tissue, Bone marrow, Umbilical cord matrix/ Blood.

1.6.2.2 Objective 2

To characterize the isolated MSCs derived from various sources according to the minimal criteria proposed by International Society for Cellular therapy (ISCT).

1.6.2.3 Objective 3

To study, compare and analyse the immunomodulatory properties of MSCs derived from the various sources under the *in-vitro* inflammatory condition to find an ideal source.

1.6.2.4 Objective 4

To analyse and compare the immunological properties of undifferentiated MSCs with immunological properties of Chondro differentiated cells in the ideal source (Umbilical cord matrix).

1.7 Rationale behind the study

The rationale behind our study is to comprehend the interaction of Mesenchymal Stem Cells derived from the various sources with Mononuclear cells (MNCs) under PHA stimulated culture conditions and thereby we extrapolate our study to understand the role of MSCs under inflammatory conditions.

1.8 Outline of the study in brief

In the present research study, an attempt was made to investigate the immunological profiling of MSCs derived from various sources such as AT, BM, and WJ source. Firstly, MSCs were successfully isolated, expanded and characterized from various sources such as Adipose tissue (AT), Bone marrow (BM), Wharton's Jelly (WJ) and Umbilical cord blood (UCB). During the isolation procedure, it was found difficult to isolate and expand MSCs from all the samples of UCB under the same culture conditions (DMEM with 10% FBS) compared to other sources of MSCs. The frequency of MSCs in UCB were too low. Since the study was proposed to isolate MSCs either from UCM (WJ) or UCB along with AT and BM sources, the study was narrowed down the sources into three (AT, BM and WJ) for further investigations to find out the ideal source of MSCs in all aspects including isolation, expansion and immunomodulatory functions. After successful isolation of MSCs from the above mentioned sources, they were characterized as per the minimal criteria proposed by International Society of Cellular therapy (ISCT).

Secondly, MSCs from AT, BM and WJ were co-cultured with immune cells (MNCs) in order to assess their cytokine profile to deepen the understanding of the role of MSCs under the inflammatory microenvironment. Conversely, understanding the responses of immune cells to MSCs will also add to the knowledge gap in this field. Most importantly, characterization of the immunomodulatory function of MSCs from different sources will aid in the identification of an ideal source for various cell therapy approaches. Therefore, this study compared three different sources of MSCs: AT, BM, and WJ and their respective cytokine profile and immunoregulatory effects. Overall, based on the outcome, the study points to WJ-MSCs, a non-invasive source, to be a superior candidate among the three sources for most cell-therapy based applications. Therefore, further investigation was narrowed down in WJ-MSCs. At present, among the tri-lineage differentiation, chondrocytes have been

considered as more effective and beneficial in the treatment of many ailments such as osteo arthritis, knee replacement etc. we were interested in comparing the cytokine profile of chondrocytes differentiated from WJ-MSCs against the cytokine profiling of undifferentiated WJ-MSCs.

Hence, finally, an attempt was made in investigating the cytokine profiling of chondrocytes by co-culturing the chondrocytes with mononuclear cells under PHA stimulated conditions that was compared with undifferentiated MSCs under the same culture condition of the WJ-source. This study is intended to bring out the variations in the secretory profile and highlight the prospect of choosing source specific MSCs for various therapeutic indications.

The goal of this study to achieve a better understanding of characteristic differences of MSCs from various sources giving prime importance to the immunomodulatory properties of undifferentiated as well as chondro differentiated MSCs as a step towards using them for clinical translation in the near future.

CHAPTER - 2
REVIEW OF LITERATURE

2.1 Mesenchymal Stem Cells

Friedenstein et al described the Mesenchymal Stem Cells also known as Mesenchymal Stromal cells in bone marrow in the year 1976. These cells have unique features to develop into fibroblastic colony forming cells (9). Also, they have unique multipotent properties with ability to differentiate into any lineage with self-renewing property (67). Because of its self-renewing properties, MSCs can be expanded into various passages (higher passage no) without any significant alterations in its major properties or characteristics. Morphologically, they are characterized as spindle shaped cells with an ability to adhere to the plastic surface under standard culture conditions. Due to its enhanced regenerative potential, it has been considered as an emerging strategy for the treatment of various immune related disorders.

2.2 Sources of Mesenchymal Stem Cells

MSCs are widely distributed in various tissue sources such as bone marrow, adipose tissue, amniotic fluid, amniotic membrane, dental tissues, endometrium, limb bud, menstrual blood, peripheral blood, placenta, synovial fluid and Wharton's jelly (10,13,17,18,30,68). The difference between the MSCs arising from the different tissues are unclear. MSCs from various origins differ in terms of colony forming capability, proliferation potential, differentiation ability, and cell surface marker expression (69). MSCs derived from the fetal source have longer expansion potential and attain senescence at later passage than the MSCs derived from adult sources. Indeed, MSCs from birth-associated tissues exhibit superior cell biological features compared to adult source such as BM-MSC in terms of proliferation, life span, differentiation potential and also immunomodulatory potential (70, 71). These differences have also been attributed to their source of origin and the quality of cells (early and late passages) (65,66). Therefore, the central need for cell based regenerative therapy is to identify a source which has high plasticity, enhanced differentiation potential and effective immunomodulatory functions for better clinical outcomes.

2.3 Minimal Criteria proposed by International Society for Cellular Therapy (ISCT)

Since the research protocols for the isolation and characterization of MSCs are varied among researchers from different laboratories, Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) has proposed some minimal criteria to define human MSCs for both research based investigation as well as pre-clinical studies (32). First, MSCs must be adherent to plastic surface of tissue culture flasks when cultured under standard culture conditions. Second, it must be positive and negative for certain cell surface markers when measured by flow cytometry (positive for CD73, CD90 and CD105; negative for CD45, CD34, CD14 or CD11b, CD79α or CD19, and HLADR). Third, it must differentiate into the tri-lineages of mesodermal origin (osteogenic, adipogenic and chondrogenic lineages) under differentiating culture conditions.

2.4 Differentiation potential of MSCs

MSCs have the ability to differentiate into mesodermal and also transdifferentiate into ecto- and endo- dermal lineages. Mesodermal differentiation includes osteogenic, adipogenic, chondrogenic, cardio and myogenic lineages. In the tri-lineage differentiation, the formation of mineralized matrices in osteogenic differentiation, production of oil droplets in adipogenic differentiation and expression of type II collagen formation in chondrogenic differentiation has been evaluated by immunocytochemical, histochemical and PCR analysis by various groups (67,72,73). Apart from mesodermal differentiation, MSCs also have the potential to differentiate into ecto- and endo- dermal lineages in the presence of suitable media and supplements that supports specific lineage differentiation.

In addition, MSCs also have the potential to transdifferentiate into endodermal lineage. When the MSCs from various sources such as umbilical cord, adipose tissue and bone marrow were treated with neuronal induction media supplemented with growth factors, they trans-differentiate into neuronal specific phenotypes such as oligodendrocytes, cholinergic and

dopaminergic neurons (74-76). The potential of MSCs to transdifferentiate into neuronal lineage can revolutionize the regenerative cell therapy in treating many neurological disorders.

Initially it was believed that hepatocytes could be derived only from the cells originating from endodermal lineages and their progenitor cells. MSCs have revealed the potential to transdifferentiate into hepatic and pancreatic lineage of endodermal origin when they are induced with specific conditioning medium. MSCs from various sources were transdifferentiated into hepatic lineages and their differentiation ability was confirmed by liver-specific transcription markers especially HNF-4α which is an essential transcription factor for the morphological and functional differentiation towards hepatocytes (77-79). The protein expression includes albumin, α-fetoprotein, nuclear factor 4 α (HNF-4α); Though it differentiates into hepatic lineage, the differentiation capacity remains inadequate for clinical application. Similarly, MSCs derived from various sources also transdifferentiate into pancreatic lineage when induced with specific conditioning medium required for pancreatic differentiation (80-82).

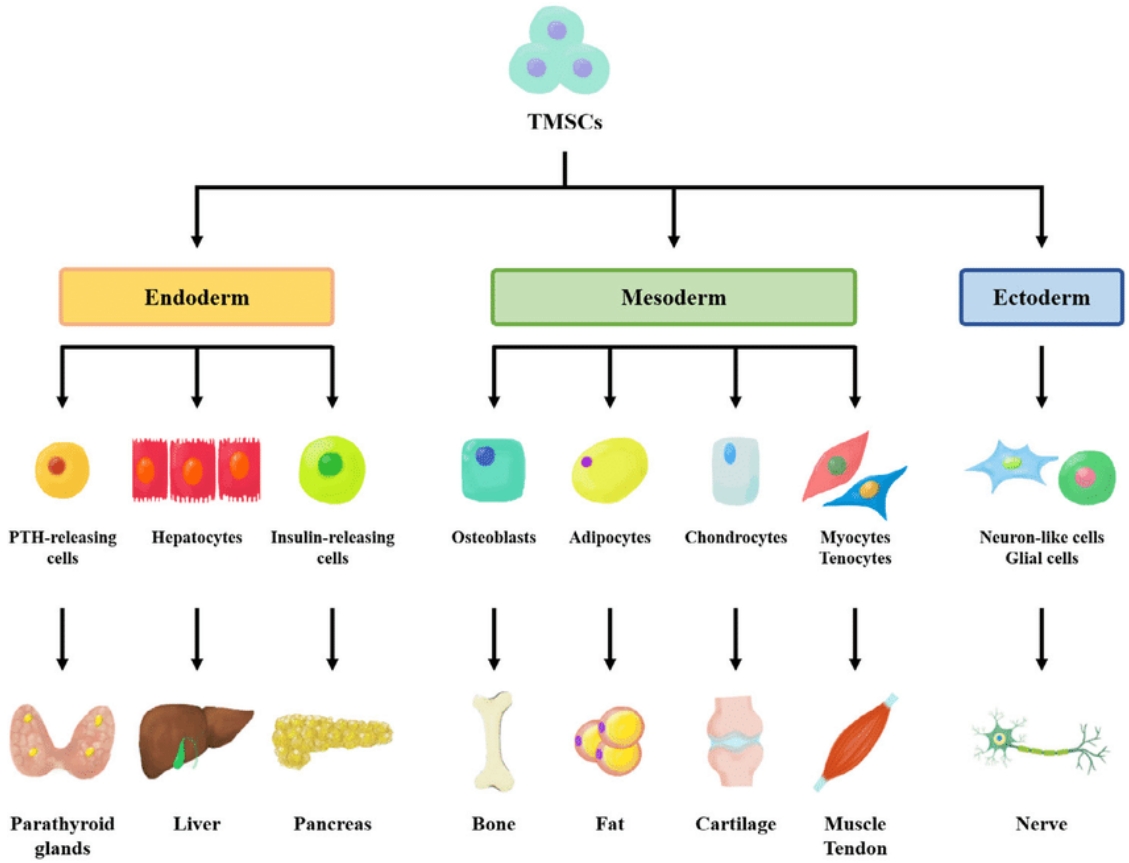


Figure 10. Differentiation of MSCs into ecto, meso and endo-dermal lineages

Reference: Oh et al Concise Review. Stem Cells. 2019.

All these studies have revealed the ability of differentiation potential of MSCs into mesodermal lineages and transdifferentiate potential into ecto and endodermal lineage that can transform the current drug therapies to a future promising cell based therapies.

2.5 Hypo-immunogenic properties of MSCs

Historically, MSCs are considered to be hypo-immunogenic or immune privileged cells due to their limited expression of MHC-I, lack of expression of MHC-II and co-stimulatory molecules (CD28, CD40, CD80 and CD 86), and ability to inhibit the proliferation of T cells and resistance against the cytotoxicity of cytotoxic lymphocytes.

2.6 Immunomodulation of MSCs

The immunomodulatory properties of MSCs was first recognized in the year 2000 by Liechy et al. They demonstrated the engraftment and site-specific differentiation of human mesenchymal stem cells after in-utero transplantation in sheep (83). Later, several studies confirmed the immunomodulatory properties both *in-vitro* and *in-vivo.* (84-87).

The immunomodulatory functions of MSCs are exerted by cell to cell interaction or through the secretion of soluble factors such as cytokines /chemokines or combination of both. The immunomodulatory properties of MSCs have effects on both cell mediated and innate immunity.

2.6.1 Cell-Cell interaction

Cell to cell interaction is an important modulatory function of MSCs through the expression of adhesion molecules on the cells surface and also by inducing regulatory T and B cell population. In an inflammatory condition, MSCs expresses few adhesion molecules on the surface like VCAM-1, ICAM-. chemokine ligands of CCR5 and CXCR3 etc. which attracts the T cells and other immune cells to the inflammation site. These adhesion molecules along with the soluble factors act on the immune cells through various mechanism and inhibit their activity. It acts on T cells and B cells by inhibiting the proliferation, inducing apoptosis and cell arrest and also converting them into regulatory phenotype as Treg and Breg. Regulatory phenotypes of T and B cells are able to produce the immunosuppressive and anti-inflammatory cytokines like IL-10 and TGF-β. It attaches to the Th17 cells via CCR6 and CD11a/CD18 and facilitate Th17 to adopt regulatory phenotype (88). In addition to this, it acts on the innate immune cells like dendritic cells, natural killer cells and shift their phenotype from pro-inflammatory to anti-inflammatory or regulatory phenotype. It Skew the monocyte towards M2 macrophage differentiation which is anti-inflammatory phenotype instead of M1 pro-inflammatory phenotype (89). Subsequently, this M2 macrophages secrete

anti-inflammatory and immunosuppressive molecules like IL-10, CCL18 and also induce Treg differentiation. When the MSCs interact with immune cells like T cells, B cells, NK cells, DCs, macrophages in an inflammatory microenvironment, it modulates the microenvironment by inducing the secretion of immunosuppressive and anti-inflammatory cytokine production and development of regulatory phenotype of the immune cells and thereby helps in the tissue regeneration and repair (90-93). The ability of MSCs to adopt different phenotype in response to sensing an inflammatory environment is crucial for understanding their therapeutic potential in treating various immune disorders.

2.6.2 Secretion of soluble factors

Secretion of soluble factors such as cytokines and chemokines by the MSCs and immune cells also plays a major role in the immunomodulation. Cytokines like transforming growth factor-β1 (TGF-β), prostaglandin E2 (PGE2), indoleamine-pyrrole 2,3-dioxygenase (IDO), nitric oxide (NO) and interleukin-10 (IL-10) are secreted by MSCs in response to inflammatory microenvironment. Though several number of cytokines and chemokines are secreted by MSCs, a few cytokines which are majorly involved in the immunomodulatory properties of MSCs are briefly discussed below. These are cytokines included in this study.

2.6.2.1 Anti-inflammatory cytokines secreted by MSCs

2.6.2.1.1 Transforming growth factor - TGF-β

TGF-β is a potent immunosuppressor secreted by MSCs in the inflammatory microenvironment. It acts on both the innate and adaptive immunity by suppressing the proliferation of T cells, the activation of B cells, the maturation and antigen presentation of DCs, the cytotoxicity effect of Natural killer cells, and phagocytic activity of macrophages. It also helps in the conversion of naïve T cell population into regulatory phenotype to enhance the regulatory effects. (94,95).

2.6.2.1.2 Prostaglandin- E2 (PGE-2)

PGE-2 is an anti-inflammatory cytokines secreted by MSCs during chronic inflammation. It acts on both the innate and adaptive immunity by inhibiting the phagocytic ability of macrophages, maturation of dendritic cells, interfering the early activation of B cells, skewing the conversion of naïve T cells from Th1 phenotype to Th2 phenotype. It also helps in the upregulation of regulatory T and B cells (96). The secretion of PGE-2 was upregulated when MSCs were co-cultured with peripheral blood mononuclear cells in the presence of inflammatory stimuli like IFN-ϒ or TNF-α or combination of both (97). When MSCs were cultured with secretory factors produced by macrophages in an inflammatory milieu, the secretion of PGE-2 was up regulated. It was demonstrated that IL-10 secreted from the anti-inflammatory macrophages induce the MSCs to secrete PGE-2 in an inflammatory microenvironment (45).

2.6.2.1.3 Indoleamine-pyrrole 2,3-dioxygenase (IDO)

IDO is an immunosuppressive cytokines produced by MSCs during inflammation. It is a key immunoregulator produced during pregnancy. IDO is a rate-limiting enzyme which catalyses the conversion or degradation of tryptophan to kynurenine via kynurenine pathway. IDO secreted by MSCs has been shown to inhibit allogenic T- cell response and induce immune tolerance. It also helps in the stimulation of secretion of IL-10 which is another immunosuppressive cytokine helps in the inhibition of dendritic cell maturation and function. When MSCs were co-cultured with mononuclear cells in the presence of inflammatory stimulus, the secretion of IDO and IL-10 was upregulated along with other soluble factors such as iNOS, PGE-2 by inhibiting the proliferation and activation of DC through down streaming cell signalling of JAK-STAT pathway (98).

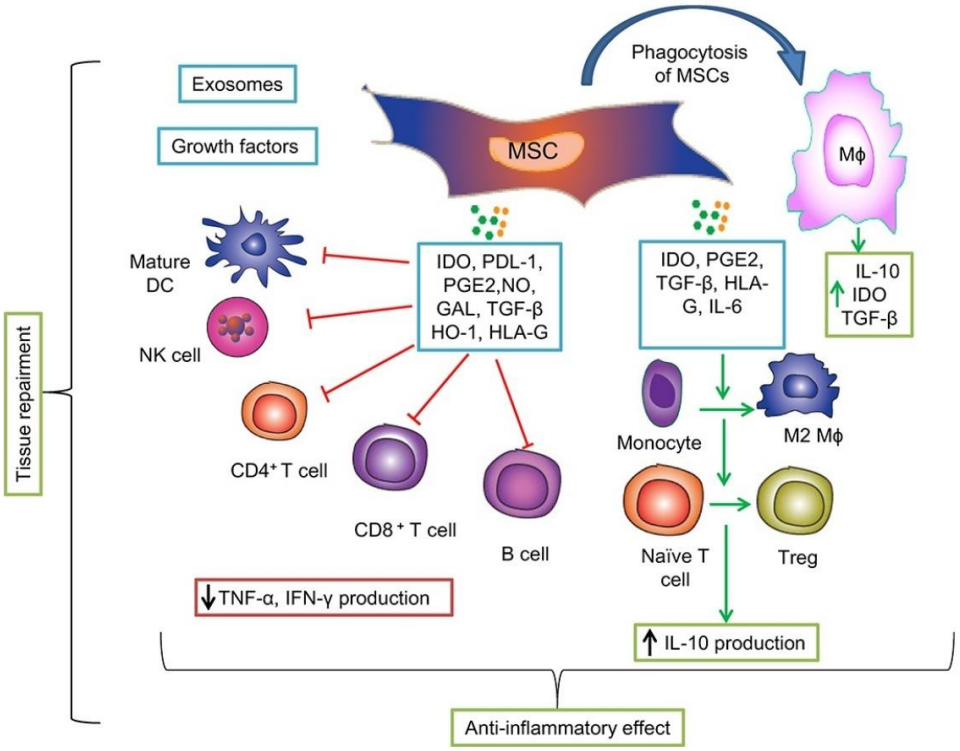


Figure 11. Immunomodulation of MSCs by cell- cell contact and secretion of soluble factors

Immunomodulatory effects of MSCs include suppression of B- and T-cell proliferation, induction and regulation of regulatory T cells, inhibition of NK cell function and inhibiting dendritic cell maturation and activation. The immunosuppressive effects of MSCs are mediated by soluble factors and cell–cell contact.

Reference: S, Pathak S, Kim JO, Yong CS, Jeong JH. Mesenchymal stem cell therapy for the treatment of inflammatory diseases: Challenges, opportunities, and future perspectives. Vol. 98, European Journal of Cell Biology. 2019.

When MSCs are co-cultured with natural killer cells, it was found that IDO and PTGS-2 are the key mediators in inhibiting the proliferation of natural killer cells and cytotoxicity (42). In addition to IDO, several other soluble factors such as HGF, IL-10, induced nitric oxide (iNOS), IL-4 also produced by MSCs in an inflammatory microenvironment.

2.6.2.1.4 Inducible nitric oxide synthase (iNOS)

iNOS is one of the major mediators of immunosuppression in combination with several other chemokines helps in the inhibition of the T cell proliferation in an inflammatory environment stimulated by IFN- γ or other pro-inflammatory stimuli.

2.6.2.1.5 Interleukin – 10 (IL-10)

IL-10 is a pleotropic cytokines secreted by immune cells particularly Th-2 cells under inflammatory conditions. It promotes anergy in T lymphocytes by effectively limiting their proliferation and cytokine production by directly blocking CD 28 phosphorylation and thereby preventing any further downstream signalling. It also acts on dendritic cells and prevent the secretion of Th1 cytokines produced by it during the inflammation and promote the generation of regulatory T cells. It considered as the anti-inflammatory, anti-fibrotic and immunosuppressive cytokines (99).

2.6.2.1.6 Human leukocyte antigen - G (HLA-G)

HLA-G is a unique immunomodulatory molecule expressed in both soluble form and membrane bound form. There are four membrane form and three soluble form. HLA-G is expressed on cytotrophoblasts at the feto-maternal interface plays major role in immunotolerance of the fetus from the mother (100). In addition to the HLA-G, feto-maternal interface also express and secrete multiple immunosuppressive cytokines such as IDO, PTGS-2 and IL-10 (101). HLA-G inhibits the proliferation of T cells and promote the generation of regulatory T cells ($CD4^+$ $CD25^+$FOXP3).

2.6.2.1.7 Vascular Endothelial Growth Factor (VEGF)

VEGF are the key regulators of angiogenesis responsible for tissue repair and regeneration after injury. It plays a major role in immunity as well as inflammation. During inflammation, it is responsible for the recruitment of inflammatory cells and expression of co-stimulatory molecules on the recruited and resident monocytes. As a result, pro-inflammatory cytokine expression at the injured site gets upregulated (44). It acts on the DC cells to produce IDO, an immunosuppressive molecule which inhibits the further proliferation and activation of DC cells, T cells and induce T cell apoptosis (102). Under the stimulation of pro-inflammatory cytokines at the site of injury, the immigrated MSCs secrete plethora of

growth factors which includes VEGF, EGF, PDGF, FGF, etc. Of which, VEGF also plays an important role in tissue repair and regeneration. These growth factors orchestrate the various cells such as endothelial cells, fibroblast, stem cells to promote the tissue regeneration by enhancing the angiogenesis, eliciting the progenitor cell differentiation and inhibiting the leukocyte trans migration (33).

2.6.2.1.8 Interleukin -4 (IL-4)

IL-4 is an anti-inflammatory cytokine which converts pro-inflammatory M1 phenotype to anti-inflammatory M2 phenotype. In an inflammatory response, it acts along with IL-6 and upregulate the regulatory phenotype (103).

2.6.2.2 Pro-inflammatory cytokines

2.6.2.2.1 Tumor Necrosis Factor- α (TNF-α)

TNF-α is a pro-inflammatory cytokine produced by macrophages during acute inflammation and responsible for necrosis and apoptosis during tissue injury. The presence of TNF-α in the inflammatory microenvironment stimulate the MSCs to exert immunosuppressive action. Hence, Pro-inflammatory stimulus is required for enhanced anti-inflammatory properties of MSCs (104).

2.6.2.2.2 Interferon - ϒ (IFN-ϒ)

IFN-ϒ is a pro-inflammatory cytokine which plays a central role in inflammatory and auto immune disorders. Priming of MSCs with IFN-ϒ induces the upregulation of immunosuppressive cytokines such as IDO. Primed MSCs stimulate the monocyte to differentiate into M2 phenotype of macrophage instead of M1 and also it enhances the production of anti-inflammatory cytokine IL-10 (105).

2.6.2.2.3 Interleukin -6 (IL-6)

IL-6 is a pleotropic cytokine involved in wide range of processes such as inflammatory immune response, haematopoiesis, cell survival, apoptosis and oncogenesis. IL-6 act as a

dual cytokine. i.e act as a both pro-inflammatory and anti-inflammatory depending upon the inflammatory microenvironment. It has been reported that pro-inflammatory effect of IL-6 are mediated by trans-signalling whereas anti-inflammatory properties are mediated by classic signalling (106). It is routinely considered as classical pro-inflammatory cytokine. In an injury, IL-6 concerted with other few pro-inflammatory cytokines such as TNF-α, IL-1 and IFN-ϒ and induce the secretion of acute phase proteins causes neutrophil recruitment, expression of cell adhesion molecule etc. It stimulates the proliferation of T lymphocytes but when combines with IL-4, it helps in the generation of Th2 response instead of Th1 response (107).

2.6.3 Immunomodulation of MSCs in earlier studies

In an earlier study, the mechanism of immunomodulatory effect of BM-MSCs were studied by co-culturing the BM-MSCs with T- cells (both allogenic and autologous) and tested the inhibitory effect of BM-MSCs by inducing the co-culture system with dendritic cells (DCs) and mitogen PHA as stimulators through mixed lymphocyte reaction assay (MLR assay). The findings of this study implies that BM-MSCs were able to efficiently inhibit the T – cell proliferation induced by both cellular and humoral stimuli. They also reported that the underlying mechanism in the inhibitory effect is due to the secretion of soluble factors such as TGF-β and HGF secreted by MSCs (108).

Likewise, the immunomodulatory effect of UC-MSCs are investigated by co-culturing them with immune cells in the inflammatory mille *in –vitro.* When the UC-MSCs were co-cultured the with PBMCs, particularly T cells in the PHA stimulated condition in *in- vitro,* it was observed that UC-MSCs primarily inhibited the division of generation 3 (G3) and generation 4 (G4) of PBMCs when studied through kinetic analysis. Also, these MSCs augmented the expression of $CD127^{+}$ and $CD45RA^{+}$ and reduced the expression of CD25+ in PBMCs stimulated by PHA. The levels of anti-inflammatory cytokines, PEG2, TGF-β, and

IL-10 were greatly up-regulated, accompanied by a significant down-regulation of pro-inflammatory IFN-γ in the co-culture. Hence, UC-MSCs are able to suppress mitogen-induced PBMC activation and proliferation in vitro by altering T lymphocyte phenotypes, increasing the frequency of CD4+CD25highCD45RA+ Tregs, and modulating the associated cytokine production (109).

It has been reported in the comparative study that UC-MSCs has the strongest inhibitory effect on PHA induced T cell proliferation compared to BM, AT and PL. This effect was due to the spontaneous secretion of IFN-γ by T-lymphocytes which in turn induce the secretion of IDO, an immunosuppressive cytokine. IDO have the ability to induce T cell apoptosis and cell cycle arrest in the S phase by inhibiting the expression of cyclin-dependent kinase 4 (CDK4) (102). FOXP3 is required for the maintenance of $CD4^+$ $CD25^+$ T reg population and this population was enhanced by the hemeoxygenase-1 (HO-1) When BM-MSCs were co-cultured with PBMCs of asthma patient. (110).

MSCs are capable of inhibiting the proliferation of T cells induced by mitogens, allo antigens CD3 and CD 28 antibodies by upregulating the regulatory T cells and through the secretion of immunosuppressive cytokines such as TGF-β1 and HGF (90).

2.6.4 Cartilage regeneration using MSCs and chondrocytes

Articular cartilage abnormalities are becoming more common around the world, and they frequently advance to osteoarthritis, causing morbidity and a lower quality of life. Autologous Chondrocyte Implantation (ACI) is currently used as a standard cell therapy for cartilage abnormalities in cell and tissue engineering approaches for cartilage regeneration. Articular chondrocytes are the only type of cell seen in this cartilage, and also they are sparsely scattered in the ECM. In the in-vitro culture condition, these chondrocytes have limited proliferation potential which leads to poorly functioning and quality extracellular matrix. Clinical use has been limited due to other factors also such as donor tissue scarcity,

donor site morbidity, chondrocyte dedifferentiation during culture, poor graft attachment to the surrounding chondral surface, and failure to restore native tissue integrity, which results in the formation of fibrocartilage, which is functionally inferior to hyaline cartilage. Mesenchymal stromal/stem cells (MSCs) are considered to be the preferred cell type for cartilage tissue engineering due to their unique properties like their i) ability to differentiate to cartilage (chondrocytes) (ii) paracrine action (iii) hypo immunogenic due to the low levels of major histocompatibility complex (MHC) and costimulatory proteins which aids them to escape from the recipient's immune system. There are several pre-clinical and clinical data are available regarding the use of undifferentiated MSCs in treating various immunological and auto immune disorders including osteo arthritis and rheumatoid arthritis. They also reported that the safety and efficacy in the treatment of OA, RA using MSCs (111).

2.6.5 Therapeutic application of MSCs in the regenerative medicine

According to the data available in clinicaltrials.gov (as of June 2021), there are 1285 clinical trials are on-going to treat various conditions of diseases using MSCs. 98 Clinical trials are ongoing for treating autoimmune disorders like Multiple sclerosis, Systemic Lupus Erythematosus (SLE), Lupus nephritis, rheumatoid arthritis (RA), Crohn's disease (CD), and Type I Diabetes mellitus (85), Graft versus host disease (GVHD). 30 clinical trials are on-going for degenerative diseases like lumbar degenerative disc disease, Lumbar Intervertebral Disc Degeneration, Osteo arthritis, Intervertebral Disc Disease etc. due to their differentiation potential, homing ability and immunomodulatory properties. Currently, more promising clinical trials are on-going for various ailments. According to clinicaltrial.gov, 1285 (as of June 2021) clinical trials are ongoing to treat various conditions using MSCs. MSCs are used in the following autoimmune disorders.

2.6.5.1 Graft versus Host Disease (GVHD)

The immunomodulatory properties of MSCs was first evaluated in GVHD patients and reported that clinical translation of MSCs are safe in GVHD (112). Severe graft versus host disease (GVHD) is a complication occurs after allogenic hematopoietic transplantation where symptoms resembles like auto immune and immunological disorders.

Allogenic MSC transplantation increased the survival rate of the GVHD patients and ameliorate the inflammation in skin, oral cavity and liver by increasing the IL-10 producing production regulatory B cells (112,113). Safety and efficacy have been proved in many clinical studies. Prochymal and TEMCELL are the two products of MSCs commercially available and approved in Canada and Japan for the treatment of GVHD. (114). MSCs were reported to be effective in children affected with GVHD than the adults (115).

2.6.5.2 Systemic lupus erythematosus (SLE)

Systemic lypus erythematosus (SLE) is an autoimmune disorder in which there is an imbalance between Th1 and Th2 cells occurs as a result of activation of T and B lymphocytes. Many clinical trials proven the safety and efficacy of MSCs for the treatment of SLE and reported that MSCs improved the clinical condition of the disease without any adverse effects by various factors. It regulates the condition by upregulation of regulatory T cells and down regulation of Th1 response through TGF-β and PGE2 dependent manners (116).

2.6.5.3 Multiple Sclerosis (MS)

MS is an autoimmune disease of central nervous system (CNS). They are characterized by perivenular accumulation of inflammatory cells, demyelination, multifocal CNS lesions and neuronal degeneration. In an earlier pilot study conducted for treating MS using autologous culture expanded MSCs suggesting that MSCs are safe, feasible and well tolerated in MS and did not cause any side effects (117). In an another study reported the safety and efficacy of MSCs in MS. They demonstrated that MSC transplantation in MS

causes reduction in the inflammatory response and upregulation of regulatory T cells population was observed after analysing the immunological parameters after 24 infusions (118). similarly, it has been used in other diseases like type I diabetes, crohn's disease, rheumatoid arthritis, osteoarthritis, etc.

2.6.5.4 Severe acute respiratory syndrome (ARDS) and covid pneumonia

Currently, Covid-19 is at critical stage and there is no targeted and effective therapy for the patients infected with Covid-19. Current treatments are only supportive approaches including oxygen therapy, fluid management, corticosteroids, broad spectrum of antibiotics in the prevention of secondary infection. Mesenchymal Stem Cells have been beneficial in the treatment of pandemic Covid-19 is mainly due to their immunomodulatory properties. Currently 79 clinical trials are on-going (clincaltrials.gov) all over the world in treating Covid-19 which includes Severe Acute Respiratory Syndrome (ARDS) (119), and Covid pneumonia (120). The safety and efficacy was proven already in many of the earlier studies in the treatment of various immunological disorders such as acute and chronic GVHD (121) and lung inflammatory diseases such as ARDS, COPD.

In the covid-19, immune system plays a major role in the pathogenesis of SARS-CoV-2 infection. In the case of mild to moderate symptoms of respiratory disease, patients recover with in few days without requiring special treatment by an appropriate immune response (innate response followed by adaptive immune response) of the body at the early stage of the disease itself. In the case of severe response, virus spread in the lungs followed by severe non-protective inflammatory response characterized by increase in the pro-inflammatory cytokines leads to cytokine storm or hypercytokenimia. It has been suggested that immunotherapy may be effective in controlling the severe inflammatory responses in covid-19 and encouraging results of various clinical trial across the world indicating that patients who are critically ill are benefiting more by MSC therapy. Hence, MSCs are considered as

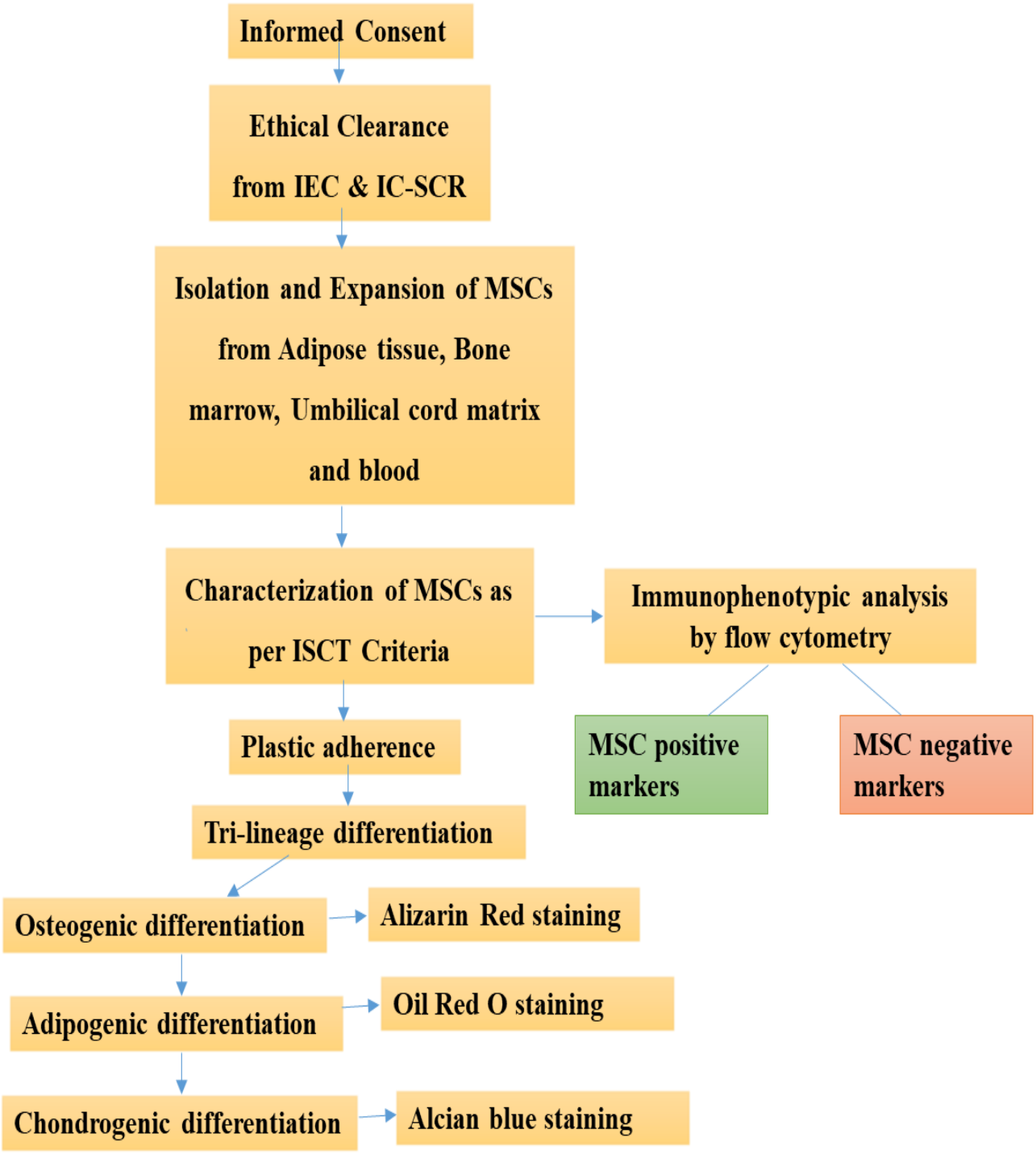
the effective candidate due to their immunomodulatory properties which is coordinated through cell to cell contact by inhibiting the proliferation and hyper activation of innate and adaptive immune response and by the secretion of anti-inflammatory and immunosuppressive molecules like PTGS-2, IDO, TGF-β and by upregulating the regulatory T and B cell population, engraftment at the site of inflammation and tissue regeneration and repair through its potential to differentiate into lung cells in the inflammatory microenvironment.

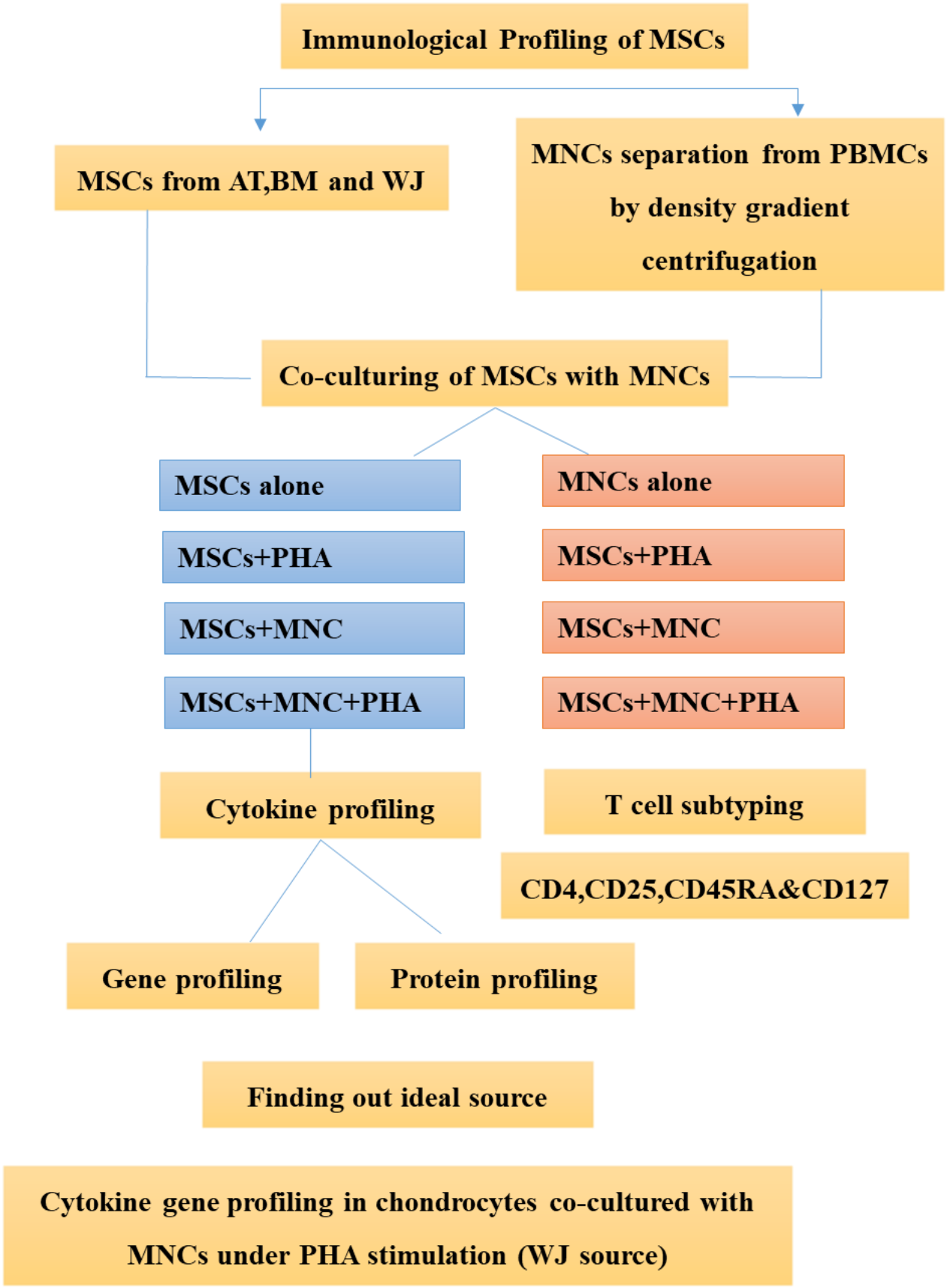
Though many clinical trials are on-going for the treatment of various diseases including covid-19 using MSCs, Our Country is far behind in the Clinical trial for treating the various ailments using cellular therapy. Since the safety and efficacy was proven in many studies MSCs can be considered as a potential candidate in the treatment of various immunological disorders including pandemic covid-19 in our country.

CHAPTER - 3

MATERIALS AND METHODS

FLOW CHART OF METHODOLOGY




Immunological Profiling of MSCs
MSCs from AT,BM and WJ
MNCs separation from PBMCs by density gradient centrifugation
Co-culturing of MSCs with MNCs
MSCs alone
MSCs+PHA
MSCs+MNC
MSCs+MNC+PHA
MNCs alone
MSCs+PHA
MSCs+MNC
MSCs+MNC+PHA
Cytokine profiling
T cell subtyping
CD4,CD25,CD45RA&CD127
Gene profiling
Protein profiling
Finding out ideal source
Cytokine gene profiling in chondrocytes co-cultured with MNCs under PHA stimulation (WJ source)

3.1 Materials

Cell culture reagents such as DMEM, RPMI, anti-coagulant citrate dextrose (ACD), Penicillin streptomycin, Phosphate buffered saline (PBS), Ficoll hypaque, phytohemagglutinin (PHA), and the growth factors used in differentiation medium were all purchased from sigma. fetal bovine serum (FBS), Collagenase, 0.25% Trypsin EDTA, CFSE cell proliferation kit, ELISA antibodies (VEGF, PTGS-2 and IDO) for functional protein assay were purchased from Invitrogen, Carlsbad, CA, USA. purchased from Thermofischer, USA. All the antibodies used in this study were purchased from BD Bioscience. Tri-reagent used for RNA isolation purchased from Sigma and cDNA synthesis kit purchased from AB Bio system. Brdu cell proliferation assay kit was purchased from Cell signalling technology. ICC kit purchased from Biogenex. Gene array kit was customized and purchased from Qiagen. The cell culture plastic wares were obtained from Corning, USA and Nunc.

3.2 Ethical clearance for this study

All protocols were approved by Institutional Committee for Stem Cell Research (IC-SCR) of Stem Cell Research Centre (SCRC), Government Stanley Hospital (GSH), Chennai (Registration No NAC-SCRT/79/20152002) /Proposal no 01/2017 & Institutional Ethical Committee (ECR/131/Inst/TN/2013/RR-16 dated 17/02/2017) of the Government Stanley Medical College and Hospital, Chennai. The samples used in this study were obtained in accordance with the Declaration of Helsinki Ethical Principles of Medical Research involving human samples. Thus, informed consent (IC) was obtained from the patients prior to sample collection.

3.3 Isolation of Mesenchymal Stem Cells

Isolation of Mesenchymal Stem Cells from various sources were carried out by the following methods.

A. Adipose tissue by enzymatic method.

B. Bone marrow by Density Gradient centrifugation method.

C. Umbilical cord matrix/ Wharton's jelly by Explant method.

D. Umbilical cord blood by density gradient method.

3.3.1 Isolation of Mesenchymal Stromal Cells from Adipose Tissue

Adipose tissue (AT) from the omentum was obtained (n=10) with consent from the patients of mean age 50±5 undergoing abdominal surgery under general anaesthesia at the Institute of Surgical Gastroenterology, GSH, Chennai, India. AT was finely minced with scalpel and further digested by Collagenase type IV (Gibco, USA) for 10 min at room temperature. The dissociated tissue was then centrifuged for 5 min at 500 g at 37^0C. The stromal vascular fraction (SVF) in the pellet was re-suspended in DMEM low glucose (Sigma) supplemented with 10% FBS (Invitrogen, Carlsbad, CA, USA) and seeded at a density of 14 x 10^4 cells/cm^2(Figure 10). The cells were incubated at 37^0C with 5 % CO_2 for 3 days and later the medium was replenished every 48 h until confluence was reached. On 80 % confluence, the cells were trypsinized (0.25 % trypsin-–EDTA Thermofischer, USA) and subsequently passaged at the cell density of 5000 cells/cm^2. MSCs of passage 3 was used for further studies.

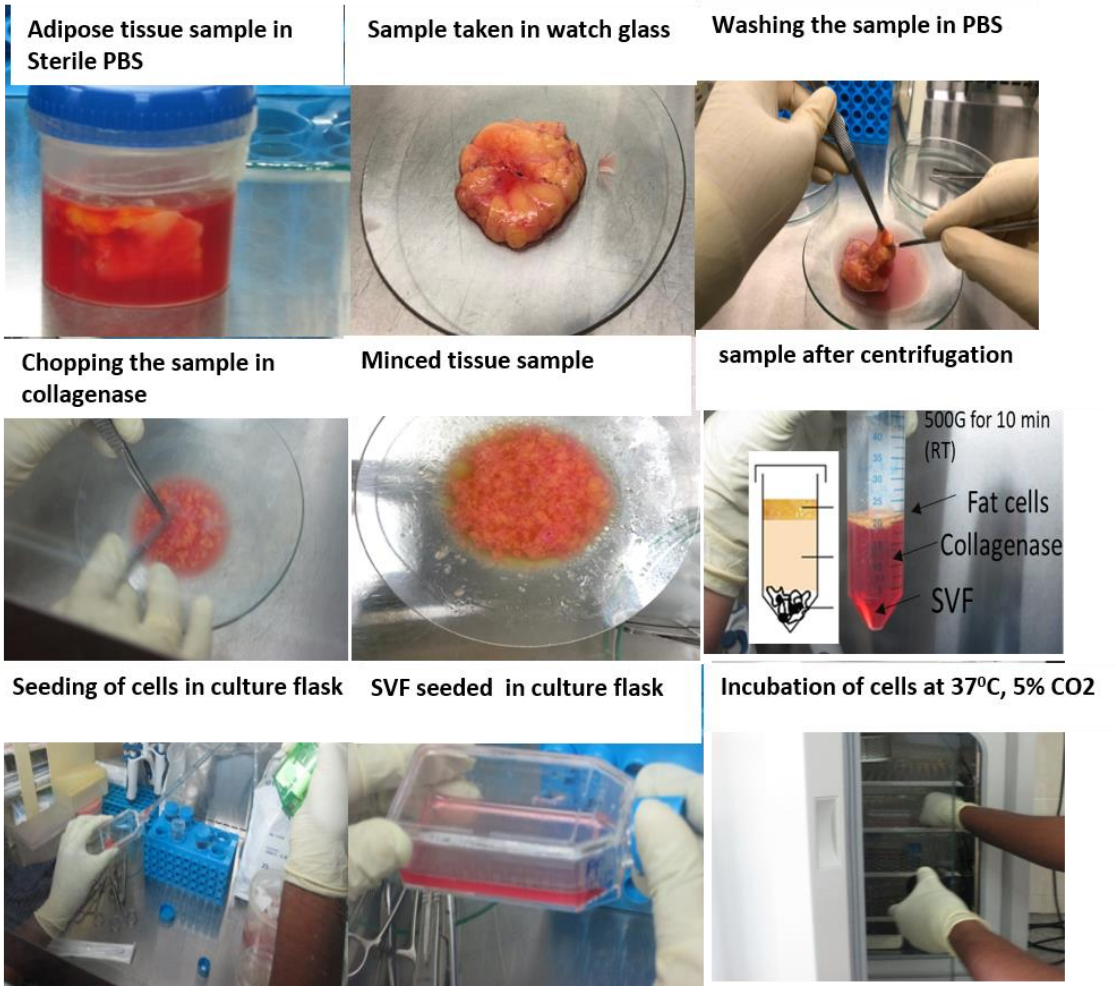


Figure 12. Isolation of Mesenchymal Stem Cells from Adipose tissue

3.3.2 Isolation of Mesenchymal Stromal cells from Bone marrow

Bone marrow sample (BM) was collected (n=15) after informed consent from the patients (with mean age and body weight of 43±5, 61.2±5kg) undergoing biopsy under general anaesthesia, at Institute of Surgical Gastroenterology, GSH, Chennai. Marrow aspirate was collected in a tube containing anti-coagulant citrate dextrose (ACD) (Sigma Aldrich, USA) and processed by Ficoll hypaque (Sigma Aldrich, USA) density gradient method. Mononuclear cells (MNCs) fraction were seeded at a density of $15x10^4$ /cm^2 in DMEM (Sigma Aldrich, USA) supplemented with 10% FBS (Invitrogen, Carlsbad, CA, USA) (Figure 11). The cells were incubated at 37^0C with 5% CO_2 for 3 days and later the medium was refreshed every 48 h until confluence was reached. On 80% confluence, the cells

were trypsinized (0.25 % trypsin–EDTA, Thermofischer USA) and subsequently passaged at the cell density of 5000 cells/cm^2.

Figure 13. Isolation of Mesenchymal Stem Cells from human bone marrow

3.3.3 Isolation of Mesenchymal Stromal cells from Wharton's Jelly

After informed consent from the mother, human umbilical Cords (WJ; n=10) were aseptically collected from the patients (mean age 26±5) who have undergone C-section mode of delivery at Government Raja Sir Ramaswamy Mudaliar (RSRM) hospital, an obstetrics section of the GSH, Chennai, India. Umbilical cord was collected in a sterile container containing transport medium maintained at 4^{o}C and processed within one hour. The blood-remains in the cord tissue was removed by washing three times with 1x DPBS containing 1x anti-mycotic and antibiotic solution following which the cord tissue was cut into small pieces under aseptic condition. The diced umbilical cord with approximate thickness of 0.5 mm were then cultured in DMEM-low glucose with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA) and incubated for 7 days at 37^{o}C in 5% CO_2 without any disturbance to allow the migration of cells from explants (Figure 12). On day 7, explants were removed and medium was changed thereafter every 48–72 h. On 80% confluence, the cells were trypsinized (0.25% trypsin–EDTA) and subsequently passaged at the cell density of 5000 cells/cm^2.

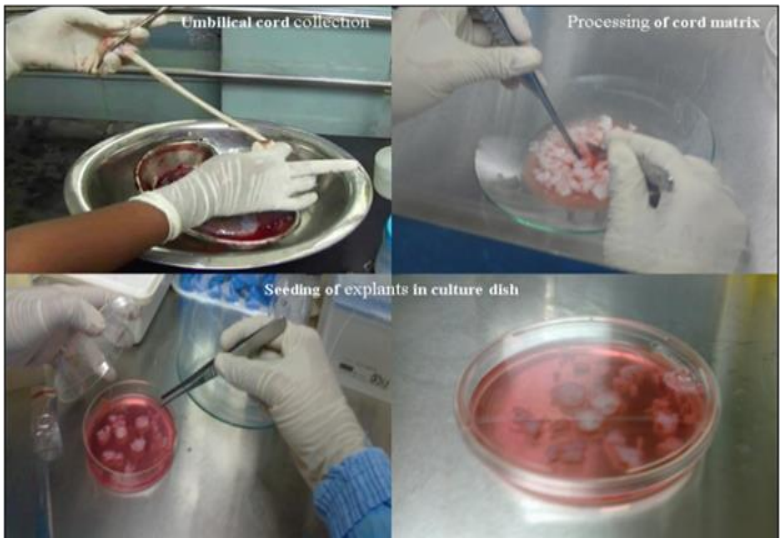


Figure 14. Isolation of Mesenchymal Stem Cells from Wharton's Jelly

A. Umbilical cord collection from operation theatre
B. Slicing of cord in sterile PBS into uniform, thin circular pieces
C. Explants were added in DMEM with 10% FBS
D. Explants cultured in Culture dish allowed to incubate at 37 ^{0}C, 5

3.3.4 Isolation of Mesenchymal Stem cells from Umbilical cord blood

Umbilical cord blood (n=10) was collected from the mothers undergoing caesarean section at RSRM hospital an obstetrics section of the Government Stanley Medical College and Hospital, Chennai and Processed by density gradient centrifugation method. The cord blood was collected into vacutainers containing EDTA (BD Biosciences, Gurgaon, Haryana, India). Mononuclear cells from the Umbilical cord blood were separated using Ficoll hypaque (Figure 13) (Sigma, St. Louis, MO, USA) gradient method and cultured using DMEM—low glucose (Sigma) with 20 % FBS (Sigma, F2442) at seeding density of 16 X10^4 cells/cm^2.Medium was changed every 48–72 h. On reaching 70 % confluency, the cells were passaged and reseeded in another flask.

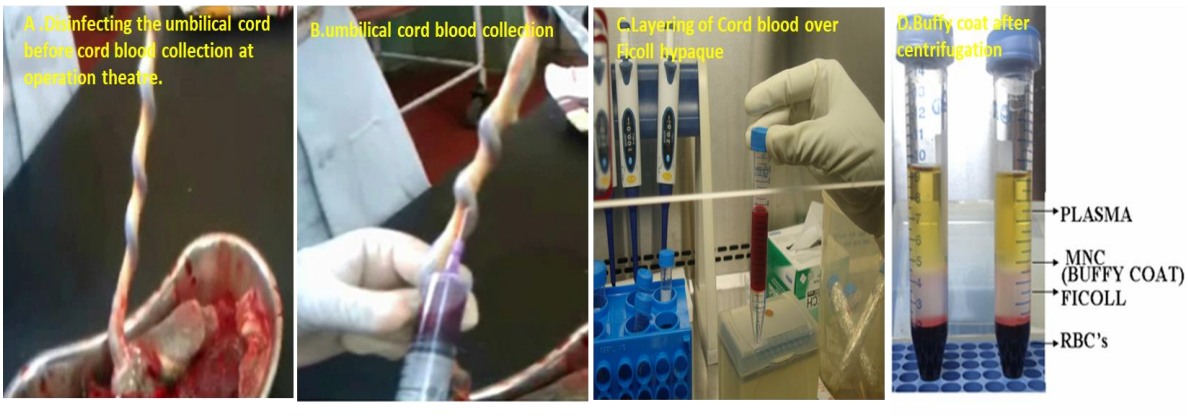


Figure 15. Isolation of Mesenchymal Stem cells from umbilical cord blood (UCB)

3.4 Expansion of MSCs -Trypsinization and Passaging

Trypsinization of MSCs was performed when the cell density in the flasks have reached 70- 80% confluency. The spent medium in the tissue culture flask was removed aseptically and the cells in the flask were rinsed gently with sterile PBS (Ca^{2+}-Mg^{2+} free) twice to remove the traces of serum. Further 2 ml (for T-25 culture flask) of 0.25% Trypsin-EDTA was added to the flask (note: Trypsin-EDTA should be pre-warmed to 37°C before addition) and exposed gently all over the surface of the culture flask. The flask with the trypsin-EDTA was incubated at 37ºC in the 5% CO_2 incubator for five minutes and viewed under the inverted microscope for the dislodging of the cells from the surface of the culture flask. To the cell suspension thus generated by trypsinization, equal amount of MSC culture medium with 10% fetal bovine serum was added to neutralize the activity of trypsin. The medium and the cell suspension were mixed evenly. The cell detachment was accentuated by gently using a sterile pipette wherein the cells medium was aspirated and blown out. The cells were then counted by hemocytometer and the viability was checked by trypan blue. The cells were reseeded at a density of 5000 cells/cm^2. Aliquots were taken at every passage for further analysis (Immunophenotyping and Cell viability check).

3.5. Characterization of Mesenchymal Stem Cells as per the criteria proposed by International Society for Cellular Therapy (ISCT)

3.5.1 Plastic adherence

The property of adherence to the Plastic surface under standard culture conditions was viewed under inverted microscope.

3.5.2 Immunophenotypic analysis by flow cytometry & Cell viability check by propidium iodide

MSCs from each source at passage 3 was characterized for the MSCs specific markers as per the criteria to define MSCs by International Society for Cellular Therapy (ISCT) (32) Cells obtained from the isolated source were trypsinized and washed with 1 X PBS. Cell density of 5 $X10^4$ cells/ml were stained with antibodies. Cells were stained with MSC positive markers like APC conjugated with anti-human CD73, FITC conjugated with anti-human CD90, PerCP Cy5.5 conjugated with anti-human CD105, and negative markers like FITC conjugated with anti-human CD14, CD45, PE conjugated with anti-human CD34 antibodies (BD Biosciences). Stained cells were taken for the flow cytometry analysis in a multi-colour flow cytometer (FACS ARIA II) using FACS DIVA software (Version 6.1.2). Compensation was performed to reduce the spectral spillage of fluorochromes and mean fluorescent intensity was calculated. Events recording more than 10,000 were considered significant for calculating the fluorescence intensity. Also cell viability was checked using propidium iodide dye.

3.5.3 Tri-lineage differentiation

Tri-lineage differentiation was performed for all the MSCs derived from all the sources as per the established protocol (30). MSCs from each source at passage 3 were subjected to osteogenic, adipogenic and chondrogenic differentiation conditions. Establishment of differentiation was confirmed by Alizarin red, Oil red O and Alcian blue staining respectively to fulfil the minimal criteria proposed by International Society for Cellular therapy (ISCT).

3.5.3.1 Osteogenic differentiation

MSCs of passage 3 were trypsinized into single cell suspension and the cells were counted manually using Neubauer chamber. The cells were reseeded at the cell density of 0.2-0.3x10^6 cells in a 6 well plate. Cells were fed with MSC growth medium for 48 hours. After 48 hours, MSC growth media replaced with osteogenic culture media (DMEM supplemented with10^{-8} M dexamethasone (Sigma, D4902), 10 mM b-glycerophosphate (Sigma, G9422), and 50 µg/ml ascorbic acid) (See appendix). The differentiation media was changed twice a week for 21 days at 37ºC in a 5% humidified CO_2 incubator. The Osteogenic differentiation was confirmed by Alizarin Red staining (Sigma Aldrich, USA) and quantitative analysis of osteogenic specific gene expression was carried out by qPCR using Applied Biosystems 7500 fast real time PCR instrument.

3.5.3.2 Adipogenic differentiation

MSCs of passage 3 were trypsinized into single cell suspension and the cells were counted manually using Neubauer chamber and plated at a density of 0.2 - 0.3 x10^6 cells in a 6 well plate. Cells were fed with MSC growth medium for 48 hours. After 48 hours MSC growth media was removed and fed with adipogenic culture media (DMEM supplemented with 10 mM of 3 isobutyl-1-methylxanthine (Sigma, 17018), 0.1 mM of indomethacin (Sigma, 17378), 10 µg/ml of insulin (Sigma, I6634), 10-6 dexamethasone) (see appendix).

Adipogenic differentiation media was changed twice a week for 21 days at 37ºC in a 5% humidified CO2 incubator. The adipocytes differentiation was confirmed by Oil Red O staining (Sigma Aldrich, USA) and quantitative analysis of adipogenic specific gene expression was carried out by qPCR using Applied Biosystems 7500 fast real time PCR instrument.

3.5.3.3. Chondrogenic differentiation

MSCs of passage 3 were trypsinized into single cell suspension and cells were manually counted using Neubaeur chamber and suspended the pellet in an appropriate volume of pre-warmed DMEM to generate a cell solution of 1.6 x 10^7 viable cells/ml. Micro mass cultures were generated by seeding 5-µl droplets of cell solution in the centre of 6 well plate. After cultivating micro mass cultures for 2 hours under high humidity conditions, warmed chondrogenesis media were added to culture vessels and incubated in 37°C incubator with 5% CO2. Media change was done twice a week up to 21 days. After 21 days, chondrogenic pellets were processed for Alcian Blue staining and quantitative analysis of chondrogenic specific gene expression was carried out by qPCR using Applied Biosystems 7500 fast real time PCR instrument.

In addition to the characterization of MSCs as per the minimal criteria proposed by ISCT, few other characterization studies such as BrdU cell proliferation, stemness gene expression, immunocytochemistry and immunofluorescence assays were also carried out for better understanding of MSCs

3.5.4 Proliferation studies

3.5.4.1 BrdU Cell proliferation assay

The Proliferation of MSCs from various sources were studied by Brdu cell proliferation assay. BrdU assay was performed to determine the rate of proliferation of MSCs by using the BrdU cell proliferation kit as per the kit protocol (Cell Signaling Technology,

Danvers, MA, USA, Cat No: 6813). Briefly, the cells were incubated for 24 h in culture medium containing BrdU, fixed, and stained with anti-BrdU detection antibody for 1 h following which HRP conjugated anti-mouse IgG antibody (both antibodies were included in the kit from Cell Signaling Technology) was added and incubated for 30 minutes. Finally, the cells were washed and 3, 3', 5, 5'- tetramethylbenzidine (TMB) substrate was added for 30 min after which stop solution was added and the readings were taken at the absorbance of 450 nm.

3.5.4.2 Population doubling time (PDT)

In order to calculate the population doubling time, MSCs were seeded at a density of 5000 cells/cm^2 in DMEM medium supplemented with 10% FBS. When the cells reached full confluency (100%), the flasks were trypsinized and viable count was recorded. Doubling time (Td) was determined using initial seeding density (N0), number of hours to reach confluency (T), and cell yield at confluency (Nt), using the standard Patterson Formula

Td = Tlg2/lg (Nt /N0) where T indicates incubation time; No refers to cell number after inoculation and Nt refers to cell number at T hour culture.

3.5.4.3 Growth curve analysis

MSCs were seeded in 6-well plates at the density of 1×10^4 cells/well and cultured in DMEM with 10% FBS. Duplicates of MSCs were harvested after trypsinisation every second day until day 12. Cell viability was checked by trypan blue dye exclusion method. The counts obtained were plotted to generate a growth curve. The experiments were performed in triplicates.

3.5.5 Stemness gene expression

MSCs from the various sources were trypsinized and the cell pellet was taken for RNA extraction. The RNA extraction procedure was performed using TRI reagent (Sigma, Cat No. T9424) and the cDNA conversion was carried out using the High-Capacity cDNA Reverse

Transcription Kit (AB Applied Bio systems, Bangalore, India, Cat No. 4368814) as per the manufacturer's protocol and PCR was carried out for the three stemness genes in a PCR apparatus (Gradient Master cycler, Eppendorf 5331). The master mix used for amplification was purchased from Thermo Scientific, Mumbai, India (Cat No. K0171). The details of the primers are tabulated as follows in the table 1

Table 1. Details of the primers for Stemness gene expression

S.No	Gene	Primer sequences
1	**Oct4**	
	Forward	5'-GAGAATTTGTTCCTGCAGTGC-3'
	Reverse	5'-GTTCCCAATTCCTTAGTG-3'
2	**Sox2**	
	Forward	5'GGCAGCTACAGCATGATGCAGGAGC3'
	Reverse	5-CTGGTCACATGGAGTTGTACTGCAGG3'
3	**Nanog**	
	Forward	5'-ACCTATGCCTGTGATTTGTGG-3'
	Reverse	5'-AAGAGTAGAGGCTGGGGTAGG-3'
4	**GAPDH**	
	Forward	5'-ATGTTCGTCATGGGTGTGAA-3'
	Reverse	5'-GTCTTCTGGGTGGCAGTGAT-3'

The PCR reaction protocol for Oct-4 and Nanog was 94 ^{0}C for 3 min, 94 ^{0}C for 30 s, 62^{0}C for 40 s, 72^0 for 45 s and 72 ^{0}C for 10 min for 35 cycles. For SOX-2, the PCR reaction protocol was 94 ^{0}C for 3 min, 94 ^{0}C for 45 s, 65 ^{0}C for 45 s, 72^0 for 1 min and 72 ^{0}C for 5 min for 35 cycles. One percent agarose gel was run and the bands were quantified by gel documentation (UVP Bio Imaging System, Upland, CA, USA, Vision works LS image acquisition and Analysis software).

3.5.6 Immunocytochemistry

The cultured cells were fixed with 4 % paraformaldehyde (Sigma, 6148), and permeabilised using Triton X-100 (Sigma, 93443). Immunohistochemical staining was performed for the markers of smooth muscle actin (SMA) and vimentin antibodies (BioGenex, Hyderabad, India). Immunocytochemical staining was performed as per the manufacturer's kit protocol (Biogenex—super sensitive polymer—HRP-IHC detection system).

3.5.7 Immunofluorescence

The cultured cells were fixed with 4 % paraformaldehyde and permeabilized using 0.25 % Triton X followed by blocking with 1 % BSA. After blocking, the cells were incubated with Anti-Vimentin antibody for overnight at 4 ^{0}C, followed by the addition of FITC-conjugated secondary antibody and incubated for 1 h. After washing the slides with PBS, the slides were mounted using glycerol and viewed under microscope.

3.5.8 Senescence assay – β- galactosidase assay

The cells seeded in the 6 well plate were washed with PBS and fixed with fixative solution provided in the kit (Senescence β-Galactosidase Staining kit- Cell signalling). The fixed cells were washed with PBS for two times and 1 ml of the β-Galactosidase Staining Solution was added to each well. The plate was sealed with parafilm and allowed to incubate overnight at 37^{0} C in dry incubator. The next day, cells were checked under the microscope (200X magnification) for the development of blue colour.

3.6 Immunological profiling of MSCs from various sources (AT, BM and WJ)

3.6.1. MSCs co-culture assay

Co-culture assay was carried out by culturing the MSCs derived from various sources (AT, BM & WJ) (3 samples from each source (3x3=9)) with the mononuclear cells in the ratio of 1:5 in the 6 well plate. Peripheral blood was collected from a healthy volunteer (3x3, n=9) (mean age 33±5; mean body weight 58±5) and mononuclear cells were isolated by Ficoll-gradient density method. MSCs of passage 3 (0.3 x 10^6) were seeded into each well containing 2.5 ml of RPMI medium (Sigma Aldrich, USA) containing 10% FBS (Invitrogen, Carlsbad, CA, USA) medium and incubated for 2 h at 37^0 C in 5%CO_2. The isolated MNCs were co-cultured with MSCs by seeding 1.5 x 10^6 MNCs onto the cultured MSCs and incubated at 37^0 C in 5% CO_2 for 3 days. The mitogen, phytohemagglutitin (PHA) (Sigma Aldrich, USA) was used for stimulation at the concentration of 10 µg/ml. The various conditions of the study are described below in the table 2 and figure 14.

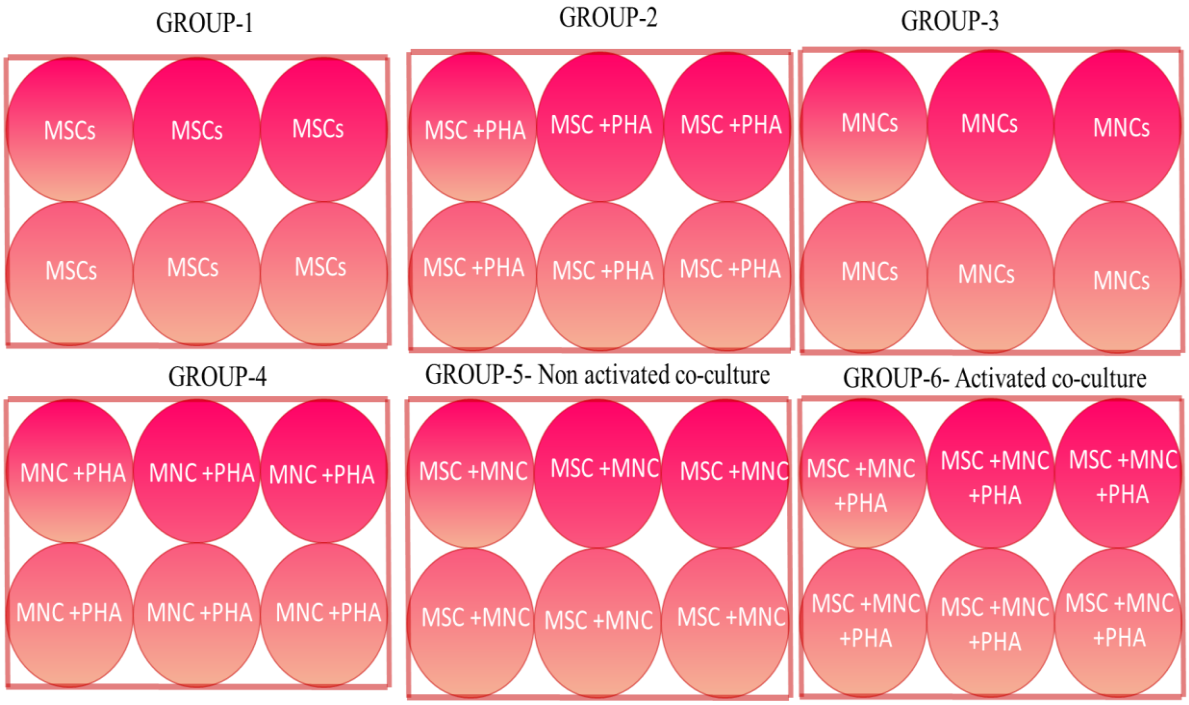


Figure 16. Schematic representation of the experimental design of the study

Table 2. Experimental design to investigate the interaction between MSCs (AT, BM and WJ) and MNCs at various conditions

S.No	Groups	No. of MSCs seeded in each well	No. of MNCs seeded in each well	Conc. of PHA	Study on MSCs	Study on MNCs
1	MSCs (Control)	0.3 $x10^6$	-	-	Cytokine profiling by gene array and Protein expression by ELISA	-
2	MNCs (Control)	-	1.5$x10^6$	-	-	CFSE Proliferation assay and T-reg expression by flow cytometry
3	MSC+PHA (PHA stimulated MSCs)	0.3 $x10^6$	-	10µg/ml	Cytokine profiling by gene array and Protein expression by ELISA	-
4	MNC+PHA (PHA stimulated MNCs)	-	1.5$x10^6$	10µg/ml	-	CFSE Proliferation assay and T-reg expression by flow cytometry
5	MSCs+MNCs co-culture group	0.3 $x10^6$	1.5$x10^6$	-	Cytokine profiling by gene array and Protein expression by ELISA	CFSE Proliferation assay and T-reg expression by flow cytometry
6	MSC+MNC+PHA (activated co-culture)	0.3 $x10^6$	1.5$x10^6$	10µg/ml	Cytokine profiling by gene array and Protein expression by ELISA	CFSE Proliferation assay and T-reg expression by flow cytometry

This above mentioned co-culture experiment was performed for all the three sources of MSCs (AT, BM and WJ) in order to understand the cultural behavioural aspects under mitogen stimulated conditions. After 3 days, MNCs in the suspension was removed from MSCs. MNCs of various groups such as activated MNCs (MNC+PHA), non-activated co-cultured MNCs (MSC+MNC) and activated co-cultured MNCs (MSC+MNC+PHA) were compared with unstimulated control MNCs for assessing their cell count, proliferation by CFSE staining and analysis of immune regulatory cell surface markers by flow cytometry. For the analysis of various immunomodulatory markers, gene and protein profiling was performed for the MSCs of the following groups: PHA activated MSCs (MSC+PHA), non-activated co-cultured MSCs (MSC+MNC) and activated co-cultured MSCs (MSC+MNC+PHA). MSCs of the above groups were compared with unstimulated control MSCs.

3.6.2 Morphological examination, Cell count, percentage distribution and cell proliferation of MNCs.

After 3 days of co-culture, MNCs of the groups mentioned in Table 1 were subjected to the following analysis: (a) Morphological analysis using Inverted Phase Contrast microscopy (b) Manual cell counting using hemocytometer (c) Determination of cell size and granularity based on FSC vs. SSC analysis using flow cytometry (d)Determination of cell proliferation by CFSE staining using flow cytometry.

To analyse the physical properties such as size and granularity, the cells (MNCs) were acquired for the forward scatter (FSC) and side scatter (SSC) parameters using flow cytometry. After FSC vs. SSC acquisition, the cells were grouped into different gates and analysed based on their size (i.e. the region from 0 – 50 was represented as P1, 50-100 as P2, 100-150 as P3, 150-200 as P4 and 200-250 as P5 respectively).

3.6.3 Cell proliferation by CFSE staining using flow cytometry

In addition to the cell counting, the proliferation of MNCs were assessed by Carboxy Fluorescein Diacetate Succinimidyl Ester assay by following the manufacturer's protocol (CFSE, Invitrogen Cat No: 34554). Briefly, MNCs required for this study were labelled with 10 µM CFSE in pre-warmed PBS at the concentration of $1x10^6$ cells / ml. The labelled cells were then allowed to incubate at 37^0C for 20 minutes in the dark. After incubation, the labelled cells were washed twice with fresh co-culture medium (RPMI containing 10% FBS) to absorb and remove any unbound dye present in the tube. CFSE labelled MNCs were then co-cultured with MSCs (AT, BM, WJ) of various sources with and without PHA stimulation for 3 days. MNCs stimulated with and without PHA were used as a positive and negative control. After 3 days, MNCs were collected from the suspension and assessed for its proliferation using flow cytometry (BD FACS Canto II) and analysed by Flowjo software.

3.6.4 Study of immunoregulatory effect of MSCs on MNCs through immunophenotypic analysis using flow cytometry

The expression of activated markers was studied in the MNCs of the following groups Group 1: MNCs alone (used as a control); Group 2: Activated MNCs (MNCs + PHA); Group 3: MNCs of non-activated co-culture (MSC + MNC); Group 4: MNCs of activated co-culture (MSC+MNC+PHA) respectively.

Briefly, MNCs of the above four groups were removed and washed twice with 1 X PBS. The washed cells ($5x10^4$ cells / ml) were stained with APC conjugated anti-human CD4, PE conjugated anti-human CD25, FITC conjugated anti-human CD45RA and PE conjugated anti-human CD127 antibodies (BD biosciences). Stained cells were acquired using a flow cytometer (FACS ARIA II) and analysed using FACS DIVA software (Version 6.1.2). Events

recording more than 10,000 were considered significant for calculating the fluorescence intensity.

3.6.5 Cytokine Profiling of MSCs under non-activated vs. activated state

3.6.5.1 RNA extraction and custom RT^2 profiler PCR array

A novel custom-made medium throughput PCR array was used to characterize the immunomodulatory properties of MSCs derived from the three sources by mRNA expression specific to selected pro and anti-inflammatory markers. The major pro-inflammatory and anti-inflammatory/immunosuppressive cytokines involved in inflammatory response and tissue regeneration were selected and included in the custom array (Qiagen Customized Cat No: CLAH2778K). The array is a 96 well microplate that contains qPCR primer for 12 genes related to immunomodulation, a housekeeping gene (β- actin), and a set of experimental/ assay controls to check the quality of cDNA (positive PCR control, reverse transcription control and genomic DNA contamination control). RNA was extracted by RNeasy mini kit and cDNA synthesis was performed using RT^2 First strand kit provided along with custom-made Qiagen cytokine array kit according to the manufacturer's instructions. Briefly, RNA was extracted from $1x10^{6}$ cells of MSCs (control, PHA activated, non-activated co-culture and activated co-culture (Table 1)) from each source (AT, BM and WJ). The RNA concentration and integrity was determined using nanodrop. The quality of RNA was assessed at a wavelength of 260/280 nm. qRT PCR was performed using Custom RT^2 Profiler PCR Array with RT^2 SYBR® Green qPCR Master mix on ABI 7500 fast. Array results were analysed using web based software provided by the manufacturer (Qiagen). Details of the list of customized cytokines for the PCR array are provided in the following table 3

Table 3. List of cytokines customized for RT^2 Profiler custom PCR array

S.No	Cytokines	Gene symbol	Gene bank
1.	Prostaglandin-endoperoxide synthase 2	PTGS2	NM_000963
2.	Transforming growth factor beta 2	TGFB2	NM_003238
3.	Interferon, gamma	IFNG	NM_000619
4.	Interleukin 10	IL-10	NM_000572
5.	Interleukin 6 (interferon, beta 2)	IL-6	NM_000600
6.	Interleukin 4	IL-4	NM_000589
7.	Platelet-derived growth factor beta polypeptide	PDGFB	NM_002608
8.	Human Leukocyte antigen G	HLA-G	NM_002127
9.	Indolamine 2,3 dioxygenase 1	IDO	NM_002164
10.	Vascular endothelial growth factor	VEGF	NM_001025366
11.	Tumour Necrosis factor	TNF-A	NM_000594
12.	Nitric oxide synthase 2, inducible	NOS2	NM_000625
13	B-Actin	ACTB	NM_001101
14	Human Genomic DNA contamination	HGDC	Control
15	Positive PCR control	PPC	Control
16	Reverse transcription control	RTC	Control

Due to the high sensitive nature of the assay and presence of various groups in the experiment, our study design reflects a pilot-scale rather than a clinical trial scale. Therefore, we obtained minimum biological replicates (3 different samples for each of the three sources (AT, BM and WJ,)) with technical duplicates to allow for generating statistical power in our inference.

3.6.6 Construction of heatmap graph and Cluster gram analysis in gene expression –

The unsupervised hierarchical cluster analysis and heatmap were constructed using the online Custom RT^2 Profiler ^{TM} PCR Array software provided by Qiagen. mRNA expression profile of the cytokines was represented as a heatmap and genes were clustered according to their expression pattern.

3.7 Quantitative protein expression studies by solid-phase sandwich Enzyme-Linked Immunosorbent Assay (ELISA)

In addition to the gene expression, protein expression for the adherent MSCs (AT, BM and WJ) of the above mentioned groups detailed in Table 1 were assessed by solid-phase sandwich ELISA. Briefly, MSCs (AT, BM and WJ) were trypsinized after 3 days of co-culture and the cell pellet was lysed using RIPA buffer supplemented with protease inhibitor cocktail (10 µg/ml). To ensure complete lysis of the cell pellet, sonication was carried out by pulsing for 10 S at frequency of 20 kHz. The cell lysate was then centrifuged at 4^0C for 45 mins at 14,000 rpm and the supernatants were collected. The total protein concentration was determined by Bradford protein estimation method. Total protein (50µg) was used to determine the expression of immunomodulatory molecules like IDO, PTGS-2 and VEGF. The concentration of immunomodulatory molecules was quantified by ELISA following the manufacturer's protocol for the respective kits (Human IDO ELISA Kit - Cat. No EH246RB, Human VEGF-A ELISA Kit – Cat No. BMS277 and Human COX-2 ELISA Kit – Cat No EH125RB, Invitrogen, USA).

Briefly, the cell lysate of all the samples (50µg of total protein diluted in diluent provided in the kit) and standards were added to the specific antibody (IDO, VEGF and COX-2) pre-coated wells and incubated for 2.5 h at 37^0C. This was followed by incubation for an hour with biotin conjugate which binds to the primary antibody. Subsequently, a secondary antibody streptavidin HRP conjugate which binds with biotin conjugate was added and incubated for 45 minutes. Unbound antibodies were removed by washing at each step. Finally, TMB substrate solution was added to the plates and incubated for 30 minutes and observed for color development to determine the amount of protein present in the sample. The reaction was terminated by the addition of stop solution and absorbance was recorded at 450 nm using ELISA reader (Robonik readwell). The concentration of the specific proteins were calculated with their respective standards. Finally, results were expressed as protein expression (IDO, VEGF and COX-2) in ng or pg per milligram of the total protein**.**

3.8 Co-culturing of chondrogenic differentiated WJ-MSCs with MNCs in non-activated and PHA activated condition

Based on the outcome of the gene array study, an attempt was made to co-culture the chondrocyte differentiated cells with MNCs (±PHA stimulation) in WJ source alone. WJ-MSCs (passage 3) were differentiated into chondrocytes by micro mass culture methods as per our established protocol mentioned above. After ensuring complete differentiation of MSCs into chondrocytes, MNCs were seeded onto the differentiated chondrocytes. Phytohemagglutitin (PHA) was used for stimulation at the concentration of 10µg/ml and the co-culture system was incubated at 37^0 C in 5% CO_2, for 3 days. The various conditions of the study were grouped as follows

Group 1: Chondrocytes alone (Control);

Group 2: MNCs alone (Control);

Group 3: PHA stimulated chondrocytes (Chondrocytes+ PHA)

Group 4: PHA stimulated MNCs (MNCs +PHA);

Group 5: Chondrocytes co-cultured with MNCs (Chondrocytes + MNC)

Group6: Chondrocytes co-cultured with activated MNCs (Chondrocytes+MNC+PHA) respectively.

The above grouped study was performed in WJ- MSCs differentiated into chondrocytes which was compared with similar co-cultured groups of undifferentiated WJ-MSCs in order to understand the cultural behavioural aspects of differentiated and undifferentiated cells under mitogen stimulated conditions. After 3 days, MNCs in the suspension was removed. Morphological changes were examined through inverted microscope. Cytokines profiling was performed using RT^2 Customized gene array. Change in the expression of cytokines between two groups were compared and analysed.

3.9 Statistical analysis

All experiments were performed in biological triplicates (n=3). The statistical analysis was performed by one-way and two-way ANOVA test using GraphPadPrism version 6.04 for Windows, Graph Pad Software, La Jolla, California, USA. Tukey's multiple comparison test and sidak's multiple comparison test were used for multiple comparisons, for all statistical analysis, $p<0.05$ was considered as significant (*$p< 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p<0.0001$).

CHAPTER - 4

RESULTS

4.1 Isolation of Mesenchymal Stem Cells

Mesenchymal Stem Cells were successfully isolated from fetal as well as adult sources. MSCs were isolated from adipose tissue (AT) by enzymatic method (Collagen digestion method), Bone marrow (BM) and Umbilical cord blood (UCB) by density gradient centrifugation, and Wharton's jelly (WJ) by explant method. MSCs derived from AT, BM and WJ were able to expand in the standard culture conditions of DMEM supplemented with 10% FBS whereas MSCs from UCB were not able to proliferate in the same culture conditions. Therefore, an attempt was made by increasing the percentage of the supplement (FBS) from 10% to 20% in the primary culture of UCB-MSCs in order to investigate the proliferation rate. Though, the growth of UCB-MSCs was better in 20% DMEM medium, it was difficult to expand the same in all the samples. Also it was not comparable with MSCs of other sources (AT, BM& UCM/WJ) for which 10% DMEM is sufficient for expansion. Therefore, the present study was confined to three sources (AT, BM & UCM/WJ) to investigate their immunological properties.

4.1.1. Morphological observation of MSCs derived from various sources

MSCs derived from all the sources showed spindle morphology when observed under the inverted microscope. The morphological observation of MSCs from various sources on different days are described in the figure 17. During initial days, heterogeneous population of cells were observed in all the sources. As the passage number increases, uniform spindle morphology of cells were observed in all the sources. But the time to reach confluency in MSCs derived from various sources varied. Bone marrow derived MSCs reached confluency in 12 days whereas MSCs from Wharton's jelly, Adipose tissue and Umbilical cord blood reached confluency in 15, 14 and 23 days respectively.

Figure 17. Morphological observation of MSCs from various sources

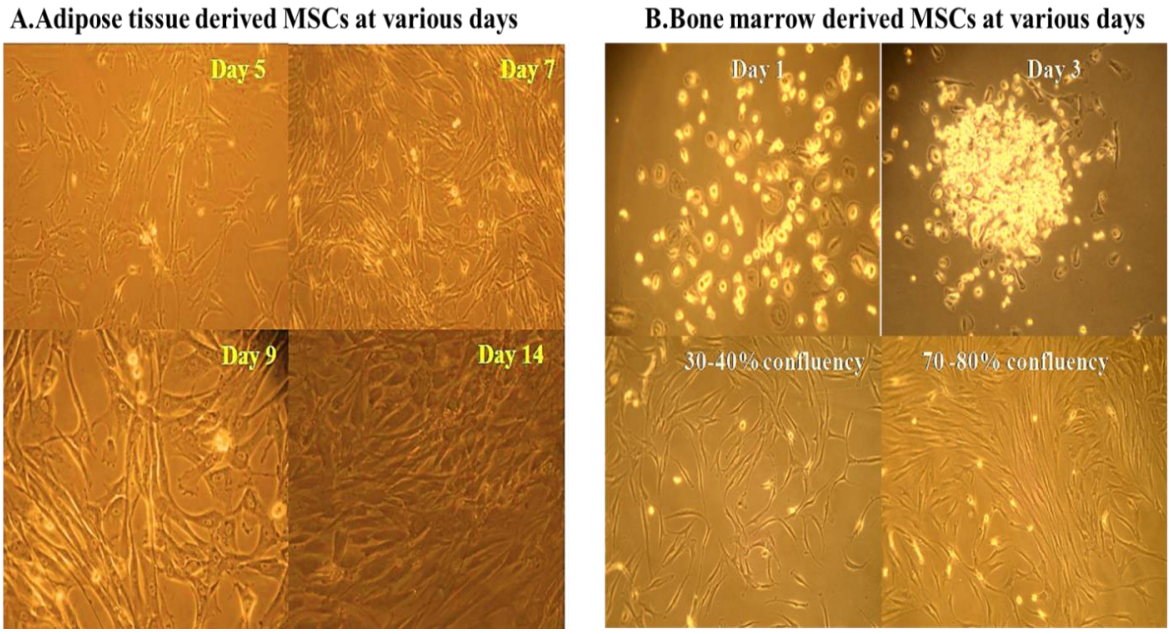


Figure 17 A. *Bright field images of Elongated Spindle and fibroblastoid structure of AT-MSCs at various days of passage P0 such as 30% confluency was observed on day 5, 50% confluency was observed on day 7, 60% confluency was observed on day 9 and 80-90% confluency was observed on day 14 (Magnification-10X).*

Figure 17 B. *Bright field images of BM-MSCs at Passage P0 such as 30-40% confluency was observed on Day 5 and 70-80% confluency was observed on day 10 (Magnification-10X).*

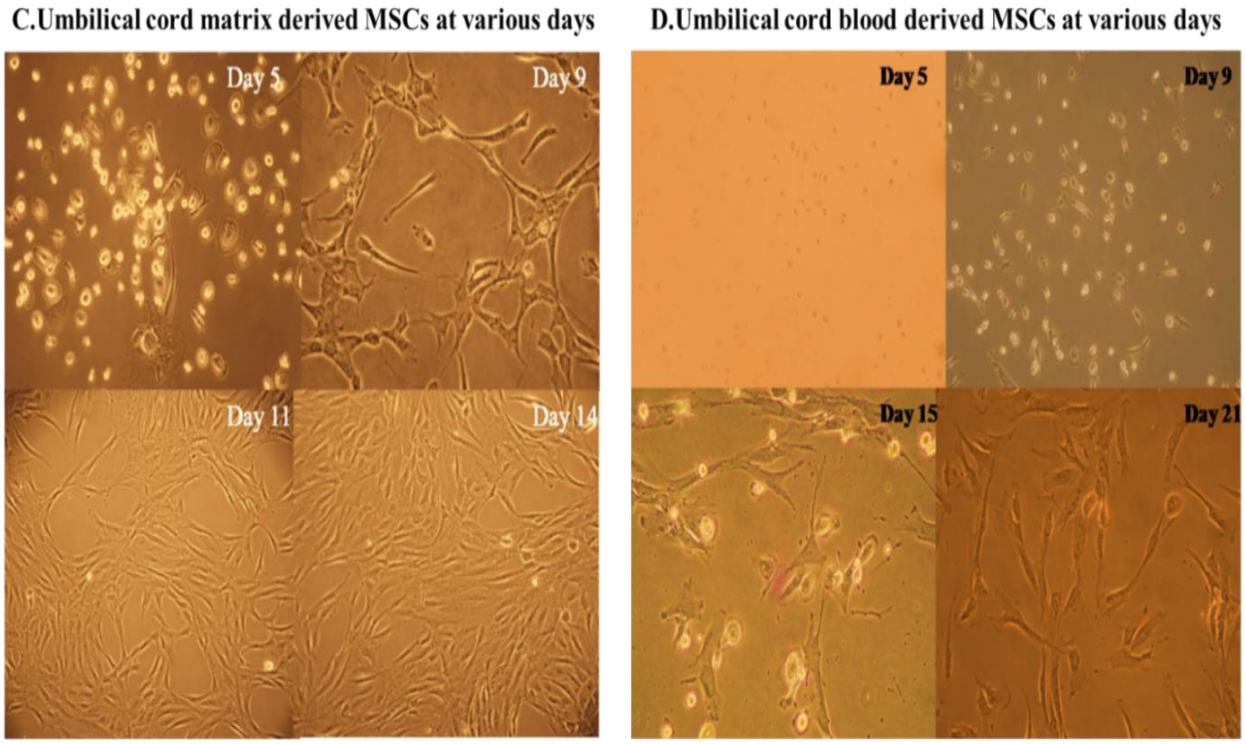


Figure 17 C. *WJ-MSCs - On day 5, heterogeneous population of cells were observed, on day 9, 30% confluency was observed, on day 11, 50 % confluency was observed and on day 14, more than 70% confluency was observed.*

Figure 17 D. *UCB-MSCs – Heterogeneous population of cells containing both round and spindle cells are observed till day 9. 40-50 % confluency was observed on day 15 and more than 60 % confluency on day 21.*

4.1.2 Expansion of MSCs -Trypsinization and Passaging

On 80 % confluence, the cells were trypsinized (0.25 % trypsin) and subsequently passaged at the cell density of 5000 cells/cm^2. MSCs of passage 3 were used for further studies. Morphology of passage 3 MSCs of all the sources with uniform spindle morphology are shown in the figure 18.

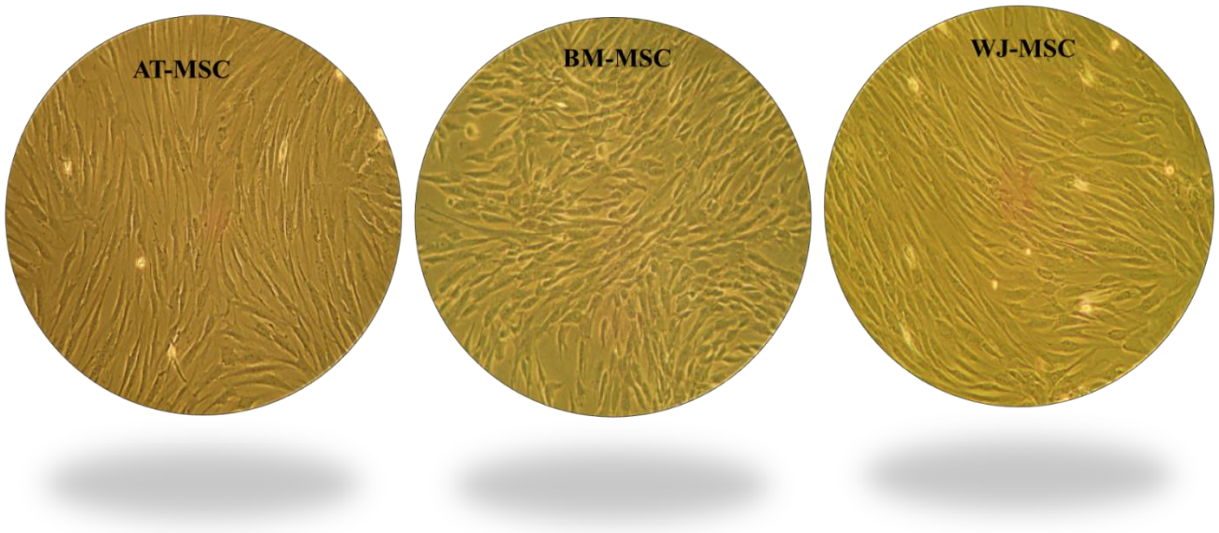


Figure 18. Morphology of MSCs derived from AT, BM and WJ at passage 3

4.2 Characterization of MSCs as per the criteria proposed by International Society for Cellular Therapy (ISCT)

4.2.1 Plastic adherence

Mesenchymal Stem Cells isolated from all the sources (AT, BM, WJ) were adherent to plastic surface under standard tissue culture conditions (DMEM with 10% FBS).

4.2.2 Immunophenotypic analysis by flow cytometry

The expression pattern of positive (CD 73, CD90, and CD105) and negative (CD45, CD14 and CD34) markers of MSCs from AT, BM, and WJ were determined using flow cytometry (FACS ARIA II) and analysed by FACS DIVA software.

A. Immunophenotypic analysis in AT-MSCs

MSCs derived from Adipose tissue at passage 3 showed positive expression for Mesenchymal stem cell markers such as CD 73 (98.6%), CD90 (93.7%) and CD105 (95.4%) whereas lower expression of hematopoietic markers CD14 (2.8%), CD34 (1.4%) and CD45 (2.3%). The expression pattern observed are shown in the figure 19A.

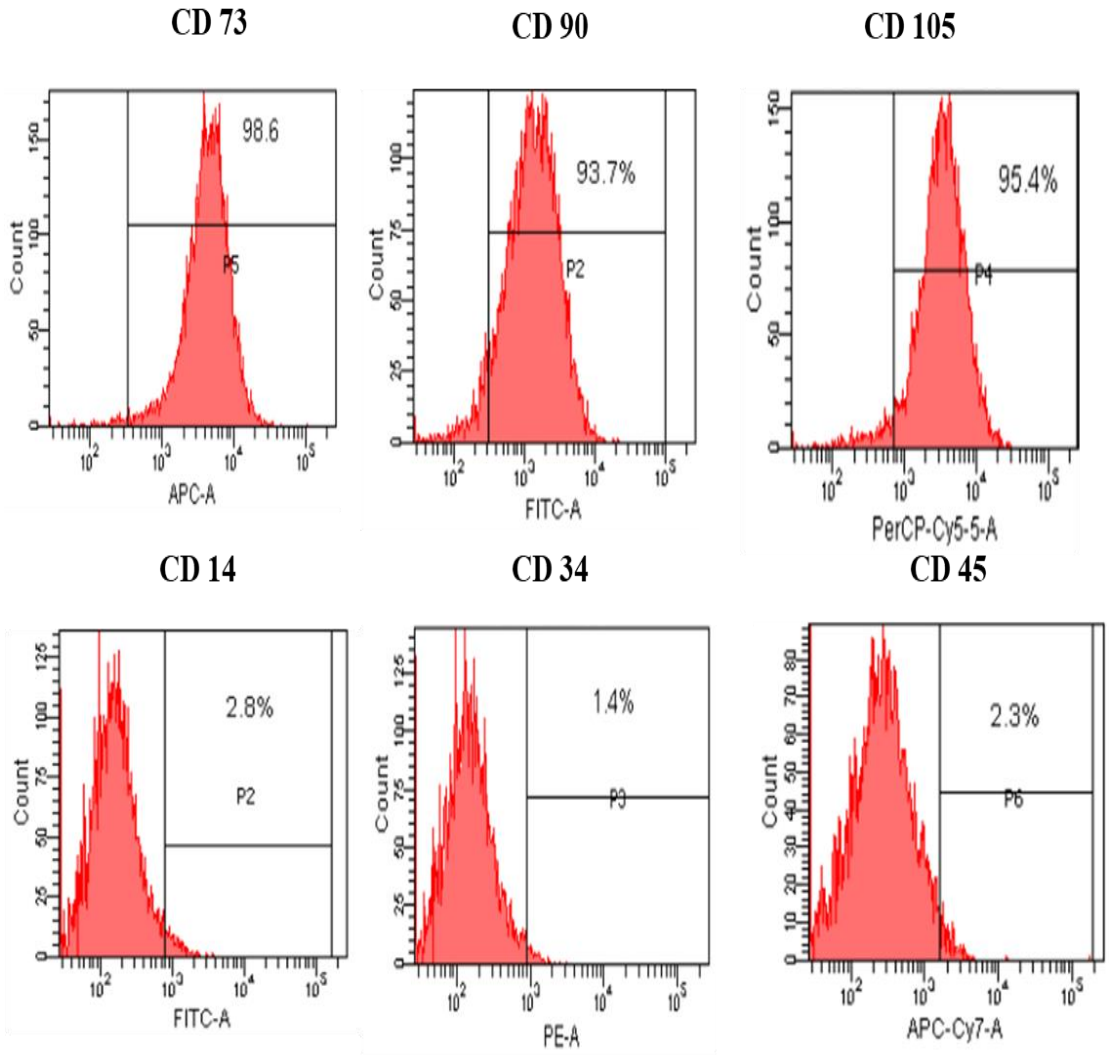


Figure 19A. Immunophenotypic analysis for AT-MSCs

B. Immunophenotypic analysis in BM-MSCs

MSCs derived from Bone marrow at passage 3 showed positive expression for Mesenchymal stem cell markers such as CD 73 (95.4%), CD90 (98%) and CD105 (99.9%) whereas lower expression of hematopoietic markers CD14 (0.6%), CD34 (0.5%) and CD45 (0.2%). The expression pattern observed are shown in the figure 19B.

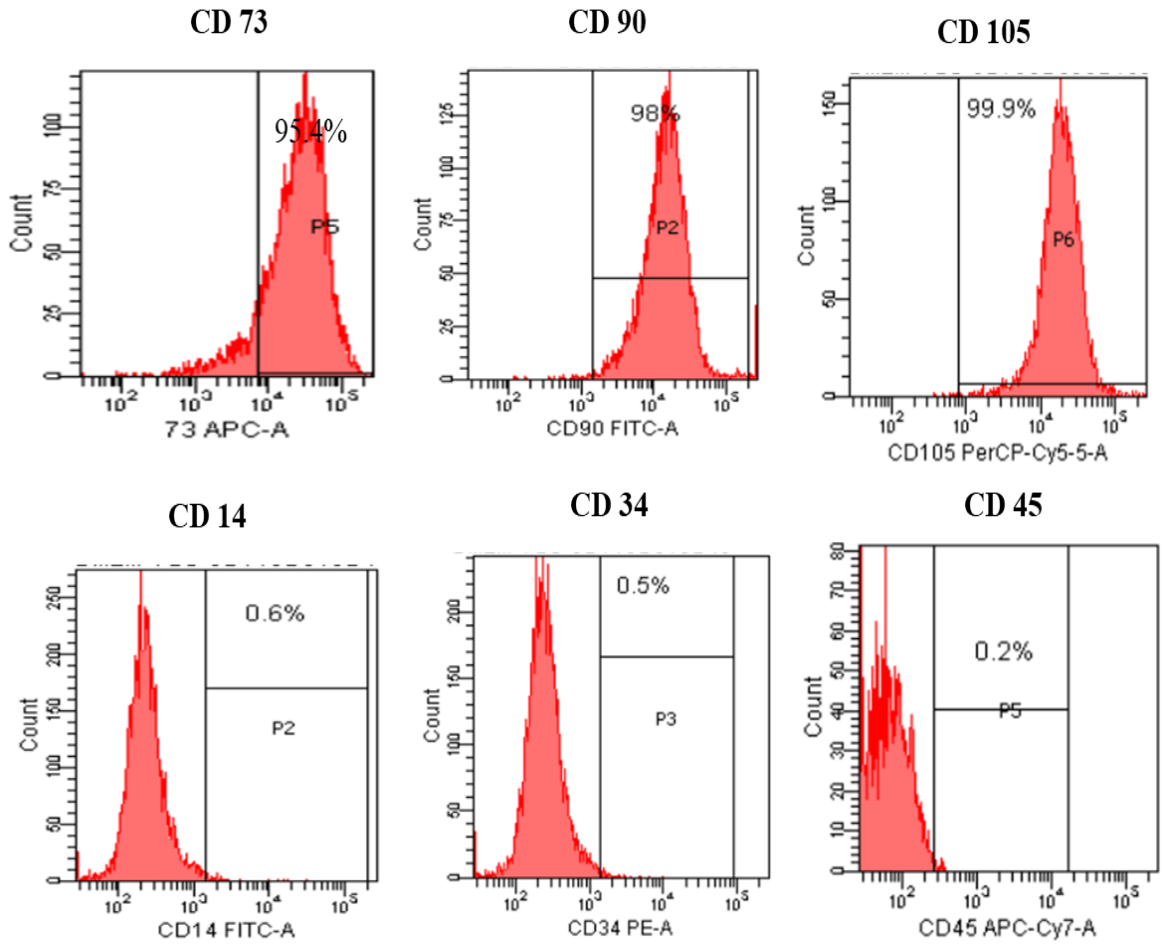


Figure 19B. Immunophenotypic analysis for BM-MSC

C. Immunophenotypic analysis in WJ-MSCs

MSCs derived from Wharton's jelly at passage 3 showed positive expression for Mesenchymal stem cell markers such as CD 73 (95.6%), CD90 (95.5%) and CD105 (95.4%) whereas lower expression of hematopoietic markers CD14 (4.0%), CD34 (2.9%) and CD45 (4.3%). The expression pattern observed are shown in the figure 19C.

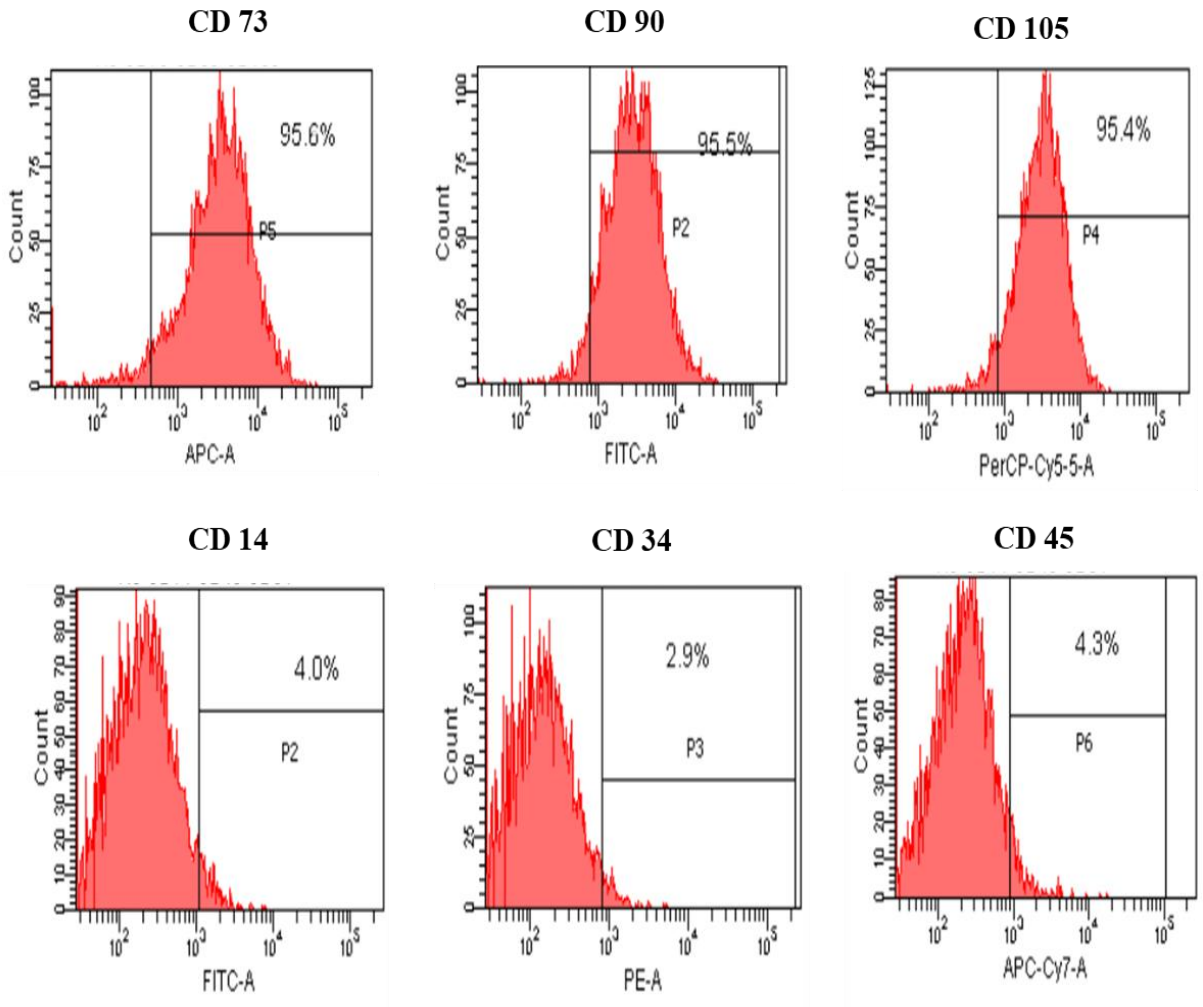


Figure 19C. Immunophenotypic analysis for WJ-MSCs

Thus, the MSCs derived from Adipose tissue, Bone marrow and Wharton's jelly were expressing the characteristic marker profiling as per the minimum criteria proposed by Dominic et al.

4.2.3 Tri-lineage differentiation

According to the minimal criteria proposed by International Society for Cellular therapy (ISCT), MSCs derived from all the sources were able to differentiate into tri-lineage differentiation but this differentiation ability varied from source to source.

4.2.3.1 Osteogenic differentiation

Osteogenic differentiation study was carried out to determine the calcium deposition in osteocytes which was confirmed by alizarin red staining. In the Osteogenic differentiation, BM-MSCs showed better osteogenic differentiation potential followed by Wharton's jelly /Umbilical cord matrix and then adipose tissue (BM>WJ>AT) as shown in the figure 20A.

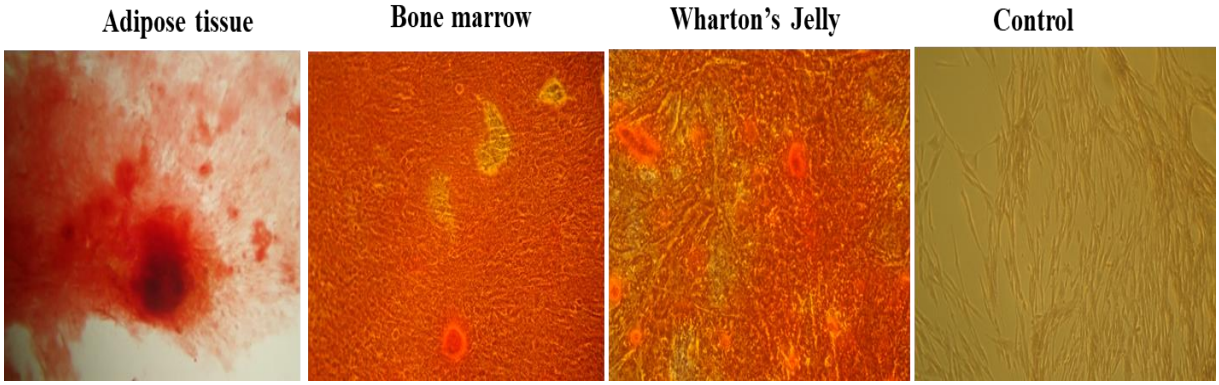


Figure 20A. Osteogenic differentiation confirmed by Alizarin red staining

4.2.3.2 Adipogenic differentiation

Lipid vacuole formation after adipogenic differentiation was confirmed by Oil Red 'O' staining. In adipogenic differentiation, AT-MSCs exhibited better differentiation compared to WJ-MSCs & BM-MSCs. (AT>WJ>BM) as shown in the figure 20B.

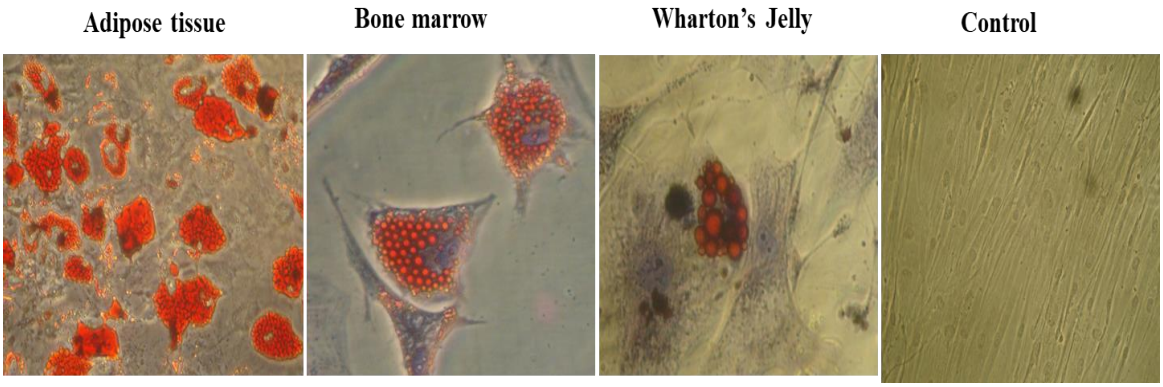


Figure 20B. Adipocytes confirmed by Oil Red 'O' staining

4.2.3.3 Chondrogenic differentiation

In Chondrogenic differentiation, MSCs derived from all the three sources differentiated into chondrocytes which was confirmed by alcian blue staining (Figure 20C).

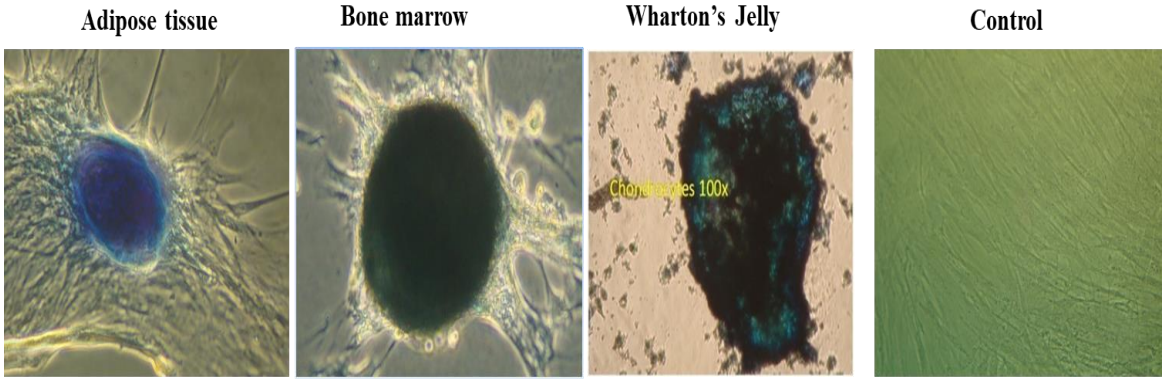


Figure 20C. Chondrocytes confirmed by alcian blue staining

4.3 Proliferation studies

4.3.1 BrdU assay

In the cell proliferation assay, MSCs derived from the AT, BM and WJ source were treated with labelling medium that contains BrdU, pyrimidine analog which is incorporated in place of thymidine into the newly synthesized DNA of the proliferating cells. The magnitude of the absorbance which is directly proportional to the cell proliferation was calculated for MSCs derived from all the three sources.

In the proliferation assay, both BM and WJ showed higher proliferation rate when compared to AT source. The main limitation with BM and AT source is that the proliferation rate decreases with age of the donor. On the other hand, Umbilical Cord a waste tissue after birth is an easily available large volume source that engages a non-invasive, easy and painless collection process. It has a fairly good proliferative potential equal to BM in the initial passages. The long term expansion of MSCs of WJ source was better than BM source. Therefore, amongst the various sources of MSCs, no significant difference between BM and WJ source was observed. However, significant difference was observed for AT when compared against BM-MSCs and WJ-MSCs (Figure 21A).

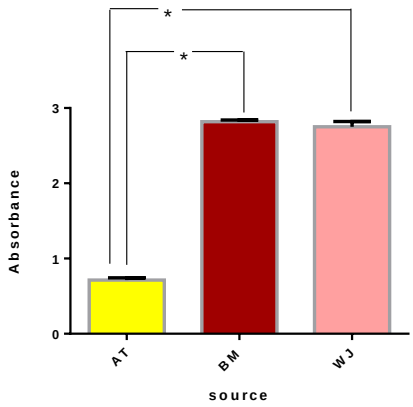


Figure 21A. BrdU cell proliferation assay

4.3.2 Population doubling time

The doubling time of MSCs from AT, BM and WJ MSCs were calculated by standard Patterson formula. The doubling time of AT-MSCs, BM-MSCs and WJ-MSCs are 45 h, 33 h and 32 h in P1, 45.5h, 29.5h and 25 h in P2, 47h,29h and 23.5h in P3, 53.5h,31.5h and 31.5 h in P4, 55.5h,34.5h and 36.5h in P5 respectively. Doubling time was high for AT-MSCs and showed significant difference compared with BM-MSCs and WJ-MSCs. There is no significant difference in doubling time between BM and WJ-MSCs from passage 1 to 5. These results were corroborated with BrdU cell proliferation in which WJ and BM showed higher

proliferation rate compared to AT source. The doubling time decreases as the growth rate or proliferation increases. Hence WJ and BM showed better proliferation rate compared to AT source (Figure21B).

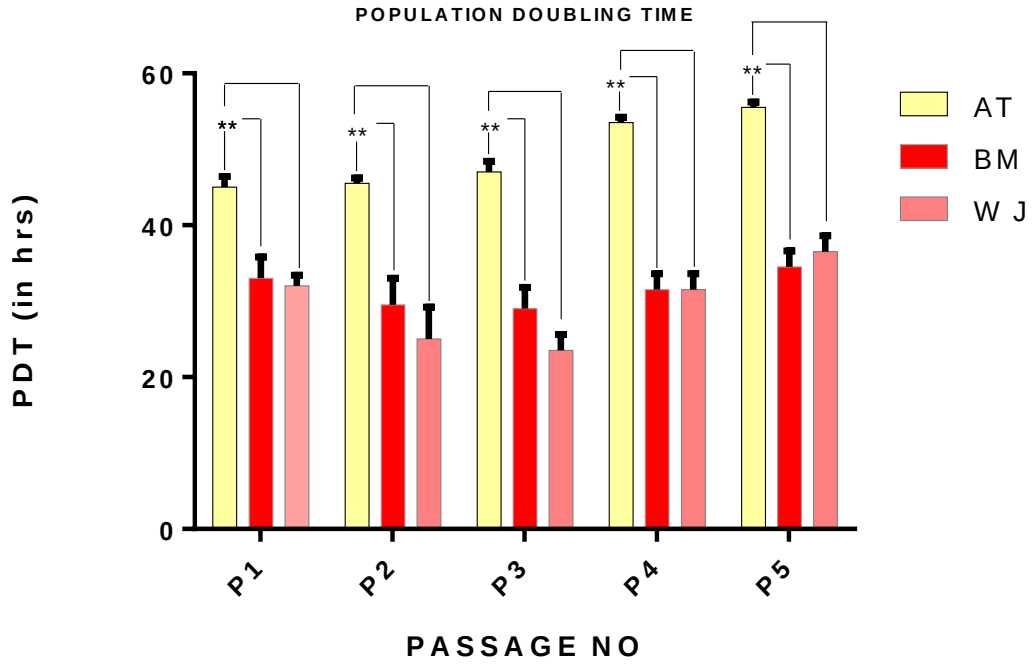


Figure 21B. Population doubling time of MSCs derived from AT, BM and WJ

4.3.3 Growth curve analysis

Growth kinetics of MSCs derived from various sources such as AT, BM and WJ are illustrated in figure. Briefly, the lag phase for AT and BM was maintained between Day 2 to 5 followed by the logarithmic phase/ exponential phase between day 5 to 9 and then immediately it reaches the decline phase. In WJ source, the lag phase was maintained between day 0 to 3 followed by logarithmic phase or exponential phase between day 3 to 7 and it reaches the plateau and it was maintained between day 7 to 9 and then decline phase started (Figure 21C)

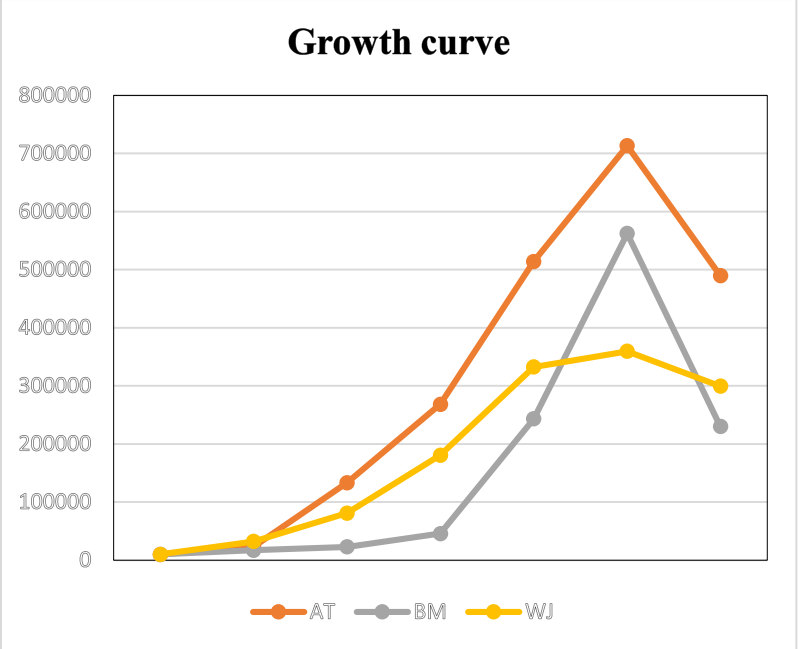


Figure 21C. Growth curve analysis of MSCs derived from AT, BM and WJ

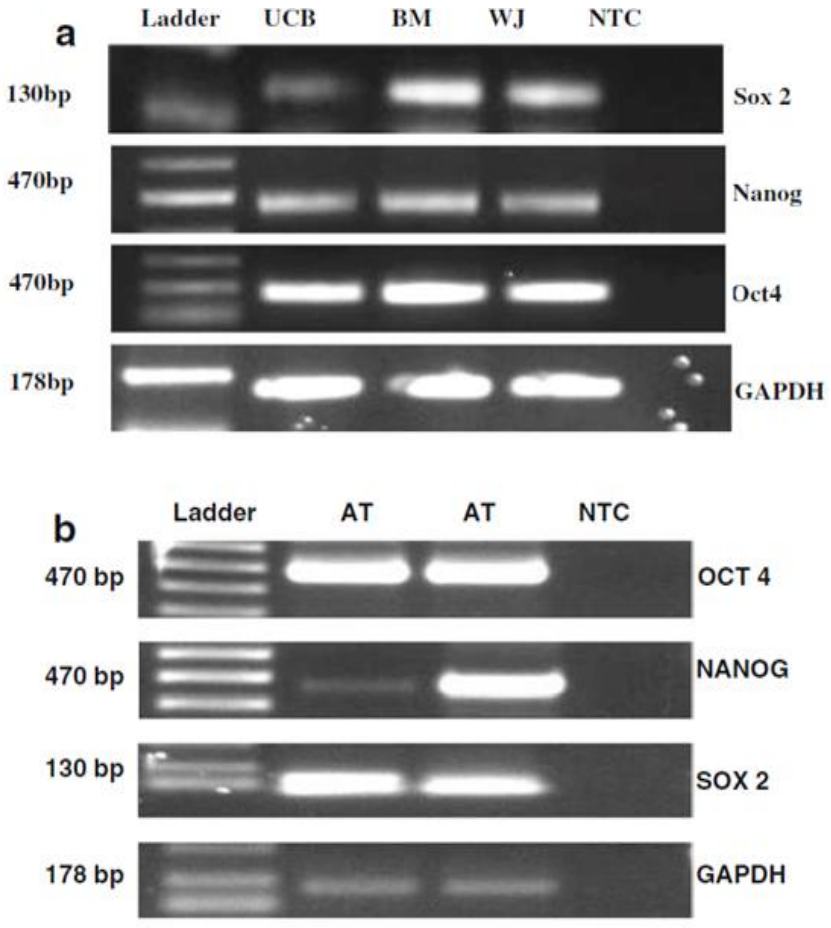


Figure 22. Gene expression of stemness markers in MSC

4.4 Stemness gene expression

In order to characterise the MSCs based on their expression of pluripotency markers, RT-PCR was performed. MSCs derived from all the sources in this study expressed Oct-4, Nanog and Sox-2. Housekeeping gene Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used for normalization of gene expression. UCB exhibited lower expression of all the three pluripotent markers compared to the other three (AT, BM and WJ) sources (Figure 22).

4.5 Immunocytochemical and Immunofluorescence staining

4.5.1 Immunocytochemistry

MSCs derived from AT, BM, WJ and UCB showed positive expression for Vimentin and SMA by Immunocytochemistry (Figure 23 A).

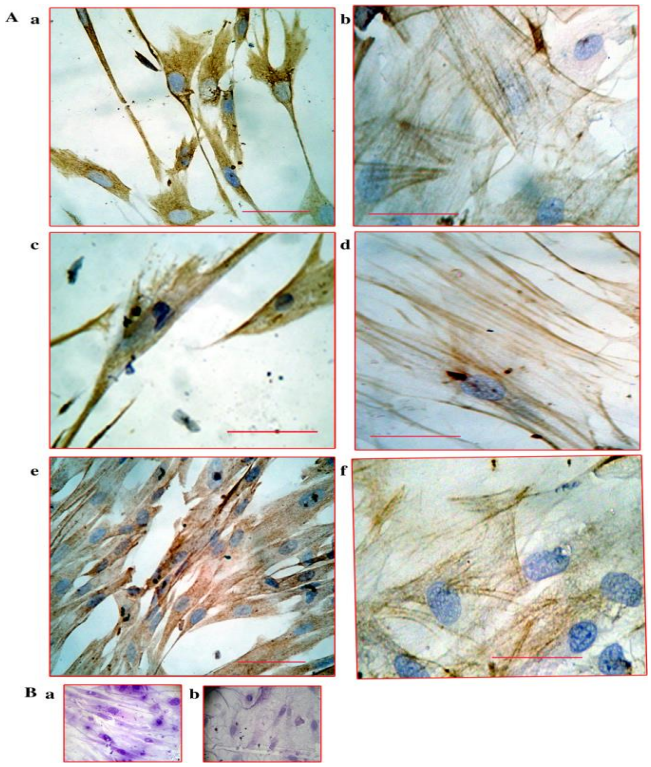


Figure 23A. Positive staining of vimentin and SMA was observed in BM (a, b), WJ (c, d) and UCB (e, f) derived MSCs respectively. B Negative controls without antibody (a) and Buccal cells (b). Bar 50 lm

4.5.2 Immunofluorescence

Immunofluorescence analysis of vimentin expression in BM-MSCs was done to further characterize MSCs. a) Phase contrast image of MSCs b) Fluorescence imaging showing vimentin expression in BM-MSCs c) Overlay of phase contrast and fluorescence imaging (Figure 23B).

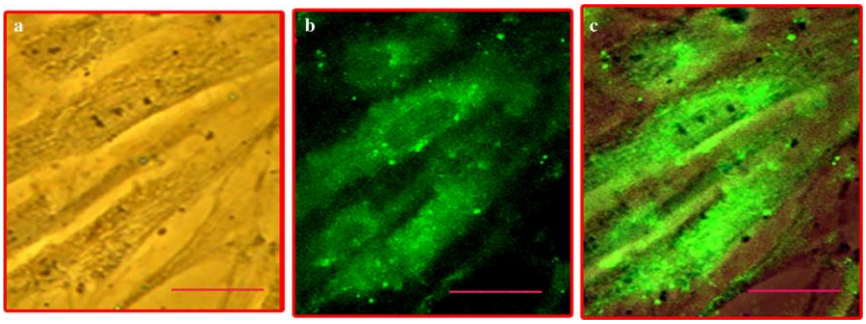


Figure 23 B. Immunofluorescence

4.6 Senescence assay – β- galactosidase assay

In the senescence study, AT-MSCs reached senescence at passage P11, BM-MSCs at passage P14, UCB- MSCs at P4 whereas WJ-MSCs were highly proliferative and did not attain senescence until P16 (Figure 24).

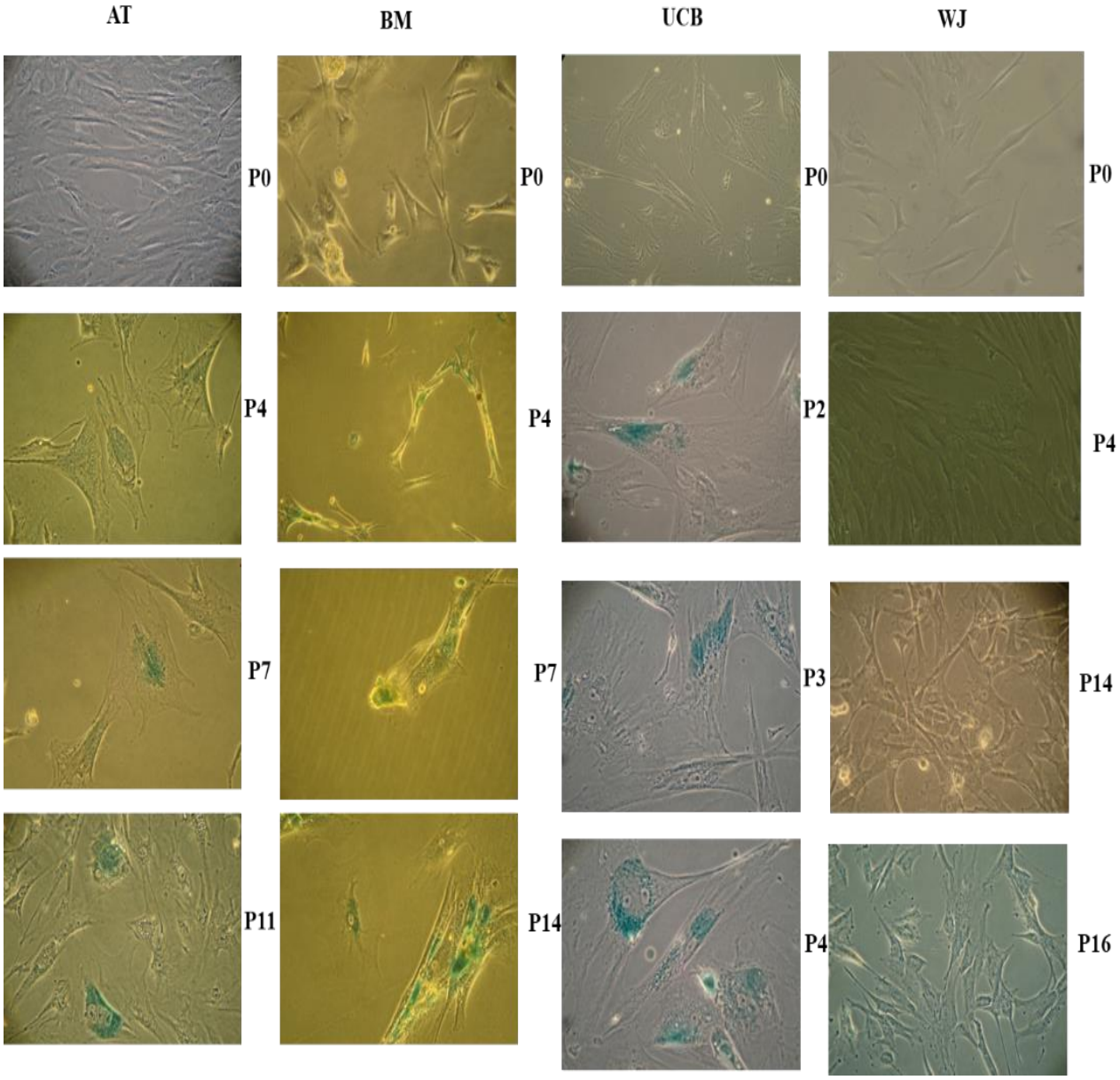


Figure 24. Senescence assay performed in MSCs derived from various sources

4.7. Immunological Profiling of MSCs using co-culture assay

4.7.1 Influence of MSCs on MNC size in co-cultured system with and without PHA stimulation

4.7.1.1 Morphological examination

After the 3 days of co-culture, the morphology of the cells was observed using inverted microscope. Morphological analysis carried out in various groups are MSCs derived from AT, BM and WJ (MSCs alone), PHA treated MSCs (MSCs+PHA) of all the three sources, MNCs, PHA treated MNCs, and co-cultured MSCs and MNCs in the presence and absence of PHA stimulation (MSC+MNC and MSC+MNC+PHA).

In the morphological examination, as shown in Figure 25 (A, B, C), AT, BM and WJ derived MSCs (controls) and PHA stimulated MSCs showed typical spindle morphology (Figure 25 A1, A2, A3 & B1, B2, B3). Whereas, the cultured MNCs appeared as single, round shaped cells with prominent single nucleus (Figure 25 C1, C2, C3). Upon activation with PHA, MNCs appeared as clumps of aggregated cells with increase in the cell size and numbers (Figure 25 D1, D2, D3). However, in the non-activated co-culture groups (MSC+MNC) (Figure 25 E1, E2, E3), a decrease in MNC cell numbers was observed, which upon activation with PHA showed an increase in the MNC cell number (Figure 25 F1, F2, F3).

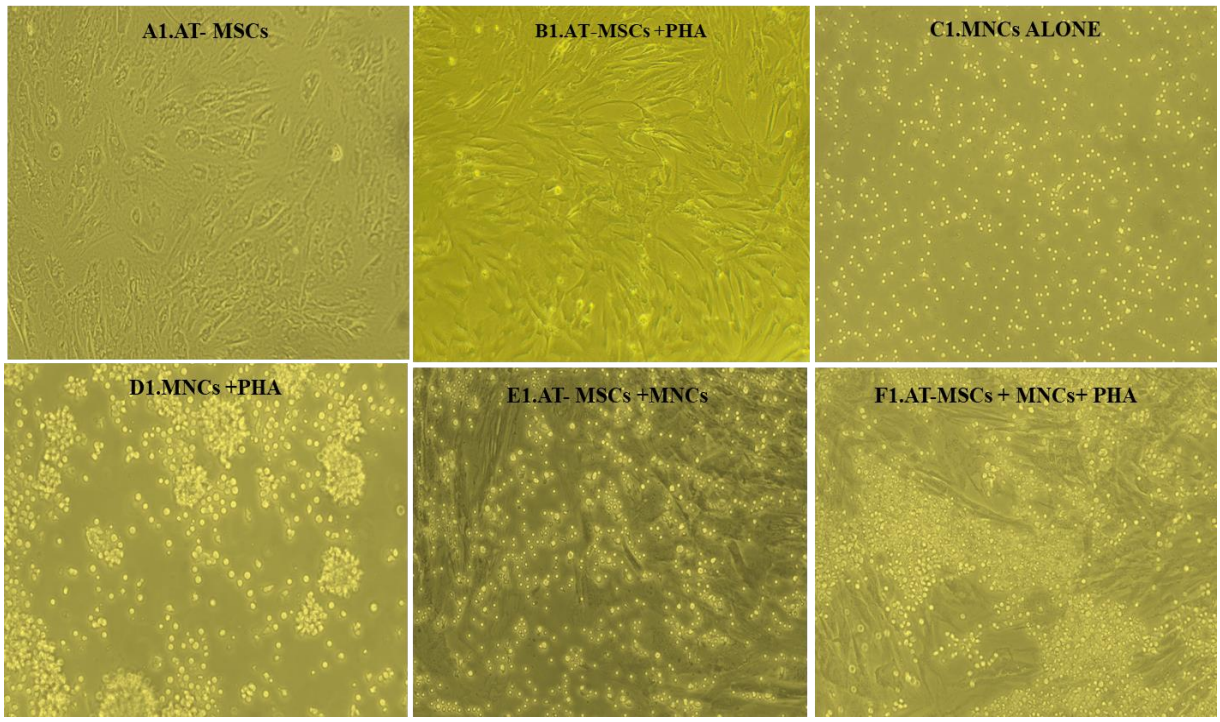


Figure 25A. Morphological analysis - Co-culture of AT MSCs with MNCs

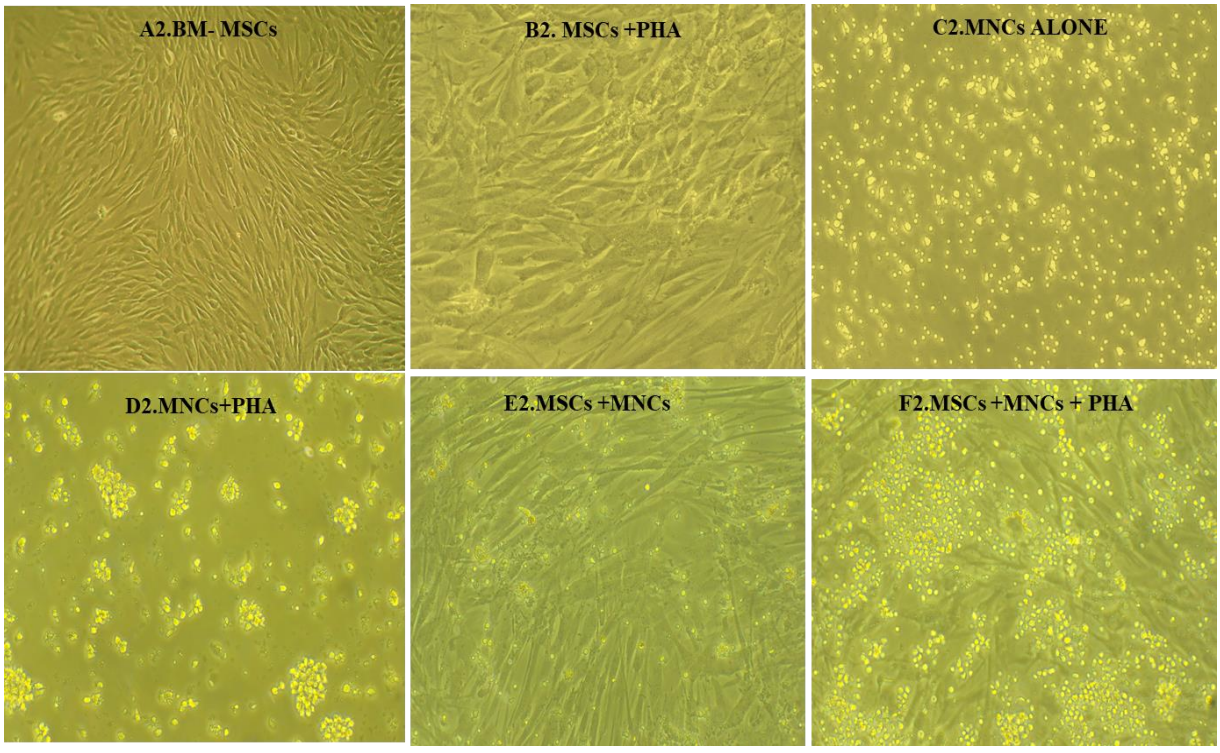


Figure 25B. Morphological analysis - Co-culture of BM MSCs with MNC

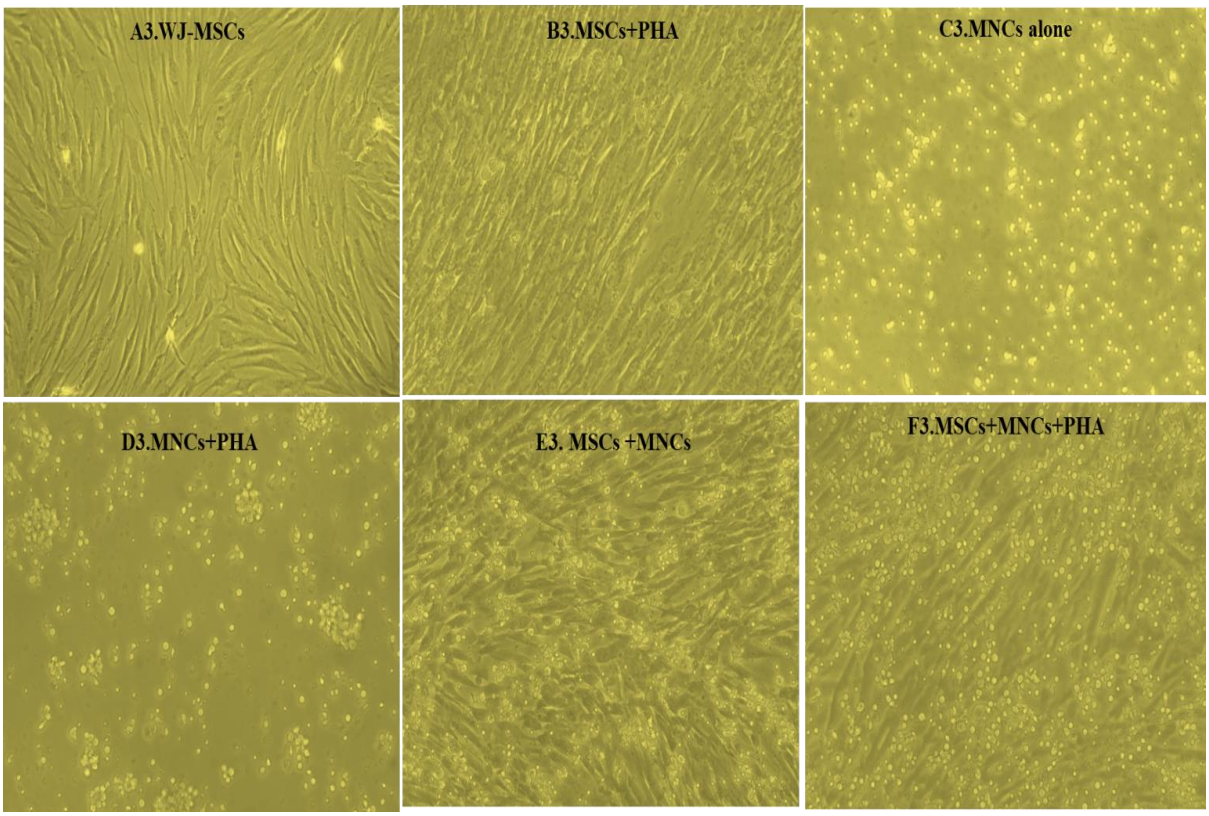


Figure 25C. Morphological analysis - Co-culture of WJ MSCs with MNCs

4.7.1.2 Cell counting

In the manual cell counting method, we observed a significant reduction of MNCs in the non-activated as well as the activated co-culture groups as compared to the activated MNCs only. The observed reduction in the numbers of MNCs even in the non-activated co-culture shows the inherent immunomodulatory property of MSCs. Though, MSCs derived from all the sources significantly reduced the numbers of activated immune cells (MNCs), BM-MSCs showed a much higher response in reducing the MNC numbers as compared to the other two sources (Figure 26).

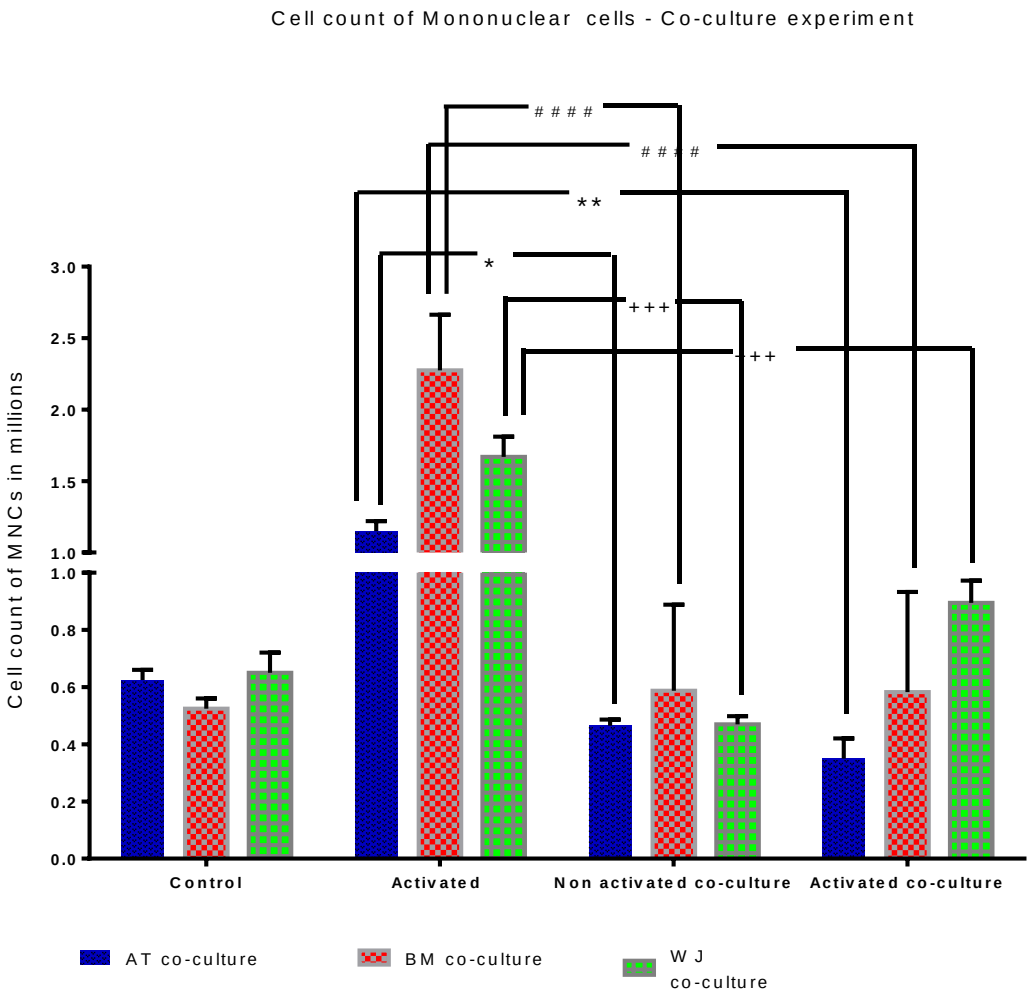


Figure 26. Cell count of mononuclear cells in the co-cultured system

4.7.1.3 Percentage distribution of PBMCs by FACS

Furthermore, we analysed the role of MSCs in influencing the cell size and complexity of MNCs in the activated and non-activated co-culture groups. The results show that there is a significant reduction in the MNC's cell size in the activated co-cultured group as evidenced by a shift in the cell population from the P3 to P5 quadrant for all the three sources. No significant difference in MNC's cell size was observed either on their own or in the non-activated co-culture group (Figure 27 and Table 4).

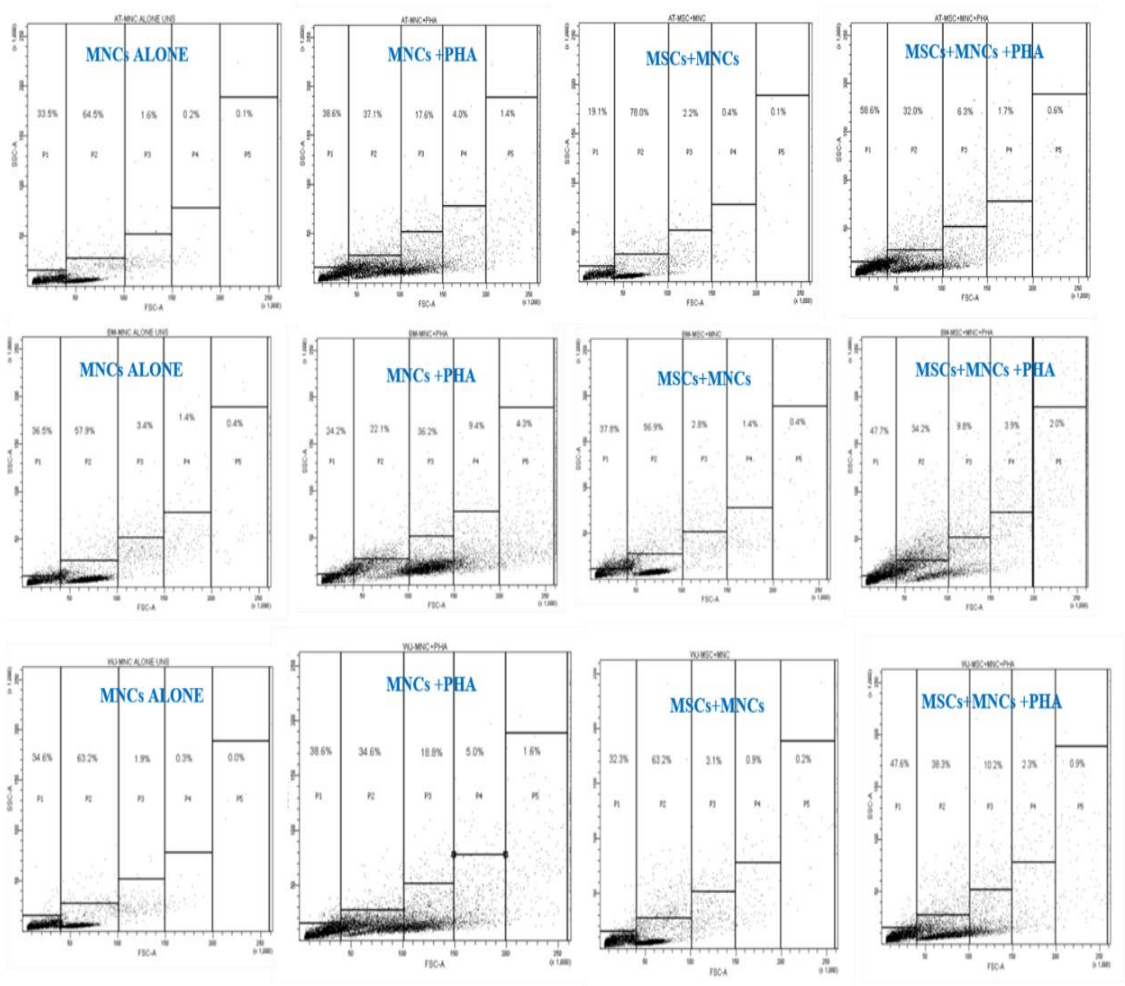


Figure 27. Flow cytometry analysis - FSC vs SSC of MNCs in co culture

Table 4. Illustrates the percentage of cells in various quadrants (FSC and SSC)

	Adipose tissue					Bone marrow					Wharton's Jelly				
	P1	P2	P3	P4	P5	P1	P2	P3	P4	P5	P1	P2	P3	P4	P5
MNCs alone	33.5	64.5	1.6	0.2	0.1	36.5	57.9	3.4	1.4	0.4	34.6	63.2	1.9	0.3	0.0
MNC+PHA	38.6	37.1	17.6	4.0	1.4	24.2	22.1	36.2	9.4	4.3	38.6	34.6	18.8	5.0	1.6
MSC+MNC	19.1	78.0	2.2	0.4	0.1	37.8	56.9	2.8	1.4	0.4	32.3	63.2	3.1	0.9	0.2
MSC+MNC+PHA	58.6	32.0	6.3	1.7	0.6	47.7	34.2	9.8	3.9	2.0	47.6	38.3	10.2	2.3	0.9

4.7.2 Effective suppression of PHA activated proliferation of MNCs by BM- and WJ MSCs

Since we observed a reduction in the number of MNCs during its co-culture with MSCs (AT, BM and WJ), we wanted to understand the role of MSCs in proliferation of MNCs using dye (CFSE) labelled MNCs. CFSE labelling has been widely used to monitor the proliferation of cells both *in-vitro* and *in-vivo* based on the progressive halving of CFSE fluorescence within daughter cells following each cell division.

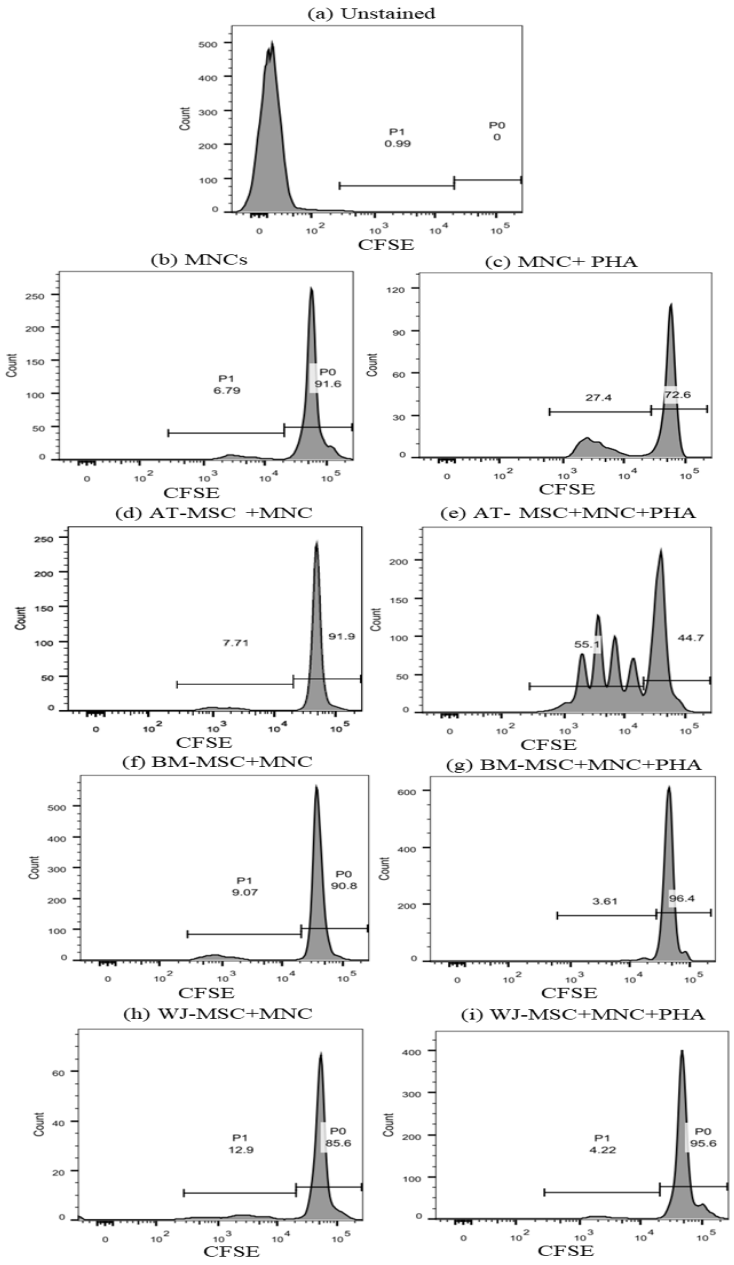


Figure 28. Cell proliferation of MNCs by CFSE staining by flow cytometry

The results show that MNCs on their own exhibited minimal proliferation as compared to the PHA activated MNCs where a significant increase in proliferation 27.4% ($p<0.0001$) (Figure 16) was observed. Upon co-culturing them with MSCs (MSC+MNC), the AT-, BM- and WJ-MSCs showed an increase in the MNC proliferation at 7.7%, 9.07% and 12.9% respectively as compared to the control MNCs (6.79%). However, in the presence of mitogen activation (MSC+MNC+PHA), both BM- and WJ- MSCs were able to effectively inhibit the proliferation stimulated by PHA to 3.61% and 4.22% when compared to the PHA activated MNCs only, which showed 27.4% proliferation as mentioned above (Figure 16). Interestingly, a significant increase in the proliferation of MNCs (55.1%; $p<0.0001$) was observed indicating that the AT-MSCs promote proliferation in the presence of a mitogen stimulus (Figure 28).

4.7.3 MSCs derived from various sources differentially modulate the immunophenotype of MNCs

To assess the immunoregulatory effect of MSCs (all three sources) on the non-activated and activated MNCs, we assessed the subpopulation of T cells in MNCs by analysing the expression of CD4 and CD25 (activated T cell markers), CD 45RA (naive T cell marker) along with CD127 (an inversely correlated activation marker for forkhead box (FOXP3) in T cells).

4.7.3.1 MSCs down regulate T cell activation upon co-culture

Here, we wanted to understand the role of MSCs in modulating the immune responses upon co-culture. Therefore, we looked at the changes in activation markers of the MNCs specifically the expression of the conventional Tcell markers CD4 and CD25 in MNCs. As such, we did not observe a significant activation or suppression of the MNCs upon co-culturing them with the MSCs of all the three sources with respect to the control MNCs.

On comparing the MNCs of non-activated (MSC+MNC) and activated co-culture (MSC+MNC+PHA) with respect to the activated MNCs (MNC+PHA), we observed a significant down regulation in the expression of the activation marker CD4 for the WJ ($p<0.0001$) source followed by AT ($p<0.001$) and BM ($p<0.05$). Whereas, in the activated co-culture group, WJ source ($p<0.001$) alone showed a significant down regulation of CD4 markers Figure 29 (A, B, C).

Likewise, CD25 expression was significantly down regulated in the MNCs of non-activated co-culture group of the WJ source ($p<0.0001$) followed by BM and AT ($p<0.01$) with respect to the activated MNCs (MNC+PHA). However, upon activation, the WJ source ($p<0.001$) showed maximum suppression of the CD25 expression of MNCs followed by BM ($p<0.05$) Figure 30 (A, B, C).

4.7.3.2 MSCs differentially modulate naive T cell population upon co-culture

In order to check if the activation of MNCs in the presence of MSCs is altering the naive Tcell population, we looked at the marker CD45RA.We found that the expression was varied: there was a significant increase of CD45RA expression in MNCs of the non-activated ($p<0.01$) and activated co-culture group ($p<0.05$) of the AT-MSC source; there was no significant change in the BM-MSC source; conversely, the WJ-MSC source showed a significant decrease in the CD45RA expression ($p<0.05$) Figure 31 (A, B, C). Collectively, our results show that MSCs from each source demonstrate their inhibitory effect on MNCs differentially by either decreasing the expression of activation markers CD4 and CD25 (WJ and BM source) or increasing the naïve Tcell population (CD45RA) (AT source).

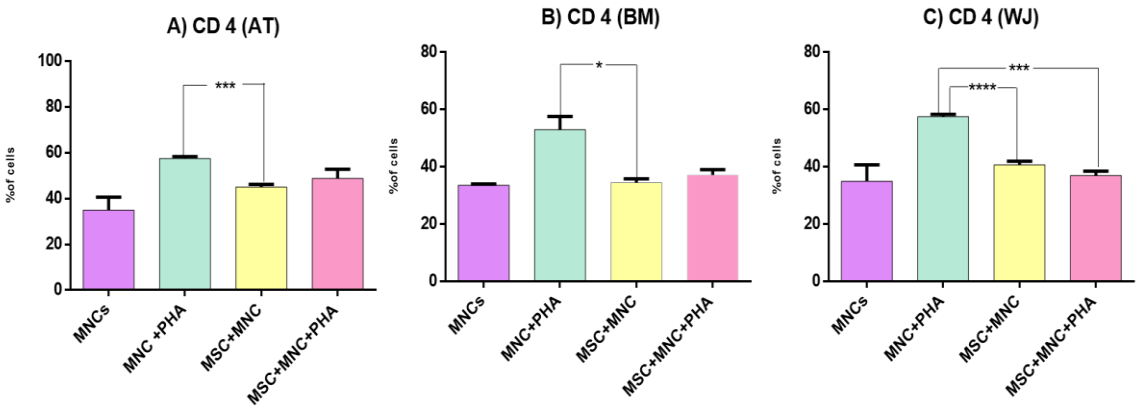


Figure 29. Effect of MSCs (AT, BM & WJ) on the expression of activated T cells (CD4)

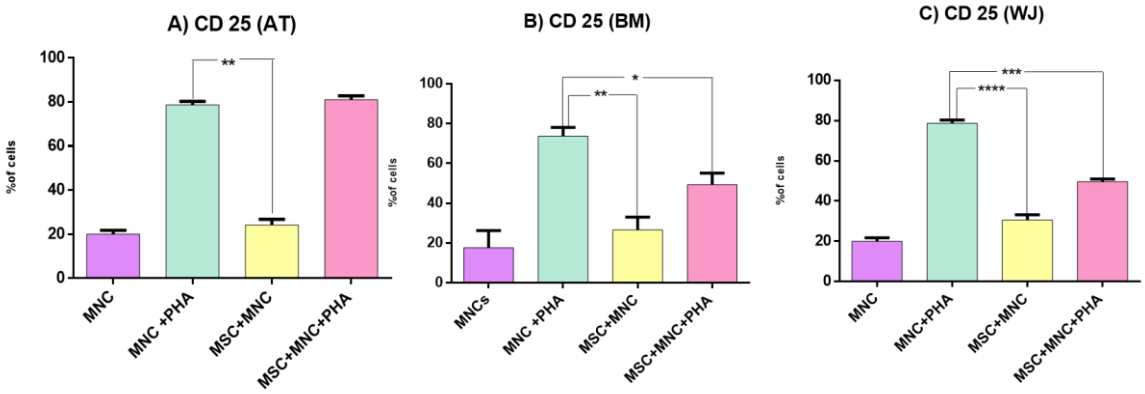


Figure 30. Effect of MSCs (AT, BM & WJ) on the expression of activation marker CD25

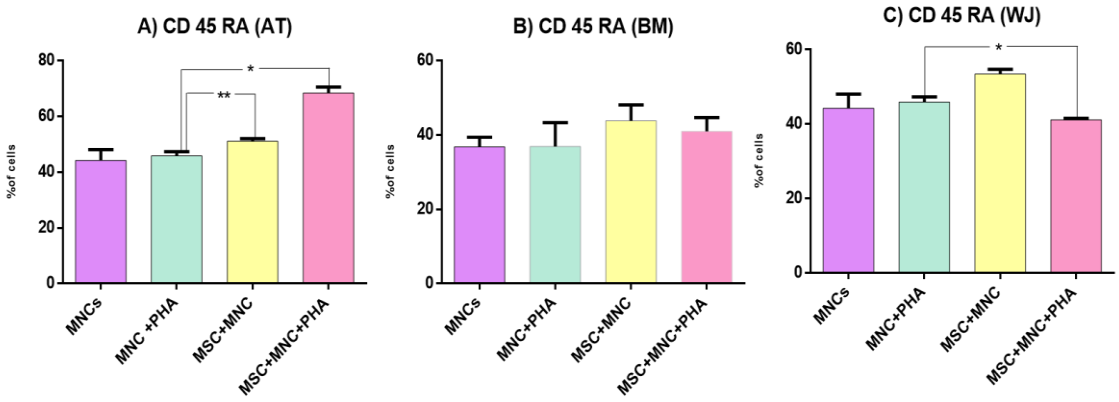


Figure 31. Effect of MSCs (AT, BM &WJ) on the expression of naïve T cell marker CD 45 RA

4.7.3.3 MSCs differentially modulate the autoimmune-preventive regulatory Tcells (Tregs)

In order to determine the regulatory effects of MSCs on the MNCs, we examined the Treg subpopulation ($CD4^+CD25^+CD127^{low/-}$). Simultaneous increase in the CD4 CD25 expression and decrease in CD127 are considered as T reg population in MNCs.

The percentage of $CD4^+CD25^+$ were compared between the MNCs of non-activated and activated co-cultured condition. The increase in $CD4^+CD25^+$ in MNC population is highly significant upon activation with PHA in all the three sources (AT ($p<0.0001$) > WJ ($p<0.01$) >BM ($p<0.05$)) Figure 32 (A, B, C).

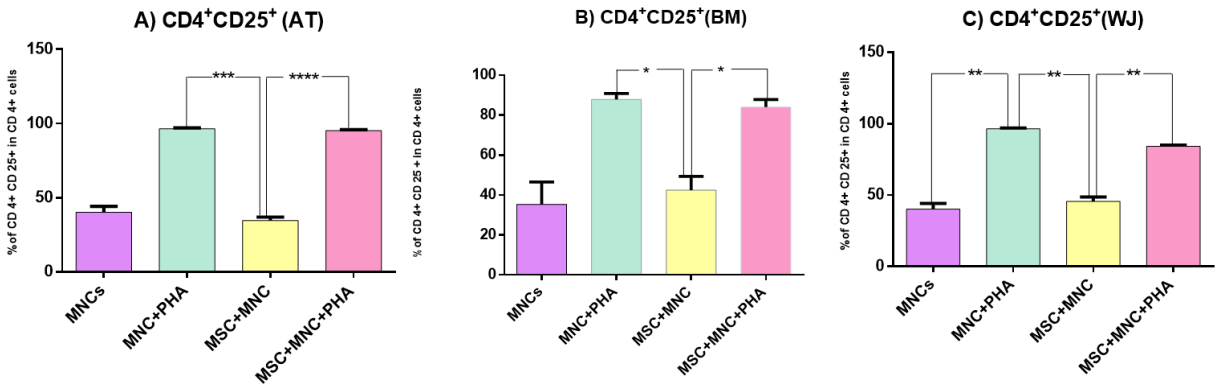


Figure 32. Effect of MSCs (AT, BM and WJ) on the expression of $CD4^+CD25^+$

This simultaneous increase in $CD4^+CD25^+$ and decrease in $CD4^+CD127^{-/low}$ was observed only when PHA was added to the MNCs and when MNCs were co-cultured with MSCs of all the three sources. No change was observed in the Treg population in the MNCs and co-cultured MNCs without PHA activation. Upon, activation of the co-cultured condition, a significant upregulation of the Treg population was observed in all the three source groups compared with their respective controls without PHA activation Figure 33 (A, B, C).

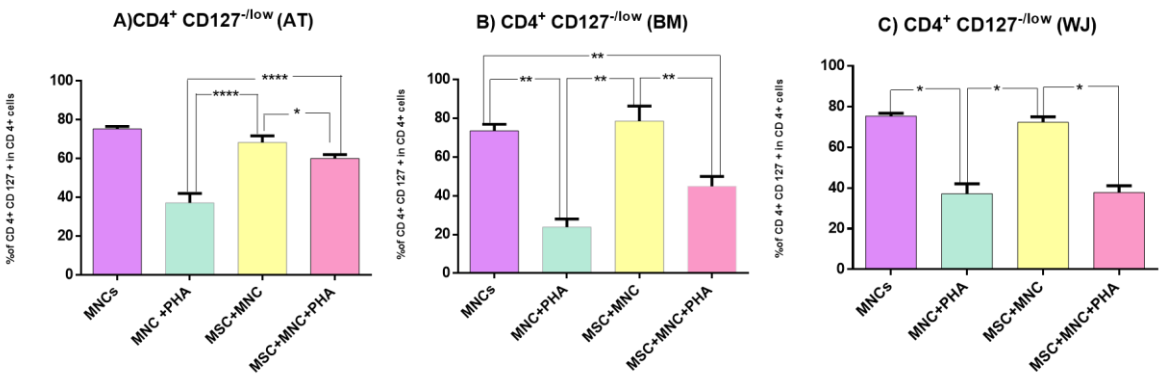


Figure 33. Effect of MSCs (AT, BM and WJ) on the expression of $CD4^{+}CD127^{+}$

Overall, our results strongly corroborate that MSCs, in general, irrespective of their sources are able to modulate the immunophenotype of MNCs in an activated co-culture system.

4.7.4 Cytokine profiling of MSCs in the activated and non-activated co-culture conditions

4.7.4.1 Custom-made PCR array for immunomodulatory cytokines

A custom-made PCR array was performed to analyse the differential gene expression profiling of MSCs (with and without PHA), MSCs co-cultured with MNCs under both PHA stimulated and non-stimulated conditions. Here, we assessed the immunomodulatory role of MSCs by comparing the expression profile of pro and anti-inflammatory cytokines under the above mentioned conditions. The heatmap representation and the cluster analysis of MSC-cytokine expression profile is represented in Figure 34.

Firstly, we observed minimal expression (not significant) of the pro and anti-inflammatory cytokines in the control (MSCs) group of all three sources. Upon co-culture (MSC+MNC), the two branches of the relative cytokine gene expression profile seem to be unchanged with the exception of TNF α (upregulation) in the BM source and PTGS-2, PDGF (upregulation) in WJ source. Activation with PHA leads to upregulation of both the pro- and anti-inflammatory cytokine expression in the MSCs from AT and BM source Figure 34 (A &

B). The pro- and anti-inflammatory cytokines expressed by activated MSCs of AT source are IFN-ϒ, TNF-α, and iNOS, PDGF, HLA-G, IL-4, TGF-β, IL-10 and IDO respectively (Figure 34A). Similarly, pro- and anti-inflammatory cytokines expressed by activated MSCs of BM source are IL-6 and TGF-β, IL-4, VEGF, iNOS, PDGF, HLA-G, and IL-10 respectively (Figure 34 B). However, the MSCs from WJ source showed an increase in the expression of the iNOS and PTGS-2 (Figure 34 C).

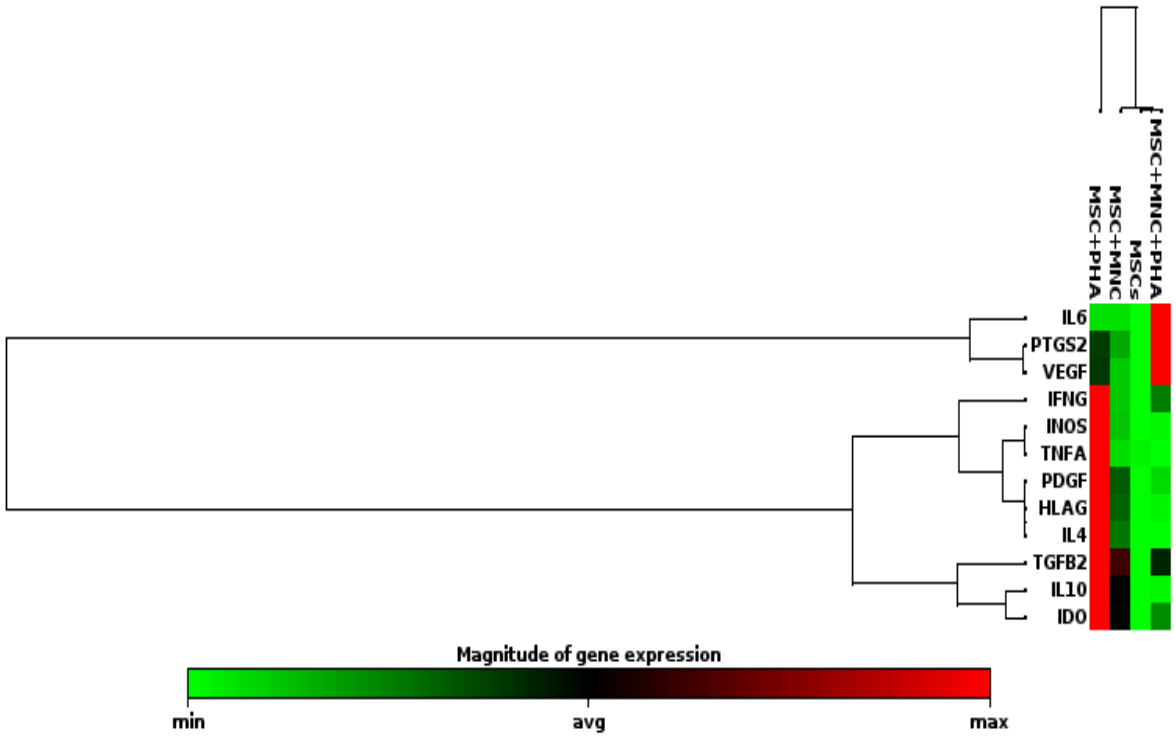


Figure 34A. Heat map representation - mRNA expression profile of pro- and anti-inflammatory cytokines of MSCs of AT source

Group1- AT MSCs alone, Group2 – activated MSCs (AT MSC+PHA), Group 3 - non-activated co-cultured MSCs (AT MSC+MNC), Group 4 - activated co-cultured MSCs (AT MSC+MNC+PHA). The genes were clustered according to their expression patterns. The numbers are represented as a bitmap and are expressed as fold changes with respect to the expression profile of control MSCs.

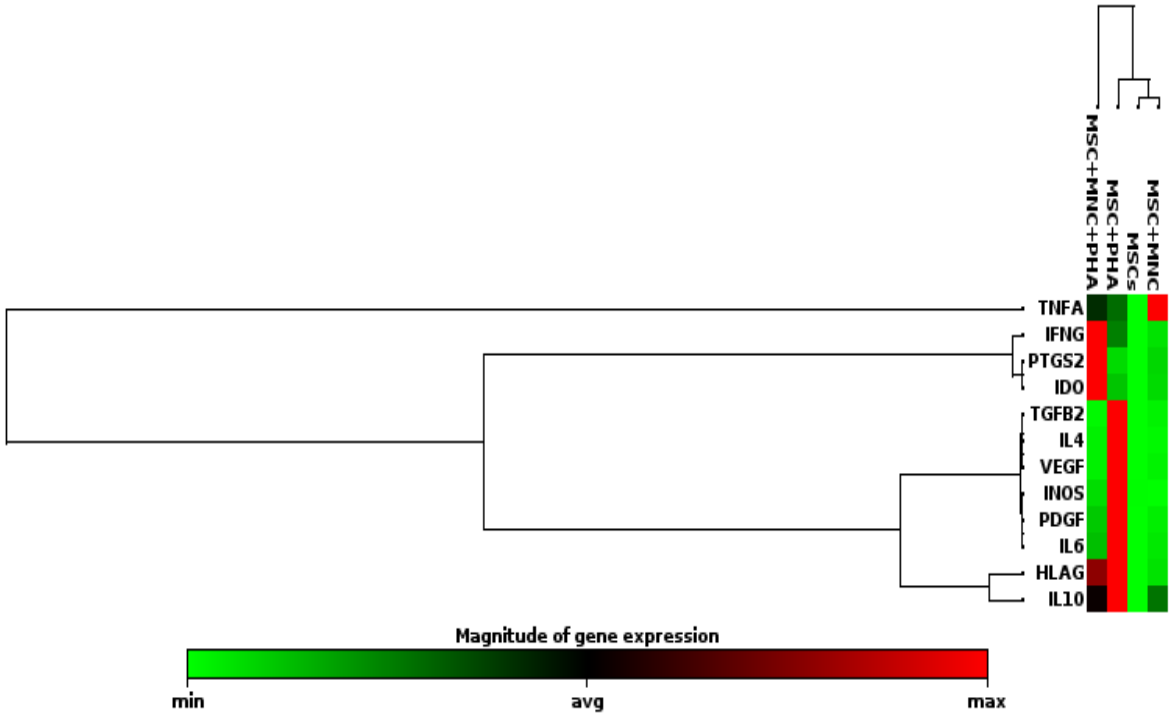


Figure 34B. Heat map representation - mRNA expression profile of pro- and anti-inflammatory cytokines of MSCs of BM source

Group 1- BM MSCs alone, Group 2 – activated MSCs (BM MSC+PHA), Group 3 - non-activated co-cultured MSCs (BM MSC+MNC), Group 4 - activated co-cultured MSCs (BM MSC+MNC+PHA) were shown on the heat map graph. The genes were clustered according to their expression patterns. The numbers are represented as a bitmap and are expressed as fold changes with respect to the expression profile of control MSCs.

Conversely, in the PHA activated co-cultured group, MSCs from all three sources showed varied expression of both pro- and anti-inflammatory cytokines (Figure 35 and table 5). Interestingly, MSCs from the WJ source showed the most responsiveness in upregulating the pro and anti-inflammatory cytokines such as IL-6, IFN- ϒ, TNF-α and PDGF, VEGF, HLA-G, IDO, IL-10, TGF-β, iNOS and IL-4 (Figure 34 C). In the AT and BM sources, both branches of the cytokines (pro and anti) that were upregulated in the activated MSCs (MSC+PHA) group exhibited a down regulatory shift in the cytokine profile in the activated co-culture group (MSC+MNC+PHA). AT-MSCs expressed pro-inflammatory cytokine IL-6 and anti-inflammatory cytokines PTGS-2 and VEGF, whereas BM expresses pro-inflammatory cytokine IFN- ϒ and anti-inflammatory cytokines PTGS-2 and IDO in the

activated co-culture condition. Based on the outcome of the study, certain cytokines such as VEGF, PTGS2, and IDO showed source specific upregulation in the activated co-cultured condition (VEGF, PTGS-2 in AT source, PTGS-2, IDO in BM source and IDO in WJ source). HLA-G, a key immunomodulator, is the only commonly expressed cytokine in all the three sources under the above-mentioned conditions.

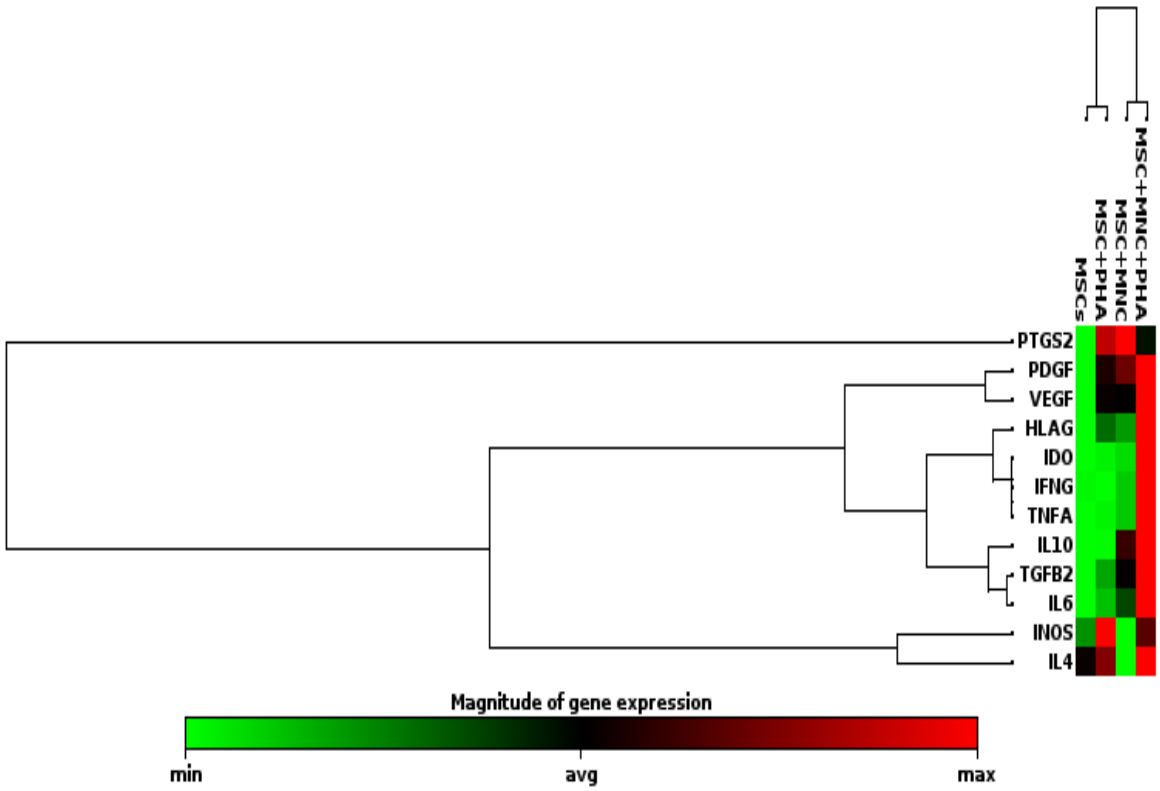


Figure 34C. Heat map representation - mRNA expression profile of pro- and anti-inflammatory cytokines of MSCs of WJ source

Group1- WJ MSCs alone, Group2 – activated MSCs (WJ MSC+PHA), Group 3 - non-activated co-cultured MSCs (WJ MSC+MNC), Group 4 - activated co-cultured MSCs (WJ MSC+MNC+PHA) were shown on the heat map graph. The genes were clustered according to their expression patterns. The numbers are represented as a bitmap and are expressed as fold changes with respect to the expression profile of control MSCs.

Overall, our results strongly corroborate that MSCs, in general, irrespective of their sources are able to immunologically respond to MNCs via heterogeneous expression of cytokine genes in a co-culture system. We noticed a difference in the pattern of cytokine gene expression for the adult derived MSCs as compared to the primitive WJ source upon encountering allogenic immune cells.

Table 5: Quantitative gene expression profile of MSCs of various sources in the PHA activated co-culture expressed in folds

Gene	**Adipose tissue**	**Bone marrow**	**Wharton's Jelly**
HLA-G	** 359	**** (~14088 fold) Exceedingly high	** (367)
IDO	Very Low (3 fold)	** Medium (249 fold)	**** Exceedingly high **(7826 fold)**
IFN-ϒ	* (77 fold)	**** Exceedingly high (3978 fold)	** (124 fold)
IL-6	** 270	* 55	Low 5
IL-4	Low 3	Low 36	Low 0.8
IL-10	* 3	* 9	* 13
iNOS	Low 10	Low 4	* 3
PDGF	* 26	* 25	* 31
PTGS-2	*** 84	*** **131**	* 2
TGF-β	* 5	* 3	* 2
TNF-α	Low 4.5	Low 0.5	**** 7593 Exceedingly high
VEGF	**** **2704** Exceedingly high	** 90	* 2.7

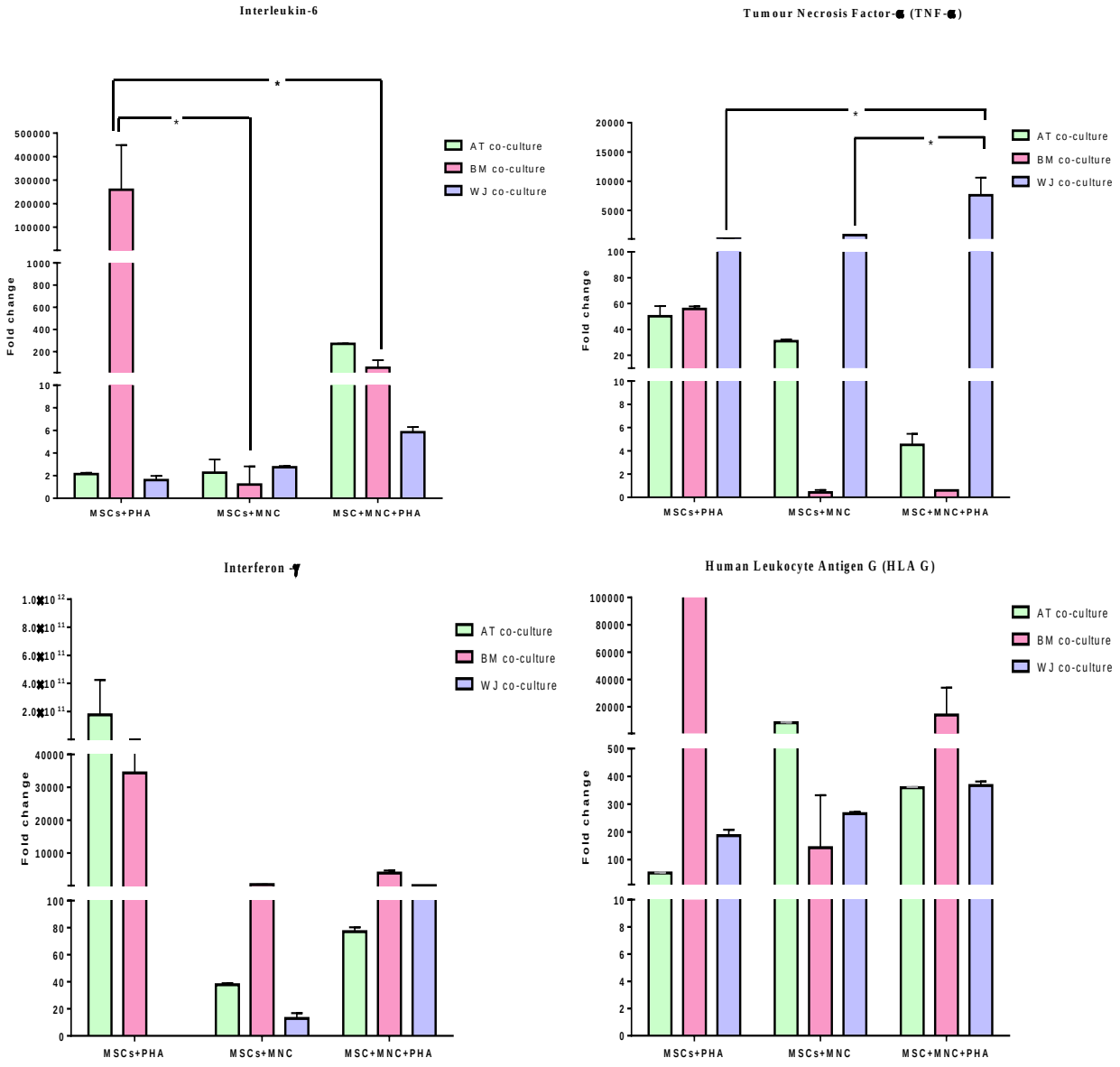


Figure 35A. Graphical representation of IL-6, TNF-α, IFN-ϒ, and HLA-G cytokine expression in MSCs (AT, BM&WJ) during co-culture by customized PCR array.

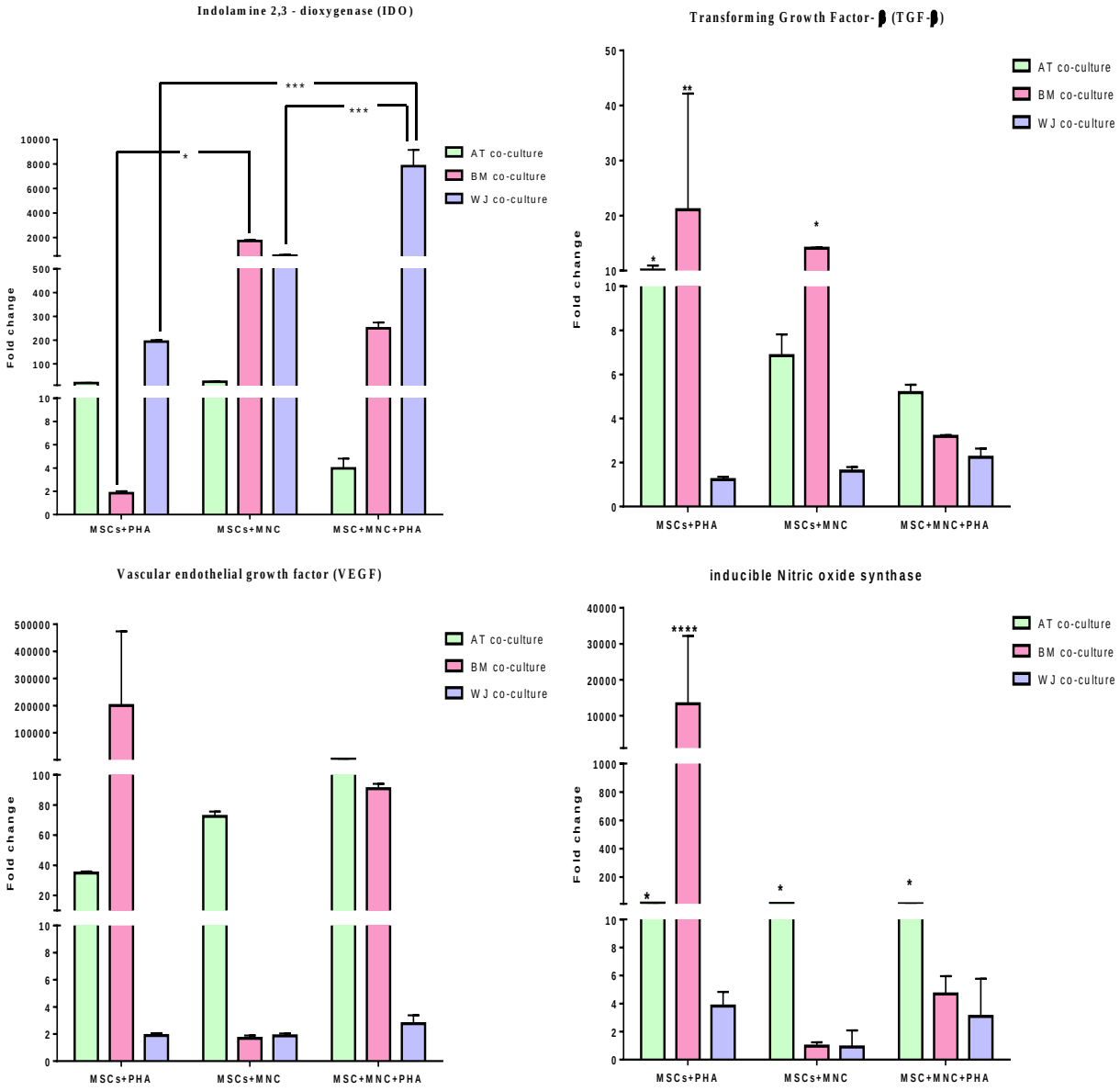


Figure 35B. Graphical representation of IDO, TGF-β, VEGF and iNOS cytokine expression in MSCs (AT, BM&WJ) during co-culture by customized PCR array

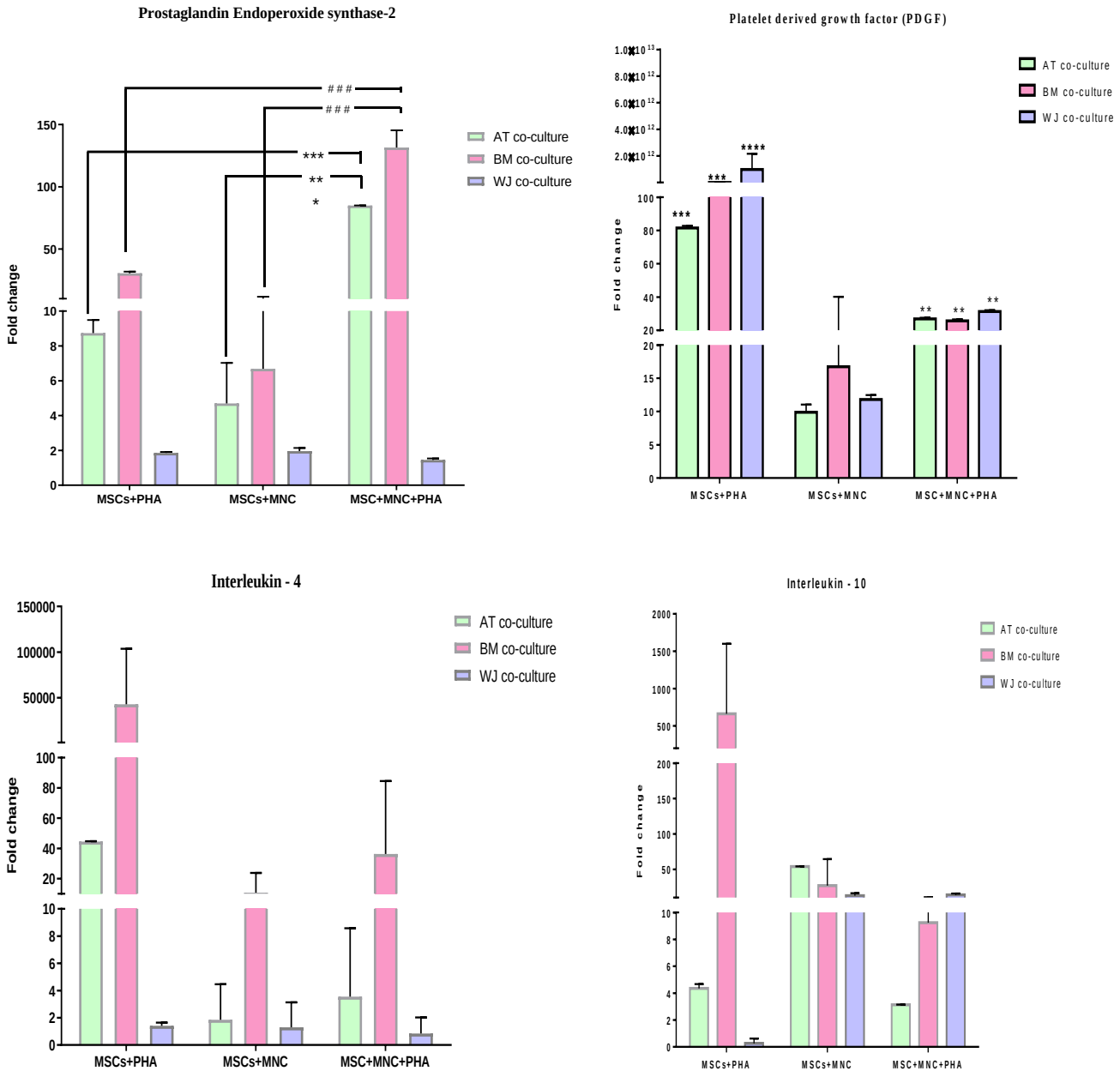


Figure 35C. Graphical representation of PTGS-2, PDGF, IL-4 and IL-10 cytokine expression in MSCs (AT, BM&WJ) during co-culture by customized PCR gene array

4.7.4.2 Functional validation of cytokine signatures of MSCs using ELISA

Solid-phase sandwich Enzyme-Linked Immunosorbent Assay (ELISA) was performed to quantify the intracellular protein expression in MSCs (with and without PHA), MSCs co-cultured with MNCs under both stimulated and non-stimulated conditions. Here, we assessed the protein levels of VEGF, PTGS-2 and IDO of MSCs from all the three sources under the above mentioned conditions in nanograms and picograms /mg of the total protein.

4.7.4.2.1 VEGF expression

VEGF was constitutively expressed in MSCs of all the three sources, however the protein level is significantly higher in MSCs from AT (12333 pg/mg of total protein) compared to BM (7491 pg/mg, $p<0.0001$) and WJ source (7590 pg/mg, $p<0.0001$). Likewise, a similar trend was observed upon PHA stimulation of the MSCs: AT (13422 pg/mg), BM (7643 pg/mg, $p<0.0001$) and WJ (6422 pg/mg, $p<0.0001$) (Figure 36 A).

In the non-activated and activated co-cultured conditions, VEGF expression was significantly higher in the AT source (13540 and 17646 pg/mg) compared to BM (7487 and 9606 pg/mg; $p<0.0001$) and WJ source (7709 and 10235 pg/mg; $p<0.0001$) respectively (Figure 7A). This is corroborating with the gene expression results where the expression of VEGF was about 2700 fold higher in the AT source when compared to the other two sources of MSCs under the above mentioned conditions (Figure 36 A).

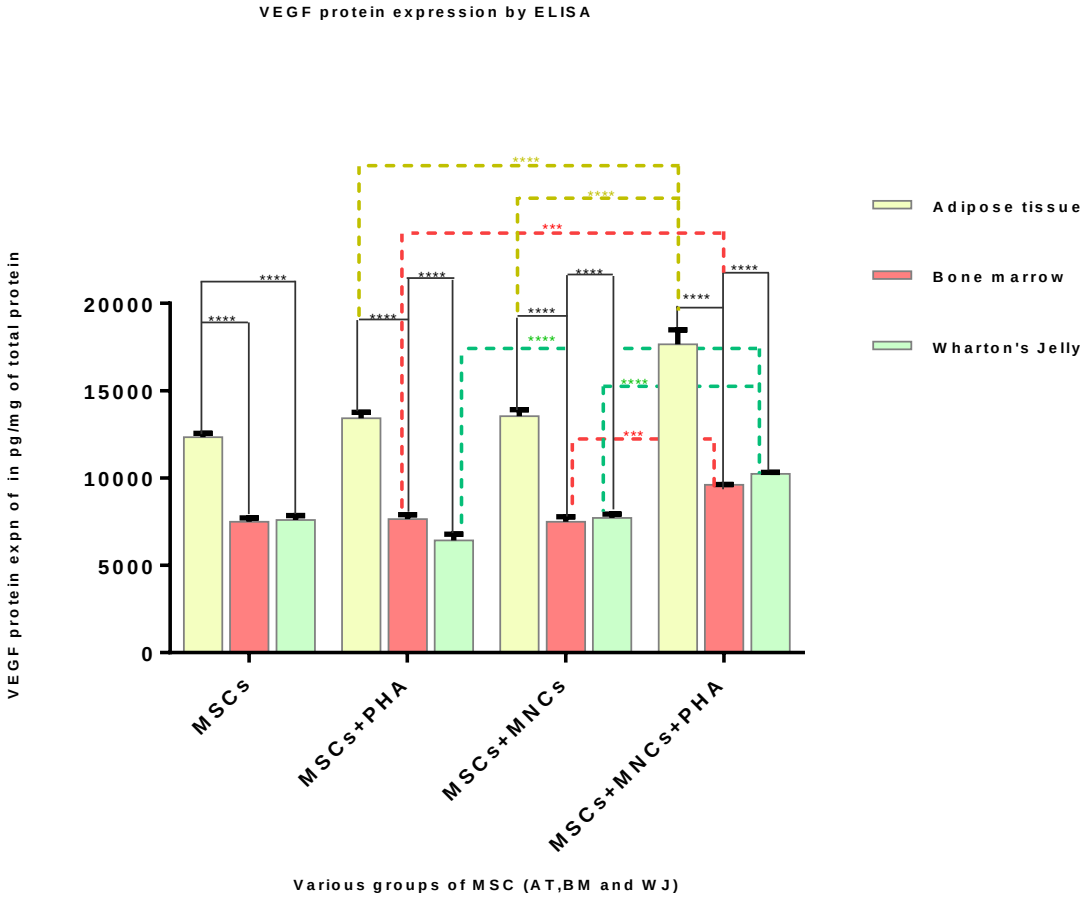


Figure 36A. Quantitative intracellular protein expression of VEGF by solid-phase sandwich Enzyme-Linked Immunosorbent Assay (ELISA)

4.7.4.2.2 PTGS-2 expression

PTGS-2 was constitutively expressed in MSCs from all the three sources, however the protein level of this cytokine is significantly higher in MSCs from AT (10.88 ng/mg of total protein) compared to MSCs from BM (10.49 ng/mg) and WJ (8.13 ng/mg, $p<0.001$). Likewise, a similar trend was observed upon PHA stimulation of the MSCs: AT (13.61ng/mg), BM (10.03 ng/mg, $p<0.0001$) and WJ (8.36 ng/mg, $p<0.0001$) (Figure 36B).

In the non-activated and activated co-culture conditions, the levels of PTGS-2 in AT (13.25 ng/mg and 13.44 ng/mg) was significantly higher than BM (10.26 ng/mg ($p<0.001$) & 10.26 ng/mg ($p<0.001$)) and WJ (9.41 ng/mg ($p<0.0001$) & 5.64 ng/mg ($p<0.0001$)). Bone marrow was significantly higher than WJ in all the groups such as control MSCs, activated

MSCs and activated co-cultured MSCs (BM>WJ: p<0.01, p<0.05, p<0.0001) except non-activated co-culture group. (Figure 36 B).

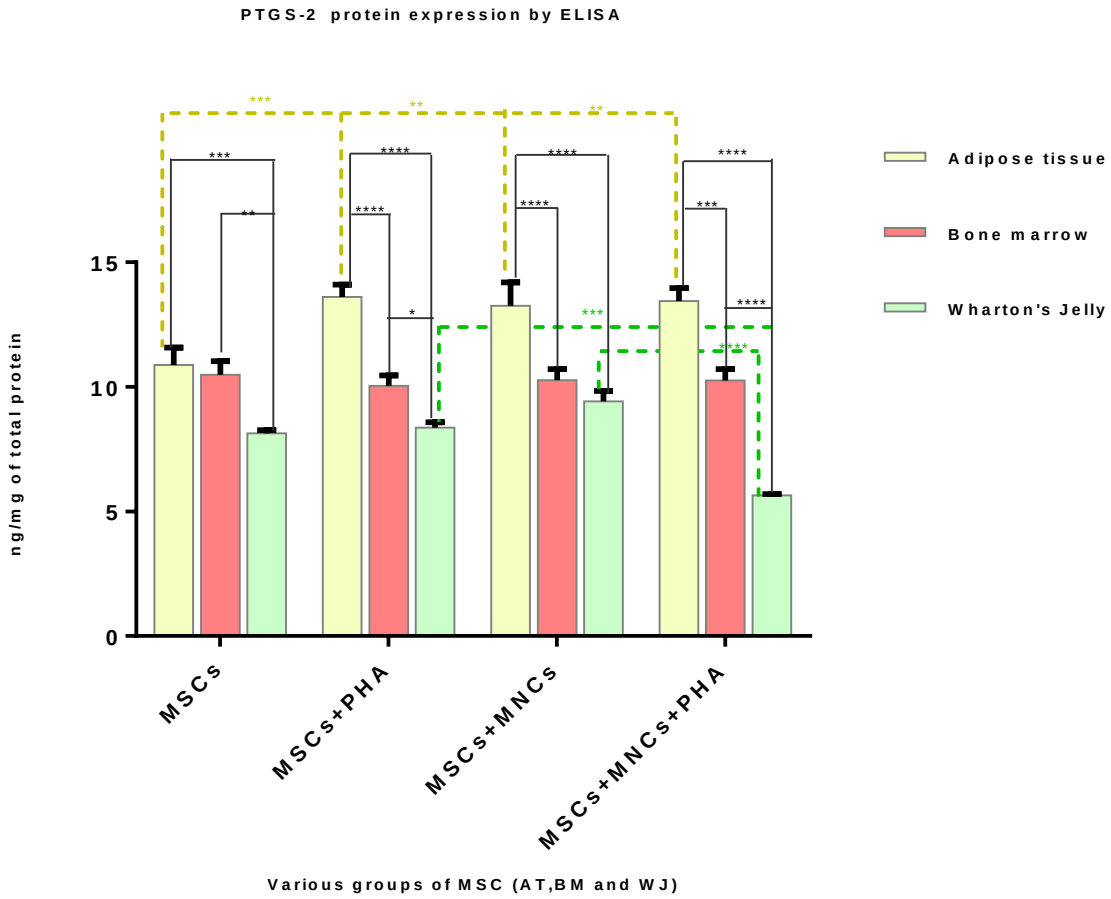


Figure 36B. Quantitative intracellular protein expression of PTGS-2 by solid-phase sandwich Enzyme-Linked Immunosorbent Assay (ELISA)

4.7.4.2.3 IDO expression

IDO was constitutively expressed in MSCs of all the three sources. However, there was no significant difference in the expression between the sources. Likewise, in the PHA stimulated MSCs and non-activated co-culture group of all the three sources, a similar pattern of expression was observed with no significant difference amongst the sources. In the activated co-cultured condition, IDO expression was significantly higher in WJ source (13.69 ng/mg, p<0.0001) compared to AT and BM (10.51,10.92 ng/mg) respectively (Figure 36C). This is corroborating with the gene expression

results where the expression of IDO was about 7800 fold higher in the WJ source when compared to the other two sources of MSCs under the above mentioned conditions (Figure 35B).

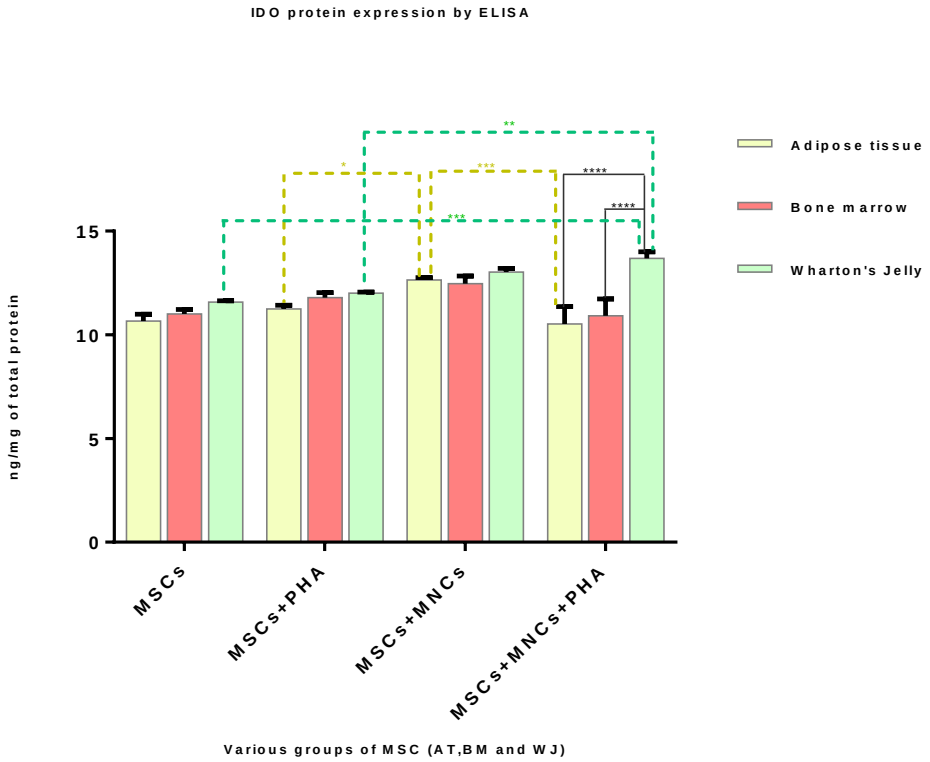


Figure 36C. Quantitative intracellular protein expression of IDO by solid-phase sandwich Enzyme-Linked Immunosorbent Assay (ELISA)

4.7.5 Co-culturing of chondrocytes with mononuclear cells with and without PHA stimulation

In order to understand the immunomodulatory properties of chondrocytes in the *in-vitro* inflammatory milieu, co-culture experiment was carried out in the same way as performed for undifferentiated MSCs. After 3 days of co-culture, morphological changes and changes in the cytokine signature were investigated to generate preliminary data on the understanding of immunomodulatory properties of chondrocytes.

4.7.5.1 Influence of PHA activation on the morphological changes during co-culture of WJ MSC derived chondrocytes with MNC

The morphological changes in the chondrocytes based on the size were measured in all the groups such as PHA activated chondrocytes, co-cultured chondrocytes with and without PHA stimulation and the control unstimulated chondrocytes. After 3 days of co-culture, we observed drastic changes in the morphology of chondrocytes among the different groups (Figure 37). The size of chondrocytes in various conditions are as follow

Chondrocytes	- 140 µm
Chondrocyte +PHA	- 697 µm
Chondrocyte + MNC	- 202 µm
Chondrocyte + MNC +PHA	- 450 µm

When the chondrocytes are stimulated with PHA, the size of chondrocyte was increased to nearly 3 times (697 µm) than the unstimulated chondrocytes (140 µm). It indicates the responsiveness of chondrocytes towards the mitogen PHA. When the immune cells are added to this inflammatory microenvironment, the size of chondrocyte was reduced by one fourth (450 µm) indicating the cross talk of chondrocytes with immune cells in the inflammatory condition. However, when the chondrocyte co-cultured with immune cells in the absence of PHA, there is no significant change but slight increase in the size compared to the control chondrocyte and decrease in size (reduced to nearly half) compared with activated co-culture condition. It indicates some responsiveness of chondrocytes towards immune cells. From these results, it is indicative that PHA has a greater role in influencing the morphological change (i.e. size). As a result of this, we expected the change in the level of gene expression of pro and anti-inflammatory cytokines.

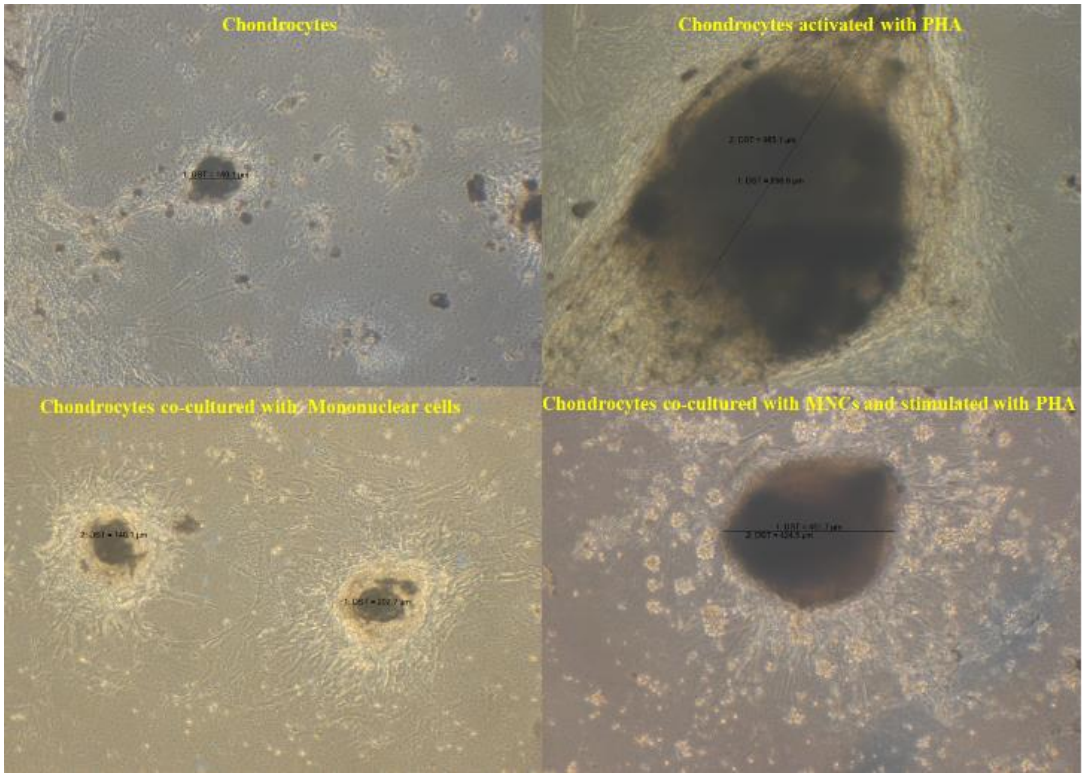


Figure 37. Morphology analysis of Chondrocytes co-cultured with immune cells under PHA activation.

4.7.5.2 Expression of Pro- and anti-inflammatory cytokines when the differentiated chondrocytes communicate with the immune cells under PHA stimulation

When the chondrocytes interact with immune cells in the presence and absence of PHA stimulation (activated and non-activated co-culture condition), the expression of major cytokines responsible for inflammation such as IL-6, TNF-α, and IFN-ϒ were significantly down regulated (Figure 38A). This indicates the effective suppression of pro-inflammatory stimulus by the chondrocytes (differentiated from WJ-MSCs) under *in-vitro* inflammatory microenvironment as well as during interaction with immune cells.

In case of anti-inflammatory cytokines, the major immunosuppressive cytokines such as IL-10, TGF-β, IL-4, and the angiogenic factor VEGF, responsible for the wound healing were upregulated only when the chondrocytes were co-cultured with the immune cells in the absence of PHA stimulation (Figure 38B).

HLA G, a checkpoint molecule that confers protection against the immune system during transplant was expressed in all the conditions of undifferentiated MSCs whereas it was not expressed after its differentiation into chondrogenic lineage. Likewise, a similar pattern was also observed for IDO expression for all the above mentioned conditions.

When the chondrocytes were stimulated with PHA, there was no changes in the expression of pro or ant- inflammatory cytokines similar to those observed in case of undifferentiated WJ-MSCs.

These results points to the fact that while chondrocytes were able to modulate immune cells by lowering the levels of pro-inflammatory cytokines, their ability to increase the levels of anti-inflammatory cytokines was not significant compared to undifferentiated MSCs. This unresponsiveness of differentiated chondrocytes and undifferentiated MSCs against PHA might be source dependent. Hence, further studies are warranted to validate our observation.

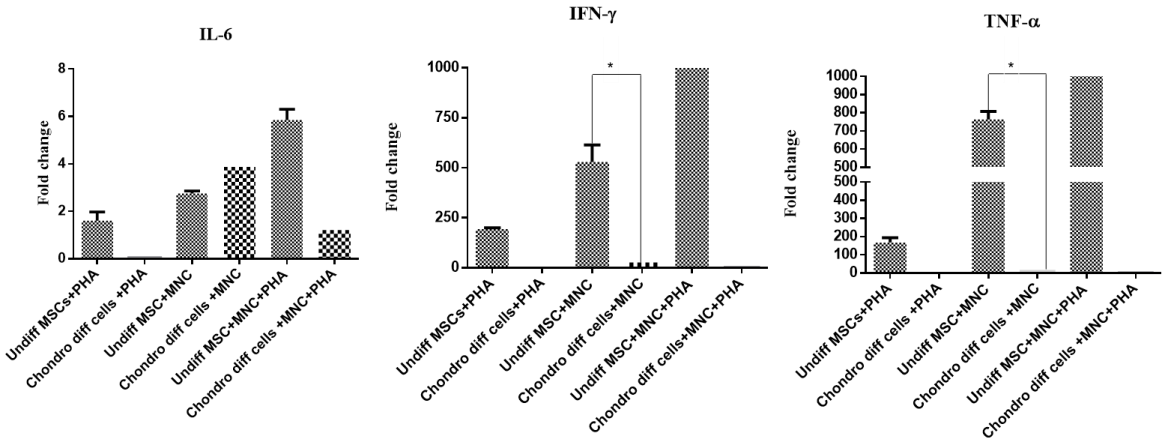


Figure 38A. Gene profiling for pro-inflammatory cytokines in Chondro differentiated WJ-MSCs Vs undifferentiated WJ-MSCs

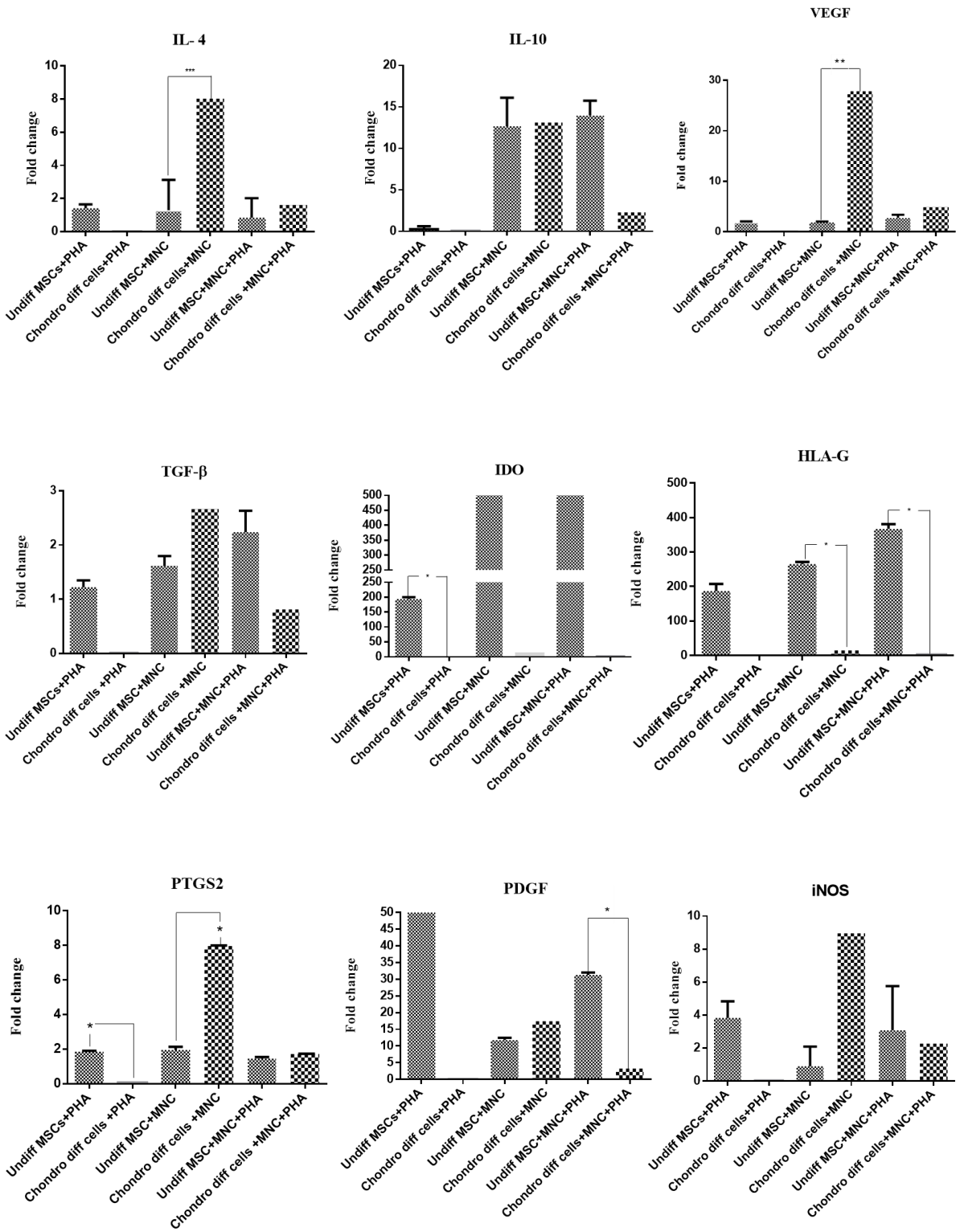


Figure 38B. Gene profiling for anti-inflammatory cytokines in Chondro differentiated WJ-MSCs Vs undifferentiated WJ-MSCs

CHAPTER - 5

DISCUSSION

This study shows that MSCs derived from various sources such as adipose tissue (AT), bone marrow (BM) and Wharton's jelly (WJ) exhibit unique properties in immunomodulation under a mitogen stimulated condition. To our knowledge, this is one of the very few studies to investigate the immunomodulatory role of hMSCs sourced from different adult tissues under an *in-vitro* stimulated inflammatory condition. Our results show that there are differences in the immunological profile when mononuclear cells were co-cultured with MSCs derived from primitive and adult sources. There is a source-specific MSC-cytokine-signature upon interaction with immunocytes under a mitogen stimulated condition. Our results also suggest that there is scope for optimal use of MSCs from different sources for various disease conditions.

Previously, it has been reported that inflammatory status determines the immunomodulatory properties of MSCs. Vigorous inflammation is required in eliciting the high level of immunosuppressive function by MSCs both *in-vitro* and *in-vivo* (51, 122). It is suggested that good therapeutic effect of MSCs can be achieved when MSCs are infused after the disease development. Hence, the microenvironment of the tissue injury plays an important role when using MSCs as a treatment option in alleviating the immune response and tissue regeneration.

Previous studies have shown that an array of cytokines are responsible for modulating the immunological niche depending upon the microenvironment (123). Here, in this study, a panel of pro-inflammatory (TNF-α, IFN-ϒ, IL-6,) and anti-inflammatory (IL-4, IL-10, VEGF, TGF-B, PDGF, PTGS-2, HLA-G) cytokines were chosen to investigate the responsiveness of MSCs to mononuclear cells under a mitogen stimulated condition. Since most inflammatory conditions involve a first-hand response by the circulating immune cells (MNCs), the interaction between MSCs and MNCs were investigated in this study. Moreover, the idea of exploiting MSCs for regenerative approaches need a prerequisite understanding

of cross-talk between MSCs and MNCs. It is also known that MSCs from different sources show difference in their immunomodulatory function. Thus, in order to investigate the immunomodulatory properties of various sources of MSCs (AT, BM and WJ), we used an *in-vitro* co-culture system involving MNCs and MSCs under a PHA stimulated inflammatory condition. The rationale behind our study of co-cultured MSCs under this mitogen stimulated state is that it mimics the *in-vivo* inflammatory condition.

MSCs are a versatile population and are considered as the key players in morphogenesis, homeostasis and tissue regeneration (124) due to the properties such as self-renewal ability, multipotent differentiation ability, homing at the site of injury and immunomodulatory properties (5-9, 65,125-128). Most importantly, MSCs are easy to isolate, expand, and manipulate under standard culture conditions. Due to the multipotent differentiation potential, much effort was made in employing the differentiation potential of MSCs for cellular therapy. However, a salient feature of the MSCs is their unique immunosuppressive and immunomodulatory nature both *in-vitro* and *in-vivo*. Another major property which attracted significant interest in the field of regenerative medicine is their ability to repopulate the damaged host tissue by secreting the immunomodulatory by cross-talking with other cells (endothelial cells, fibroblasts and immune cells) in the inflammatory microenvironment (122). Also, they are hypo-immunogenic and called as immune privileged cells. These features may be attributed to their stemness and lack of MHCII, low expression of MHC-I and absence of co-stimulatory molecules (CD40, CD80 and CD 86) on their cell surface, thereby rendering them immune-privileged. Nevertheless, several studies have shown a role for MSCs in immune-modulation during disease conditions. Therefore, we wanted to understand the role of MSCs in immunomodulation of mononuclear cells under an *in-vitro* inflammatory condition.

Though MSCs are widely distributed in various tissues such as adipose tissue, bone marrow, amniotic fluid, placenta, dental pulp, Wharton's jelly etc, their biological properties can greatly vary depending on the multiple parameters such as source, isolation method, culture conditions (10,31,129,130). The difference between the MSCs arising from the different tissues are unclear. MSCs from various origins differ in terms of colony forming capability, proliferation potential, differentiation ability, cell surface marker expression and immunomodulatory properties. These differences have been attributed to their source of origin and the quality of cells (early and late passages) (65, 66). Therefore, the central need in regenerative therapy is to identify a source which has high plasticity, enhanced differentiation potential and effective immunomodulatory functions for better clinical outcomes.

In this study, MSCs were successfully isolated from adipose tissue, bone marrow, umbilical cord blood by density gradient method and from Wharton's jelly by explant method using the standard culture conditions as per the established protocol of our laboratory (30). MSCs isolated from three sources (AT, BM and WJ) were able to form spindle shaped fibroblast like cells in the plastic surface under standard culture conditions (DMEM supplemented with 10% FBS) but at different time points. In the same culture condition, MSCs from UCB were not able to proliferate. Therefore, an attempt was made by increasing the percentage of the supplement (FBS) from 10% to 20% to check the proliferation rate of UCB-MSCs. Though, the growth of UCB-MSC was better in 20% DMEM medium, it was difficult to expand the same in all the samples. It has been reported that frequency of MSCs in UCB is very low and the reason may be due to the different culture conditions (131). Hence, the present study was confined to three sources (AT, BM and WJ) in investigating the immunological profile of MSCs.

Secondly, the isolated MSCs were characterized as per the criteria proposed by International society for Cellular Therapy (ISCT) (32). MSCs isolated from all the three sources (AT, BM and WJ) fulfilled the ISCT criteria i.e., i) showed spindle shaped morphology in the plastic surface under standard culture conditions ii) able to differentiate into tri-lineages (osteo, adipo and chondrogenic lineages) and iii) showed positive expression for CD73, CD90 and CD105 and negative expression for CD14, CD 34 and CD 45 in the cell surface analysis by flow cytometry. In addition to this, few other characterization studies were performed to investigate the difference between the adult and fetal derived sources. Proliferation studies (BrdU cell proliferation assay, population doubling time and growth curve analysis), stemness gene expression, senescence assay and immunocytochemical analysis were also carried out to characterize the MSCs derived from the three sources.

In the proliferation assay, both WJ and BM showed higher proliferation rate when compared to AT source. The main limitation of BM and AT source is their proliferation rate decreases with age of donor. Whereas WJ is an extra embryonic primitive source and hence the chance of variation in the proliferation rate is minimal depending on the donor. Also, WJ has a longer expansion potential compared to the other two sources. In the determination of population doubling time also, WJ had shorter doubling time followed by BM and AT source. It indicates that MSCs derived from fetal source have longer expansion potential compared to adult sources (AT and BM). The proliferation potential of MSCs is very important with regard to the application of stem cells in cellular therapy and tissue engineering. Our findings are consistent with few earlier studies also in which they reported that MSCs derived from the primitive neonatal source like Umbilical cord, placenta have higher proliferation rate compared to the adult BM and AT source (71,132). The population doubling time of WJ-MSCs are shorter than BM and AT source. Supporting our results, there are few studies in which they reported that MSCs derived from neo-natal tissues have shorter doubling time

compared to adult sources (132). In addition to this, the results were substantiated by β-galactosidase senescence assay to determine the senescence of the MSCs at various passages. In the senescence assay, AT-MSCs reached senescence at passage P11 and BM-MSCs at passage P14, whereas WJ-MSCs was more proliferative even at passage P16. It has been demonstrated that MSCs derived from the fetal source have longer expansion potential and attain senescence at later passage than the MSCs derived from adult sources. Indeed, MSCs from the birth-associated tissues exhibited superior cell biological features (proliferation, differentiation potential and delayed senescence) when compared to adult source BM-MSC (70,71). In addition to the proliferation study, MSCs derived from all the sources showed positive expression for the stemness genes (oct-4, nanog and sox-2) and other markers such as smooth muscle actin (SMA) and vimentin.

MSCs have also exhibited differences in their immunomodulation potential. It has been already mentioned that their microenvironment plays a crucial role in their immunomodulatory function (50). The immunomodulatory properties of MSCs are tightly regulated by the microenvironment or stem cell niche to maintain the homeostasis and regeneration during tissue injury. In response to tissue injury, MSCs play a major role in promoting the regeneration through cell to cell contact. Increased evidence from previous studies indicate that MSCs are able to produce factors such as extracellular matrix components, growth factors, cytokines, chemokines, extracellular vesicles and organelles which can modulate or temporarily replace regulatory signals from missing niche cells and limit their injury-induced responses at the inflammatory site (133). In an *in- vitro* condition, MSCs exhibit the immunomodulatory properties by significantly inhibiting the proliferation of PHA-stimulated PBMCs by modulating the phenotype of PBMCs and altering their cytokine secretory profile.

In line with this, our study also shows that MSCs derived from all the three sources were able to modulate cell size of the activated immune cell population as evidenced through morphological observations and flow cytometric analysis of MNCs (Figure 25(A, B, C), 26 & 27). Our proliferation and cell-size analysis results revealed that BM- and WJ-MSCs have a better potential to inhibit mitogen induced MNC proliferation. These results were consistent with few previous studies indicating that MSCs could regulate the proliferation of activated immune cells in different microenvironment (134, 109) . The inhibitory effect seen in the MNC proliferation, indicate that MSCs prevent activation of the immunocytes even under a PHA activated scenario. The biological effects of MSCs on other cells especially MNCs of the blood has been documented even in other species (135). A common observation is that MSCs suppress proliferation of stimulated MNCs, which is profound when cells are in contact. A paracrine effect was also observed to bring about the above mentioned changes in the *in-vitro* system. One plausibility for this response could be due to the expression of cytokines or other signalling molecules by the MSCs in the co-culture that directly influence MNC cell size (109). This could also mean that in the presence of MSCs, the immunocytes, both naïve and PHA activated cells, could be deprimed or deactivated resulting in the maintenance of the immunocytes in their un-proliferative state.

Further, in order to understand the activation profile of MNCs, activation markers such as CD4, CD25 were assessed in the T cells. In addition to this, the naïve T cell marker CD45RA was also assessed. It has been previously shown that $CD4^{+}25^{+\,high}$ Tcells which also express the forkhead box P3 (FOXP3) maintain phenotypic and functional characteristics of regulatory T cells (Tregs) (136). Notably, FOXP3 (an intracellular protein) expression was reported to be inversely correlated with CD127 (surface marker). Therefore, CD127 expression levels can be used for studying Treg sub population ($CD4^{+}25^{+high}CD127^{low}$) (137). Thus, the expression of $CD4^{+}CD25^{+}CD127^{low,}$ regulatory T cell markers were also studied.

In line with this, a significant upregulation of the aforementioned activation markers in the PHA activated MNCs was observed (Figure 29). Upon co-culturing them with MSCs, a significant reduction in the activation of $CD4^+$ (WJ source) and CD25+ (WJ and BM source) was observed. No apparent change in MNCs was observed for the AT source (Figure 29). However, in the naïve T cell population (CD 45RA), varied expression was observed for all the three sources: a significant increase (AT source), decrease (WJ source) and no apparent change (BM source). $CD4^+CD25^+CD127^{low}$, regulatory T cell marker expression was considered as an indicator for immunosuppressive profile of T cells. PHA activation of the co-culture system led to an increase in the T regulatory population. A similar modulation of the Treg cell population was also reported earlier (109). Together, these results show that MNCs exhibited a varied activation pattern upon co-culturing them with MSCs sourced from various human adult tissues.

Next the interest was to understand how MSCs from these three sources responded with respect to their cytokine signatures in the presence of the effector MNCs during unstimulated and stimulated conditions. It is known that MSCs are capable of modulating both systemic and local inflammatory responses (138). Here, immune modulation may happen as a two-stage process: 1) MSCs altering their own signals in accordance to the environmental milieu and 2) the effector cells responding to this altered MSC- signal overcoming the cues received from the shared milieu. Whether the MSCs alter their regulatory signals specifically to the MNCs or during PHA activation or in the presence of both effectors is of importance for the immune outcome. Indeed, recent evidence also shows that co-culturing MSCs with MNCs lead to an increase in the regulatory subset (FOXP3) indicating an immunosuppressive role for MSCs (139). There is also evidence that mitogen activation or inflammation leads to a change in the MSC cytokine profile. Paracrine factors play a central role in bringing about such differences in cellular responses. Cytokines are an

active part of the paracrine factors. Inflammatory conditions lead to an increase in the expression of these molecules in the MSCs. Additionally, it makes them (MSCs) more responsive to activation agents such as LPS, IL1-alpha, PHA etc... accordingly shifting their cytokine profile to a pro-inflammatory mode (140). At the molecular level, activation of the Toll Like Receptors (TLR) on the MSCs have been linked to increased expression of the inflammatory cytokines in the MSCs from AT and BM sources, but WJ-MSCs remained unresponsive (141). A similar response was observed for the PHA stimulated MSCs sourced from AT, BM and WJ (Figure 34A, 34B &34C).

Therefore, a need for careful consideration of the MSC source in designing immunomodulating cell-therapy is important. Irrespective of the source of origin, the role of inflammation in eliciting a response in the MSCs seems to be universal. It is, however, not clear how the interplay of signals between specific cells in an environmental milieu and the MSCs is brought about. Evidence also point to a feed forward loop for e.g. in the case of Transforming growth factor-beta1 (TGF-β1)—a cytokine that plays dual-role in inflammation. Where, upon stimulation by TGF- β1, AT-MSCs were shown to secrete more TGF- β1 and -β 2 among other immune suppressive and anti-allergic cytokines (142). Based on this, it has been posited that the pro-inflammatory environment created *in-vitro* by the addition of PHA may modulate the MSC's autocrine response to activate their immune pathway, consequently leading to an overexpression of the anti-inflammatory cytokines. Indeed, an autocrine activation mechanism has earlier been reported for WJ-MSCs in activation of the SHH pathway (143)

In this *in-vitro* study, most of the pro and anti-inflammatory cytokines were upregulated in MSCs derived from AT and BM source upon PHA activation suggesting that the *in-vitro* activation of MSCs by PHA mimics an *in-vivo* inflammatory milieu. Accordingly, up regulation of gene expression of pro- and anti- inflammatory cytokines in the PHA

activated MSCs (AT and BM) might suggest a direct response of these cells to an inflammatory milieu. In line with this, an increase in the gene expression of IFN-ϒ, TNF-α, iNOS, PDGF, HLA-G, IL-4, TGF-β, IL-10 & IDO cytokines in the PHA activated MSC group of AT source and increased expression of IL-6, iNOS, PDGF, HLA-G, IL-4, TGF-β,IL-10 and VEGF cytokines in BM source (Figure 34A &34B) was observed. Of note, a responsive cytokine profile was not observed for the WJ-MSCs in our customized array, which is consistent with the previously reported results (141) indicating that the MSCs from this extra-embryonic primitive source may have other defensive signalling mechanisms in place to contain inflammatory responses.

Then, the role of MNCs in modulating the MSCs under such inflammatory condition was observed in the co-culture system (Figure 34). The results show that there is a shift in the cytokine profile i.e., most anti-inflammatory cytokines that were originally highly expressed (in the MSC+PHA only condition) were now down regulated in the presence of MNCs (Figure 34A&34B). This pattern was observed in both the AT and BM sources. Now, interestingly, addition of MNCs to the WJ-MSCs, under activated conditions, led to a significantly high expression of both pro- and anti-inflammatory cytokines. It should be noted that the above mentioned WJ-MSC response is also a shift from its respective independent activation condition (WJ-MSC+PHA only). Nevertheless, it was observed that the presence of MNCs in an inflammatory condition is differentially modulating the MSC's responses. One plausible explanation for such observed differences in the immune responses of the MSCs from three different sources to both MNCs and PHA is their inherent differences in their surface reactive molecules

Overall, it was observed that there are MSC-cytokine-signatures representative of the source: 1) AT-MSCs show an increase in VEGF expression (~2700 fold), 2) BM-MSCs show an increase in PTGS-2 expression (albeit only ~100 fold) and a dramatic decrease in IL-6

expression ($2x10^5$ fold) signifying the role of MNCs in down regulating this pro-inflammatory cytokine in BM-MSCs, 3) WJ-MSCs show an increase in IDO gene expression (~7800 fold), when compared to their respective MSC+PHA only conditions (Table 5).

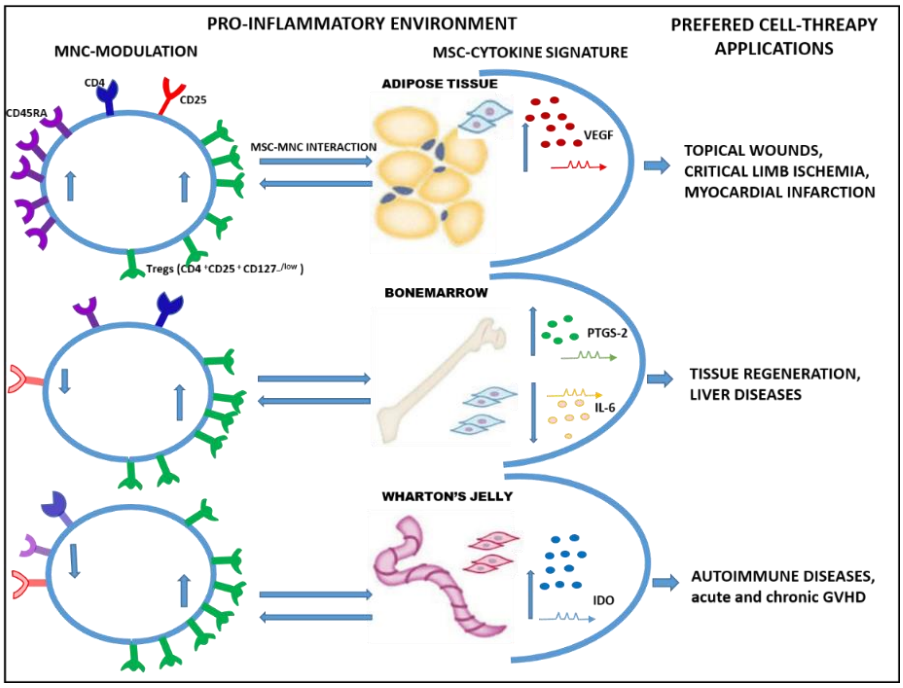


Figure 39. Diagram representing differential immunomodulation of MSCs and MNCs.

Likewise, a relative increase in gene expression of PTGS-2 in BM-MSCs under inflammatory co-culture condition signifies its role in enabling the MSCs with efficient homing abilities by virtue of its ability to modulate cell motility. In the case of WJ-MSC, the increase in IDO gene expression reflects its immune tolerance enabling characteristics: ability of IDO in protecting the fetus from the maternal immune system (144, 145). Such an increase in the IDO gene expression by WJ-MSCs observed in this study is corroborated with previous studies as well, where pro-inflammatory conditions stimulate the expression of the immunosuppressive molecule, IDO, in enhancing immune tolerance (146). This shows that MSCs from various sources are capable of differentially responding to stimulus in a context dependent manner. HLA-G, another key immunomodulator is selectively expressed on cytotrophoblasts at the feto-maternal surface and is responsible for the maternal tolerance of the fetus (100). It is highly expressed under activated conditions (co-culture as well) in MSCs

from all three sources. This shows that constitutive and inducible expression of immunomodulators act as first-line defense in immune suppression function of MSCs.

In order to validate the cytokine-signature (gene expression) of MSCs, key source-specific targets (VEGF, PTGS-2 and IDO) were quantified at the protein level. Constitutive expression of VEGF, PTGS-2, and IDO was observed in MSCs of all the three sources. The intracellular level of VEGF protein was significantly high for the AT source compared to BM and WJ. The low levels of VEGF expression at the gene and protein level in the MSCs from highly vascularized tissues such as BM and WJ show that MSCs have a unique stemness signature, which is partly determined by their homing environment. The lack of such robust VEGF gene expression in the MSCs from highly vascularized tissues such as BM and WJ shows that MSCs have a unique stemness signature, which is partly determined by their homing environment. Because adipose tissues are not highly vascularized, it is only logical to think that MSCs from this tissue might have an increased propensity to express genes that would enable increased vasculature. Thus, we propose that AT-MSCs can be sourced to treat diseases that require neo-vascularization such as topical wounds and Critical limb ischemia (CLI).

Even though BM-MSCs showed an increased gene expression (~100) for the cytokine PTGS-2 the intracellular levels of PTGS-2 protein did not show a corroborating increase upon activation in the presence of MNCs. Of course, this indicates that a post-transcriptional and or post-translational regulation is in place to limit the presence of functional PTGS-2 in this condition. It remains to be further investigated whether a complex *in-vivo* situation might enable a more functional role for PTGS-2 in suppressing immune activation by these cells. Especially, PTGS-2 has been shown to enable MSCs to efficiently home to the site of inflammation by virtue of its ability to modulate cell motility (147). In the case of the AT-MSCs the protein level of this cytokine is increased under the activated (MSC+PHA) and

activated co-culture conditions (MSC+MNC+PHA), which corroborate with the gene expression levels, indicative of a need for the presence of such immune-suppressive proteins in the inflammation-prone adipose tissue.

IDO was expressed at the highest level in the activated co-culture condition of the WJ source. However, no significant difference in the protein expression was observed between sources of different culture conditions (MSCs, MSC+PHA, MSC+MNC). In the case of WJ-MSC, the increase in IDO at the gene and protein expression levels reflects its immune tolerance enabling characteristics: ability of IDO in protecting the fetus from the maternal immune system (144, 145). Such an increase in the IDO gene expression by WJ-MSCs observed in this study is corroborated with previous studies as well, where pro-inflammatory conditions stimulate the expression of the immunosuppressive molecule (IDO), in enhancing immune tolerance (146). This shows that MSCs from various sources are capable of differentially responding to stimulus in a context dependent manner where constitutive and inducible expression of immunomodulatory molecules (VEGF, PTGS-2 and IDO) act as first-line defense in an inflammatory milieu.

In summary, this study demonstrates that the immunomodulatory properties of MSCs varies from source to source. Heterogeneous expression of cytokine was observed in the MSCs of the extra embryonic primitive and adult sources. It has been observed that modulation of T cells is synchronous with the expression of pro- or anti-inflammatory cytokines by the MSCs under activated co-culture condition, which mimics an inflammatory environment (Figure 8). Overall, this study highlights that MSCs sourced from different tissues may exhibit unique cytokine signatures and thus may be suitable for specific regenerative applications.

Articular cartilage abnormalities are becoming more common around the world, and they frequently advance to osteoarthritis leading to increased morbidity and decreased quality

of life. Autologous Chondrocyte Implantation (ACI) is currently used as a standard cell therapy for cartilage abnormalities in cell and tissue engineering approaches for cartilage regeneration. Articular chondrocytes are the only type of cells seen in the cartilage that are sparsely scattered in the ECM. In the *in-vitro* culture condition, these chondrocytes have limited proliferation potential which leads to a poor functioning and quality of the extracellular matrix. Clinical use has been limited due to other factors also such as donor tissue scarcity, donor site morbidity, chondrocyte dedifferentiation during culture, poor graft attachment to the surrounding chondral surface, and failure to restore native tissue integrity, which results in the formation of fibrocartilage that are functionally inferior to hyaline cartilage. Mesenchymal stromal/stem cells (MSCs) are considered to be the preferred cell type for cartilage tissue engineering due to their unique properties like their i) ability to differentiate to cartilage (chondrocytes) (ii) paracrine action (iii) hypo immunogenic due to the low levels of major histocompatibility complex (MHC) and costimulatory proteins which aids them to escape from the recipient's immune system. There are several pre-clinical and clinical data are available regarding the use of undifferentiated MSCs in treating various immunological and autoimmune disorders including osteo arthritis and rheumatoid arthritis. The safety and efficacy of MSCs in the treatment of OA and RA has been widely reported. The immunomodulatory and immunosuppressive properties of MSCs have been well documented but the immunomodulatory properties of differentiated MSCs are not well studied. When the MSCs are differentiated into chondrocyte, it become immunogenic and stimulate the expression of MHC I, MHC II molecules and co-stimulatory molecules (148, 149). In the recent study, it has been reported that WJ-MSCs remained hypoimmunogenic and possess immunomodulatory properties even after chondrogenic differentiation (150). Contradictory results are available regarding the immunomodulatory and immunosuppressive properties of MSCs after chondrocyte differentiation.

In this study, an attempt was made to understand the interaction of chondrocytes with immune cells in the presence of PHA stimulation. This was compared with gene profiling of undifferentiated MSCs in WJ source alone due to the ready availability of source, non-invasive collection procedure, no ethical concern, and also based on the outcome of this study. This is a preliminary data generated to understand the immunomodulatory properties of chondrocytes differentiated from MSCs of WJ source since fetal derived MSCs have longer expansion potential and plasticity compared to the adult sources. In adult source, the proliferation and differentiation capacity decrease with age. Also, UC-MSCs have potent chondrogenic potential and they were able to produce collagen thrice as much when compared to BM- source (151).

Current clinical trials using chondrocytes are at the infant stage with preliminary aim to evaluate safety, feasibility and efficacy of MSCs in cartilage regeneration. Careful consideration is mandatory regarding the expression of both pro and anti-inflammatory cytokines when the chondrocytes interact with immune cells under inflammatory conditions. It also requires to validate the expression of the same cytokines at the protein level. Pre-clinical study in large sample size using the chondrocytes in an animal model of cartilage injury is required before clinical translation. They might provide a better clarification about the immunomodulatory properties of chondrocytes in *in-vivo* condition.

SUMMARY
AND
CONCLUSION

- Mesenchymal stem cells were successfully isolated, and expanded from both primitive and adult sources.
- Isolated MSCs were characterized as per the criteria laid down by ISCT.
- In the proliferation studies, WJ have better proliferation, shorter doubling time and constant growth curve compared to AT and BM source.
- MSCs derived from all the sources showed stemness gene expression (oct-4, sox-2 and nanog).
- In the senescence assay, WJ MSCs did not reach senescence till passage 16 whereas AT reached senescence at P11 and BM at passage 14. Also in AT and BM it varies upon the age of donor.
- This study involves the investigation of human MSCs from three different sources: adipose tissue (AT), bone marrow (BM) and Wharton's jelly (WJ) with respect to phenotypic responses of human peripheral blood mononuclear immune cells (MNCs) to MSCs and concurrent changes in cytokine expressions in MSCs, under a mitogen stimulated condition by co-culturing them *in-vitro*
- There are unique differences in the immunomodulatory properties of MSCs sourced from primitive and adult sources upon crosstalk with MNCs.
- In CFSE proliferation assay, WJ and BM derived MSCs effectively suppress the proliferation of mitogen stimulated MNCs.
- In this study, flow cytometric analysis was used for studying the immunoregulatory properties of MNCs. Customized PCR array and ELISA were used to investigate the cytokine profile of the MSCs in terms of gene and protein expression.
- MSCs suppress the proliferation of mitogen stimulated MNCs and modulate the immune cells differentially: towards a more immune-suppressive phenotype.

- Results from this study reveal the differential modulation of immune cells as well as MSCs of all the three different sources upon activation by the mitogen PHA, independently and during co-culture.
- This study observes source-specific MSC-cytokine-signatures of gene and protein expression under such stimulated co-culture conditions.
- AT-MSCs up-regulate VEGF over tens of thousands of folds, BM-MSCs up-regulate PTGS-2 by hundred-fold and dramatically down regulate IL-6, and WJ-MSCs increase expression of IDO over several thousand folds through gene expression.
- HLA-G, a key immunomodulator, is the commonly expressed cytokine in all the three sources under the above-mentioned conditions.
- The source specific cytokines (VEGF, PTGS-2 and IDO) are validated and quantified at the protein level by ELISA.
- Based on the outcome of the study and considering other advantages of the WJ source such as large volume availability, non-invasive collection process without ethical concern, high proliferation and differentiation potential (especially chondrogenic), an attempt was made in investigating the immunomodulatory properties of chondrocytes differentiated from WJ-MSCs under the same co-culture condition.
- This study points to the fact that while chondrocytes were able to modulate immune cells by lowering the levels of pro-inflammatory cytokines, their ability to increase the levels of anti-inflammatory cytokines was not significant compared to undifferentiated MSCs. It requires further validation at the level of intracellular or secretory protein expression to confirm this results.

- Hence, further studies are warranted to validate the study observation in protein expression and pre-clinical studies.
- The source-dependent specificity of MSCs in their immunomodulatory abilities will enable optimal use of MSCs in cell-based therapies.
- The results also highlight the prospect of choosing source specific MSCs for various regenerative treatments.
- Since clinical trials using chondrocytes or undifferentiated MSCs for the treatment of cartilage degeneration are at the infant stage with preliminary aim to evaluate safety, feasibility and efficacy of MSCs in cartilage regeneration.
- Hence, more studies are required to understand the immunogenicity and immunomodulatory properties of chondrocytes after differentiation.
- Extensive investigation regarding the expression of both pro and anti-inflammatory cytokines when the chondrocytes interact with immune cells under inflammatory conditions in *in-vitro* is mandatory before clinical translation.
- Pre-clinical study in large sample size using the chondrocytes in an animal model of cartilage injury is required before clinical translation. They might provide a better clarification about the immunomodulatory properties of chondrocytes in *in-vivo* condition.
- Overall, although AT- and BM-MSCs can be sourced autologously and do exhibit characteristic immunomodulatory properties, this study points to WJ-MSCs, a non-invasive source, to be a superior candidate among the three sources for most cell-therapy based applications
- Further clinical research using Wharton's jelly derived MSCs as a therapeutic option is worth pursuing.

Future directions

Mesenchymal Stem Cells have been considered as the potential candidate of regenerative medicine in treating various autoimmune and immunological disorders are mainly due to their differentiation potential, homing potential and immunomodulatory properties. This study finds out the unique differences in the immunomodulatory potential of MSCs derived from the primitive and adult sources. The study generated some preliminary data on the immunomodulatory potential of chondrocytes after differentiation from the WJ-MSCs by co-culturing them *in-vitro* with immune cells. The study also points out the advantage of WJ source to be used in future studies involving clinical translation. Clinical trial for the treatment of any diseases will be requiring huge volume of cells and it is possible if we consider the WJ source for the isolation compared to AT and BM which requires invasive collection from the patient who are already ill. It is also possible to generate allogenic stem cell bank using WJ-MSCs by cryopreserving the millions of MSCs which might be helpful for the clinical translation which requires multiple infusions.

According to clinicaltrials.gov, there are 1285 (as of June 2021) clinical trials are on-going by using Mesenchymal Stem Cells (MSCs) as a candidate for cellular therapy in treating various ailments such as GVHD, Crohn's disease (CD), Type I Diabetes mellitus, rheumatoid arthritis (RA), osteoarthritic, Multiple sclerosis, SLE etc... Most of the studies reporting the safety and efficacy of MSCs are mainly due to their immunomodulatory potential. The current study can be taken to the next level by investigating the immunomodulatory potential of MSCs in *in-vivo* pre-clinical animal model such as inflammatory bowel diseases (Crohn's disease and ulcerative colitis). Since our research Centre is attached with surgical gastroenterology department, the outcome of the future studies in animal model will be helpful in clinical trial involving the treatment of patients affected with IBD and hence will benefit the low-socio economic group.

The immunomodulatory and immunosuppressive properties of MSCs has been well documented but the immunomodulatory properties of differentiated MSCs are not well studied. It has been reported in few studies that when these MSCs are differentiated into chondrocyte, it become immunogenic and stimulate the expression of MHCI, MHCII molecules and co-stimulatory molecules. Current clinical trials using chondrocytes are at the infant stage with preliminary aim to evaluate safety, feasibility and efficacy of chondrocytes in cartilage regeneration. Careful consideration is mandatory regarding the expression of both pro and anti-inflammatory cytokines when the chondrocytes interact with immune cells under inflammatory conditions. It also required to validate the expression of the same cytokines at the protein level. Pre-clinical study using the chondrocytes in an animal model of cartilage injury is required before clinical translation. They might provide a better clarification about the immunomodulatory properties of chondrocytes in in-vivo condition. Therefore, further studies are warranted to investigate the chondrocytes differentiated from MSCs to understand mechanism of action in an *in-vivo* environment.

In addition to this, the recent emerging interest in the regenerative medicine now focusses on cell free therapy using the secretome secreted by MSCs. Hence, the variations in the composition of growth factors, membrane vesicles, proteasomes, exosomes, microRNA etc in the secretome of MSCs derived from various sources can be studied as an extension of this project which might be helpful in investigating the potential of cell free therapy for wound healing such as non-healing ulcers, diabetic foot ulcers etc.

From this study, it is concluded that further pre-clinical and clinical studies can be pursued by using the MSCs derived from the WJ source.

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ANNEXURES

1. Institutional Ethical Committee (IEC) clearance for the study

INSTITUTIONAL ETHICAL COMMITTEE
STANLEY MEDICAL COLLEGE, CHENNAI-1

Title of the Work	: Characterization of Adult Human Mesenchymal stem cells.
Investigator	: Raja Sundari
Designation	: Research fellow
Guide	: Dr. Rosy Vennila MD, Prof of Microbiology & Professor Research
Department	: Stem Cell Research Centre Government Stanley Medical College, Chennai-01

The request for an approval from the Institutional Ethical Committee (IEC) was considered on the IEC meeting held on 02.07.2014 at the Council Hall, Stanley Medical College, Chennai-1 at 2PM

The members of the Committee, the secretary and the Chairman are pleased to approve the proposed work mentioned above, submitted by the Principal Investigator.

The Principal Investigator and their team are directed to adhere to the guidelines given below:

1. You should inform the IEC in case of changes in study procedure, site investigator investigation or guide or any other changes.
2. You should not deviate from the area of the work for which you applied for ethical clearance
3. You should inform the IEC immediately, in case of any adverse events or serious adverse reaction.
4. You should abide to the rules and regulation of the institution(s).
5. You should complete the work within the specified period and if any extension of time is required, you should apply for permission again and do the work.
6. You should submit the summary of the work to the ethical committee on completion of the work.

K Vasanthi
MEMBER SECRETARY,
IEC, SMC, CHENNAI

2. Institutional Committee for Stem Cell Research (IC-SCR) clearance for the study

INSTITUTIONAL COMMITTEE FOR STEM CELL RESEARCH

STEM CELL RESEARCH CENTRE, GOVT STANLEY HOSPITAL, CHENNAI-01.

1. a. Proposal Number: 01/2017

b. Date of Receipt: 10/07/2017

c. Title of the Proposal: **Characterization of Adult Human Mesenchymal Stem Cells - Immunological Profile of Adult Human Mesenchymal stem cells derived from various sources –Bone marrow, Adipose tissue, and Umbilical cord Matrix/Blood.**

d. Date of Review: 25/07/2017

2. Institutional Ethical Committee clearance obtained: Yes

3. Status of the Proposal: APPROVED / NOT APPROVED

3.1. If Not Approved, reason for Not Approval:

3.2. Suggestions:

Proposal approved by : (Details of the IC-SCR members)			
Name	**IC-SCR Designation**	**Area(s) of Expertise**	**Signature**
Dr. T. S. Surendran	Chairperson	Vice Chairman, Medical Research Foundation	
Dr. R. Ravi	Member Secretary	Expert Surgical Gastroenterologist	
Justice Thiru. Masilamani	Law Expert	Law	
Thiru. R. Swaminathan	Ethics Expert	Expert in Organ sharing boards	
Esther Mariaselvam	Social Scientist	Social Sciences	
Dr. T. A. Kannan	Stem Cell Expert	Expert Stem Cell Research	
Dr. P. Ravichandran	Member	Expert Surgical Gastroenterologist	
Dr. M. S. Revathi	Member	Expert Hepatologist	
Dr. R. Murali	Member	Expert Medical Gastroenterologist	

3. Informed consent for Adipose tissue Resection

Stem Cell Research Centre

Govt. Stanley Hospital

I am aware that the excess accumulated fat tissue in my body (Subcutaneous & Omentum fat) will be resected during the surgical treatment. I consent for this procedure, and I also give consent to use the resected portion for any research purpose including Stem Cell Research. I am aware that any cell or its derivative in the sample will be used only for research and may or may not be applicable for my treatment.

நான் எனது உடலில் இருக்கும் மிகுதியான கொழுப்பு திசு அறுவை சிகிச்சை போது அகற்றப்பட வேண்டும் என்பதை அறிவேன். என்னுடைய அகற்றப்பட்ட கொழுப்பானது மூல செல் ஆராய்ச்சி உட்பட ஆராய்சிகளுக்கு பயன்படுத்த சம்மதிக்கிறேன். மற்றும் என்னில் இருந்து பெறப்பட்ட கொழுப்பானது ஆராய்ச்சி பணிகளுக்காக மட்டும் பயன்படுமே தவிர என்னுடைய சிகிச்சைக்கு உதவாது என்பதையும் நன்கு அறிவேன்.

Doctor’s Signature Signature

Date:

Place:

4. Informed consent for Bone Marrow
Stem Cell Research Centre

Govt. Stanley Hospital

I consent for the aspiration of my bone marrow for research purpose. I am informed that tests for detecting the presence of stem cells will be done. I have been informed by the treating doctors that stem cell treatment for such conditions is at the research stage. I am aware that any cell or its derivative in the sample will be used only for research and not for my treatment.

நான் ஆராய்ச்சி நோக்கதிற்காக என்னுடைய எலும்பு மஜ்ஜையை எடுத்துகொள்ள ஒப்புதல் அளிக்கிறேன். என்னுடைய எலும்பு மஜ்ஜையானது மூல செல் ஆராய்ச்சி உட்பட ஆராய்சிகளுக்கு மட்டுமே தவிர என்னுடைய சிகிச்சைக்கு அல்ல என்பதையும் மருத்துவர்கள் மூலம் நன்கு அறிவேன்.

Doctor's Signature Signature

Date

Place:

5. Informed Consent for Umbilical Cord and Cord Blood

Stem Cell Research Centre

Govt. Stanley Hospital,

I am aware that after delivery the Umbilical cord and placenta serves no purpose to me and hence will be discarded and I have been informed that the Umbilical cord and its contents can be used for research purposes. I hereby give consent to use the umbilical cord and its contents for Stem Cell Research at Stem Cell Research Centre, Government Stanley Hospital, Chennai.

எனது பிரசவத்தின் போது வெளியேறும் நஞ்சுக்கொடியானது எனக்கு உபயோகமில்லை எனவும் அது கழிவாக ஒதுக்கப்படுகிறது என்பதையும் அறிகிறேன். அதனை பல்வேறு ஆராய்ச்சிகளுக்கு பயன்படுத்த முடியும் என்பதால் ஸ்டெம் செல் ஆராய்ச்சி போன்ற ஆராய்ச்சிகளுக்கு, சென்னையில் உள்ள அரசு ஸ்டான்லி மருத்துவமனையில், ஸ்டெம் செல் ஆராய்ச்சி மையத்தில் பயன்படுத்திக்கொள்ள சம்மதிக்கிறேன்.

மருத்துவரின் கையொப்பம்

நோயாளி, உறவினர்
கையொப்பம் மற்றும் பெயர்

6. Adipogenic differentiation medium

Preparation of Stock Dexamethasone Solution (Concentration: 10^{-4}M)

Dissolve 1mg of the Dexamethasone powder in a small volume of absolute ethanol and make up with ethanol to a final volume of 25.5 ml. Aliquot in multiple 500µl vials and store at -20°C.

Preparation of Stock Indomethacin Solution (Concentration: 10 Mm)

Dissolve 89.45mg of Indomethacin powder in 5 ml of ethanol. Aliquot in 100µl vials. Store at 4°C.

Preparation of Stock IBMX Solution (Concentration: 40 Mm)

Dissolve 88.88mg of IBMX in 10 ml of ethanol. Aliquot in 625µl vials. Store at 20°C.

Preparation of Stock Insulin Solution (Concentration: 1.4mg/ml)

Aliquot the insulin in 370µl vials. Store at 4°C

Media Preparation

DMEM	-	44.0ml
FBS	-	5.0ml
Dexamethasone	-	500µl
3-Isobutyl–1–methyl xanthine	-	625µl
Insulin	-	370µl
Indomethacin	-	100µl
Penicillin Streptomycin	-	50µl
Total Volume		**50ml**

All the above components are mixed well and filtered through a 0.22 µm filter into a sterile container.

7. Osteogenic differentiation medium

Preparation of Stock Dexamethasone Solution (Concentration: 10^{-4}M)

Dissolve 1mg of the Dexamethasone powder in a small volume of absolute ethanol and make up with ethanol to a final volume of 25.5 ml. Aliquot in multiple 500µl vials and store at -20°C.

Preparation of Stock Ascorbate Solution (Concentration: 10mg/ml)

Dissolve 100mg of ascorbic acid powder in 10 ml of sterile water. Aliquot in 250µl vials. Store at -20ºC.

Preparation of Stock β-Glycerophosphate Solution

Concentration: 1M

Dissolve 2.16gm of beta Glycerophosphate powder in 10ml of water. Aliquot in 500µl vials. Store at -20ºC.

Media Preparation

DMEM LG	-	42.0 ml
FBS	-	7.5 ml
Dexamethasone	-	5.0 µl
β – Glycerophosphate	-	250 µl
Ascorbic acid	-	250 µl
Penicillin Streptomycin	-	50 µl
		50 ml

All the above components are mixed well and filtered through a 0.22 µm filter in to a sterile container.

8. Chondrogenic differentiation Medium

Chondrocyte Differentiation Basal Medium (A10069-01) - 90ml

Chondrogenesis Supplement (A10064-01) - 10 mL

Penicillin streptomycin - 50 µl.

Preparation of staining solution

1. Oil Red O Stain preparation

(i) Preparation of stock:

Oil Red O Stain - 0.25g

Isopropanol - 50ml

(ii) Preparation of working solution

Mix 3:2 ratio of Oil Red O stock stain and distilled water. (Working stain solution is stable only for 2 hours at 25^{o}C)

Incubate the stain/water mixture for 10 minutes at 25^{o}C.

Wet the filter paper with water and filter the stain solution using a filter paper.

2. Alizarin Red Stain Preparation:

Alizarin Red Stain - 2.0 g

Distilled water - 50 ml

Adjust the pH to 4.2 using Ammonium hydroxide.

3. Alcian blue Stain preparation

Alcian blue -0.1g

0.1N HCL-10ml

4. 4% Paraformaldehyde

4 grams of paraformaldehyde dissolved in 100 ml of PBS and dissolve it by keeping in water bath at 75^{0}C for 20 minutes.

5. 0.1% Triton X-100

0.1 ml of Triton X -100 dissolved in 99.9 ml of PBS.

9. Paper publication

1. **Differential immunomodulation of Human Mesenchymal Stromal Cells from various sources in an inflammation mimetic milieu** – Manuscript under second revision in an international journal *Cytotherapy* **Raja Sundari Meenakshi Sundaram[1],** Kadapakkam Nandabalan Sangeetha[1], Secunda Rupert[1], Prasanna Srinivasan[1], Pavithra Sankar[1], Bamdeb Patra[2], Rama Shankar Verma[2], Rosy Vennila[3], Jeswanth Sathyanesan[1], Surendran Rajagopal[4]

   (Impact factor – 4.297)

2. **Effect of Human Platelet Lysate in Differentiation of Wharton's Jelly Derived Mesenchymal Stem Cells**

   Rosy Vennila, **Raja Sundari M. Sundaram** , Sakthivel Selvaraj, Prasanna Srinivasan , Surajit Pathak, Secunda Rupert, and Surendran Rajagopal *Endocrine, Metabolic & Immune Disorders - Drug Targets*, 2019, 19, 1177-1191 PMID: 30819088 DOI: 10.2174/1871530319666190226165910. **(Impact factor -1.821)**

3. **Isolation, expansion and characterisation of mesenchymal stem cells from human bone marrow, adipose tissue, Umbilical cord blood and matrix-a comparative study**

   R. Secunda, Rosy Vennila, A.M. Mohanashankar, **M. Rajasundari** S. Jeswanth,

   R. Surendran October 2015, Volume 67, Issue 5, pp 793-807 PMID: 24798808 PMCID: PMC4545441 DOI: 10.1007/s10616-014-9718-zdoi:10.1007/s10616-014-9718-z **Impact factor (1.891)**

4. **Human Mesenchymal stem cells: A comparative study of their isolation, expansion and characterization from the Blood and Matrix of the Umbilical cord,**

   Rosy Vennila, R. Secunda, **M. Rajasundari**, S. Jeswanth, R. Surendran *Stanley Medical Journal, Vol 1, Issue 1* Sep 2014

5. Presented poster in ISCT 2021 New Orleans VIRTUAL Meeting held between May 26 – 28 (2021) on the topic **"Use of Platelet lysate as an alternative for FBS in generating Clinical Grade Mesenchymal Stem Cells – A Pilot study"**

10. Seminars and Workshop

- Attended the short term course sponsored by AICTE on **"Recent Advances in Stem Cells and Tissue Engineering"** organized by the Department of Biotechnology, Indian Institute of technology, Madras (IIT-M) from 24.08.2020 – 28.08.2020
- Completed training in cGMP facility of Stempeutics Research Pvt Ltd, Manipal from 06th May to 17th May 2019 and observed the manufacturing of Clinical grade Human Mesenchymal Stem Cells(CGMSCs) from Bone marrow.
- Completed the course on "Research Methodolgy and Bio-statistics" conducted by Tamilnadu Dr.M.G. R Medical University.
- Completed the Online certificate course on "Good Clinical Practice" by NIDA Clinical trials Network.
- Attended the Seminar on "National Ethical guidelines For Biomedical and Health Research involving Human Participants –ICMR 2017 Guidelines" Conducted by Tamilnadu Dr.M.G.R Medical Universtity.
- Attended CME on "Good Clinical Practice and Clinical Trials" Conducted by Department of Pharmacology, Govt. Stanley Medical College.
- Presented paper in National conference on "Recent Advances in Applied Sciences" organized by Dr.M.G.R Educational and Research Institute on the topic " Isolation, expansion and Characterization of Mesenchymal Stem Cells from Umbilical cord matrix by non-enzymatic method".
- Actively participated and involved in the demonstration during CME cum Workshop on "Flow cytometry and its applications" organized by Stem Cell Research Centre.
- Attended workshop on "A to Z in Stem Cells" conducted by Dr.Iqbal Ahmed,PhD, University of Nebraska Medical Centre, Omaha, USA on 17.06.19 at Centre for Medical Genetics.
- Presented Poster in International Stem cell summit India on the topic **"Isolation of amniotic derived Mesenchymal stem cells and its protein Profiles"** organized by Indian Institute of Technology Madras &Lifeline hospital, held at IITM, Chennai from 14.09.2008-16.09.2008.
- Attended 30 lectures at the Seminar cum workshop series from 21.09.2013 to 30.11.2013 organised by Stem Cell Research Centre, Govt. Stanley hospital, Chennai.
- Presented paper in National conference on "Recent Advances in Applied Sciences" on the topic "Isolation, expansion and characterisation of Mesenchymal stem cells from Umbilical cord matrix by non-enzymatic method" organised by Dr.M.G.R Educational and Research Institute, Maduravoyal, Chennai from 10.02.2012-11.02.2012.

DOCTORAL COMMITTEE

Guide : **Prof. R. Surendran M. S, MNAMS**
Director, Department of Hepatobiliary Sciences,
MIOT International Hospital, Chennai &
Former Principal Investigator, Stem Cell Research Centre (SCRC),
Government Stanley Hospital (GSH), Chennai

Co-guide : **Prof. Rosy Vennila M.D.,**
Dean, Karur Medical College (retired) &
Former Principal Investigator, Stem Cell Research Centre (SCRC),
Government Stanley Hospital (GSH), Chennai

Members : **Prof. Jeswanth Sathyanesan, M. S, M. Ch (GI Surgery).,**
Professor and Head,
Institute of Surgical Gastroenterology (SGE),
Government Stanley Hospital, Chennai

: **Prof.K. Kumanan M.V.Sc, Ph.D.(Stem Cell Research expert member)**
Dean of Basic Sciences,
Tamilnadu Veterinary and Animal Sciences University (TANUVAS),
Madras Veterinary College (MVC), Chennai

CURRICULUM VITAE

NAME : RAJA SUNDARI .M

DATE OF BIRTH : 07-07-1985

EDUCATION n

Master of Science in Biotechnology **2006 – 2008 (76%)**

Bharathiyar University, Coimbatore, Tamilnadu- 641 046?()?) ()FFFFFFF()()F

Bachelor of Science in Microbiology **2003 – 2006 (79%)**

(University third rank and Gold Medalist)

Madurai Kamaraj University, Madurai, Tamilnadu-625016

Diploma in Clinical nutrition and Dietetics **(2003-2006)**

Madurai Kamaraj University, Madurai, Tamilnadu-625016

RESEARCH EXPERIENCE e

Senior Research Fellow **07/2011 – till date**

Stem Cell Research Centre, Government Stanley Hospital,

Chennai -600 001

Projects involved

1. **Clinical Trial-Use of Adult Stem Cells in Patients with End Stage Liver Disease** funded by Tamilnadu Innovation Initiatives (TANII) (on-going)
2. **Hepatocyte progenitor cells isolation from various sources, characterization expansion and transplantation** funded by Indian Council of Medical Research (ICMR), New Delhi.

Senior Research Fellow **10/2010 – 06/2011**

Department of Aquaculture,

Fisheries College and Research Institute (FCRI, TANUVAS),

Thoothukudi, Tamlinadu.

Project worked - **Quantitative Infectivity potential assessment of WSSV isolates and genotyping of virulent strains** funded" funded by Department of Biotechnology (DBT), New Delhi.

Senior Research Fellow **09/2008 -09/2010**

Department of Animal Biotechnology

Tamil Nadu University of Veterinary & Animal Science,

Madras Veterinary College, Chennai, Tamilnadu

Project worked - **Application of Mesenchymal stem cells in induced Mice skin and burn wound healing funded by** Department of Biotechnology (DBT), New Delhi.

M.SC Dissertation work - "Isolation of Amniotic Membrane Derived Mesenchymal Stem Cells and its Molecular Profile" in the Department of Animal Biotechnology, Tamil Nadu University of Veterinary & Animal Science, Chennai, Tamilnadu (Feb 2008).

Number of publications in the area of stem cell biology – 10