

Faculté de médecine et médecine dentaire

In vitro analysis of immunomodulating properties of dental stem cells : A comparative approach to the transcriptome and secretome of four dental stem cells populations

Auteur : Haulot Thomas
Promoteur·rices : Pr. des Rieux et Pr. Leprince
Année académique 2022-2023
Master 180 en médecine, à finalité spécialisée

Abstract :

Since several years, the newly discovered immunomodulatory properties of mesenchymal stem cells (MSC) have interested scientists. Studying those properties allow scientists to better understand immunological processes and to generate a new therapeutic arsenal. The aim of this project was to compare the immunomodulatory properties of different MSC in a pro-inflammatory environment versus their quiescent form. For this purpose, we compared MSC of dental origin : RP89, MSC of the apical papilla (SCAP), of the dental pulp (DPSC) and in a novel way, of pathological dental tissues affected by dental granuloma (Granuloma-derived stem cells). Their properties were compared to adipose tissue stem cells (ADSC), the current gold standard in regenerative therapy. In our research, the presence of immunomodulation was observed in all tested modalities. The enzymatic test carried out showed a statistically significant increase in the activity of indoleamine-2,3-dioxygenase (IDO). Transcriptome analysis by real time-quantitative polymerase chain reaction (RT-qPCR) of two genes (TSG-6 and TGF- β) involved in immunomodulation also showed an increase in TSG-6 mRNA level, similarly between ADSC and dental MSC. No statistical difference was found TGF- β mRNA despite a significant biological upregulation in activated conditions. The study of the secretion of interleukin (IL)-6 and 10 was allowed by ELISA study. Interestingly, no IL-10 secretion in the supernatant was detected from MSCs contrasting whilst IL-6 was found to be significantly increased in every activated condition supernatant, especially in activated GDSC. In conclusion, this project highlights the non-inferiority of dental MSC of healthy and of pathological origin in terms of *in vitro* immunomodulation compared to ADSC. Dental MSC, even of pathological origin, could thus complement the arsenal of available MSC and provide unique and promising properties in regenerative and immunological research.

Acknowledgments :

My work in biomedical research started five years ago in the LNR laboratory of Pr. Nicaise at the Namur University, which allowed me to become familiar with this universe. At the end of my three years as a bachelor, I had the great opportunity to continue my scientific training by joining the AADB team of Pr. des Rieux and Pr. Leprince.

Naturally, I wanted to sincerely thank Pr. des Rieux for accepting me in her team, for directly supervising me throughout my project, for giving me precious advices and especially for transmitting me her unique vision of research that I aspire to have. Of course, I wanted to thank Prof. Leprince for being present during this project, for passing on his passion for dental research and for his enthusiasm throughout the project.

I was very lucky to meet passionate colleagues who were able to guide me in my project despite my discontinuous presence. I would like to thank every PhD student of the AADB laboratory and particularly Viridiane, Marie, Auriane and Floriane who were able to guide me during this project and answer my numerous questions. My sincere thanks go to every member of the team of the AADB including Bernard, Kevin, Nathalie and Pierre who helped me without hesitation in the good realization of my experiments.

Finally, I would like to thank Maxime and my family for their attentive listening and without whom this would not have been possible. During my studies, their presence helped me to persevere in the most difficult moments and for that, I will be forever grateful.

The illustrations in this thesis were made with the help of Biorender.com.

Table of contents :

I.	INTRODUCTION.....	1
I.1	STEM CELLS BIOLOGY	1
I.1.1	<i>Identity of stem cells</i>	1
I.2	DIFFERENTIATION MECHANISM.....	2
I.3	USAGE OF STEM CELLS.....	3
I.4	MESENCHYMAL STEM CELLS.....	4
I.4.1	<i>Definition of mesenchymal stem cells</i>	4
I.4.2	<i>hMSC potency</i>	5
I.4.3	<i>Types of mesenchymal stem cells</i>	5
I.4.3.1	Distribution of MSC in the organism.....	5
I.4.3.2	Bone marrow stem cells.....	6
I.4.3.3	Adipose tissue stem cells	7
I.5	TEETH ANATOMY AND HISTOLOGY	7
I.5.1	<i>Histo-anatomy of the tooth and its annexes</i>	7
I.5.2	<i>Dental stem cells</i>	8
I.5.2.1	Healthy tooth stem cells	8
I.5.3	<i>Granuloma stem cells</i>	10
I.6	APPLICATIONS OF MESENCHYMAL STEM CELLS	11
I.6.1	<i>Regenerative therapies</i>	11
I.6.2	<i>Cell biology study models</i>	12
I.7	IMMUNOMODULATING PROPERTIES OF MESENCHYMAL STEM CELLS	13
I.7.1	<i>Immunomodulation of mesenchymal stem cells</i>	13
I.7.1.1	Discovery of MSC immunomodulation	13
I.7.1.2	Immunological properties	13
I.7.2	<i>Emphasis on immunomodulators</i>	15
I.7.2.1	The Kynurenine pathway	15
I.7.2.2	Cytokines	16
I.7.2.2.1	Interleukine (IL)-10	16
I.7.2.2.2	Interleukin (IL)-6	16
I.7.2.2.3	Tumor growth factor- β (TGF- β)	17
I.7.2.2.4	Tumor growth factor-inducible protein 6 (TSG-6).....	17
II.	OBJECTIVES	18
III.	MATERIALS AND METHODS	19
III.1	ETHICS STATEMENT	19
III.2	CONSERVATION OF HUMAN TISSUES SAMPLES	19
III.3	SCAP ISOLATION FROM FRESH EXTRACTED WISDOM TEETH.....	20
III.4	SCAP INCUBATION AND OVERGROWTH	20
III.5	CELL FREEZING PROTOCOL	21
III.6	CELL DEFROSTING AND CULTURE PROTOCOL.....	21
III.7	REAGENTS AND CYTOKINES	21
III.8	FLUORESCENCE ACTIVATED CELL SORTING (FACS) PROTOCOL.....	22
III.9	TUMOR-NECROSING FACTOR- α AND INTERFERON- γ STIMULATION PROTOCOL.....	22
III.10	INDOLEAMINE 2,3-DIOXYGENASE (IDO) ACTIVITY ASSESSMENT	24
III.11	REAL-TIME QUANTITATIVE POLYMERASE CHAIN REACTION (RT-QPCR)	24

III.12	INTERLEUKINE-6 (IL-6) AND INTERLEUKINE-10 (IL-10) ENZYME-LINKED IMMUNO SORBENT ASSAY (ELISA)	25
III.13	STATISTICAL ANALYSES.....	26
IV.	RESULTS	26
IV.1	SCAP ISOLATION FROM FRESH EXTRACTED WISDOM TEETH.....	26
IV.2	TNF- α + IFN- γ CELL STIMULATION PROTOCOL	26
IV.3	ANALYSIS OF MSC MARKERS BY FACS	26
IV.3.1	<i>Side scattering (SSC) and forward scattering (FSC).....</i>	27
IV.3.2	<i>Mesenchymal stem cells positive markers</i>	28
IV.3.3	<i>Mesenchymal stem cells negative markers.....</i>	29
IV.4	IMPACT OF PRO-INFLAMMATORY STIMULATION ON IDO ACTIVITY	30
	IMPACT OF PRO-INFLAMMATORY STIMULATION ON TSG-6 AND TGF- β GENES EXPRESSION.....	31
IV.5.....		31
	IMPACT OF PRO-INFLAMMATORY STIMULATION ON IL-6 AND IL-10 SECRETION.....	33
IV.6.....		33
V.	DISCUSSION:	35
V.1	DENTAL STEM CELL ISOLATION AND CHARACTERIZATION.....	35
V.2	INCREASED IDO ACTIVITY UPON PRO-INFLAMMATORY STIMULATION.....	36
V.3	MODULATION OF TSG-6 AND TGF- β GENES EXPRESSION UPON PRO-INFLAMMATORY STIMULATION	38
V.4	INCREASED IL-6 SECRETION IN ACTIVATED STEM CELLS SUPERNATANTS WITH SURPRISING ABSENCE OF IL-10	39
VI.	CONCLUSIONS AND PERSPECTIVES	40
VII.	REFERENCES	42

Abbreviations :

(c)DNA : (complementary) deoxyribonucleic acid
(i)PSC : Pluripotent stem cells
(m)RNA : (messenger) ribonucleic acid
3-HAA : 3-hydroxyanthralinic acid
3-HK : 3-hydroxykynurenine
ADSC : Adipose tissue-derived stem cells
ANOVA : Analysis of variance
APC : Allophycocyanin
BM- MSC : Bone marrow mesenchymal stem cells
BSA : Bovine serum albumin
CD : Cluster of differentiation
CFU-F : Colony-forming units-fibroblastic
CTL : Cytotoxic T lymphocyte
DAB : 4-dimethylaminobenzaldehyde
DAMP : Damage-associated molecular patterns
DMEM : Gilco's Dulbeco's Modified Eagle Medium
DMSC : Dental mesenchymal stem cell
DMSO : Dimethylsulfoxide
DPBS : Dulbeco's phosphate buffered-saline
DPSC : Dental pulp stem cell
EDTA : Ethylenediaminetetraacetic acid
ELISA : Enzyme-linked immuno-sorbent assay
ESC : Embryonic stem cell
FACS : Fluorescence activated cell sorting
FBS : Fetal bovine serum
FITC : Fluorescein isothiocyanate
FSC : Forward scattering
GDSC : Apical Granuloma derived stem cells
GF : Gingival fibroblast
hAM-MSC : Human amniotic membrane mesenchymal stem cell
HLA : Human leucocyte antigen
HRP : Horseradish peroxidase
IDO : Indoleamine 2,3-dioxygenase
Ig(M/G) : Immunoglobulin (M/G)

IL : Interleukin
INF- γ : Interferon-gamma
ISCT : International society for cells and gene therapy
KYNA : Kynurenic acid
KYNA : Kynurenic acid
L-Kyn : L-Kynurenine
M/V : Mass-to-volume
MCF-7 : Mammary carcinoma cells – 7
MHC : Major histocompatibility complex
MLR : Mixed lymphocyte reaction
MSC : Mesenchymal stem cells
NK : Natural killer
NO : Nitrogen monoxide
PBMNC : Peripheral blood mononuclear cells
PBS : Phosphate – buffered saline
PDLSC : Periodontal ligament stem cells
PE – A : Phycoerythrin
PerCP : Peridinin chlorophyll
PRR : Pathogen-recognition receptors
QQ : Quartile-quartile
QUIN : Quinolinic acid
RPL19 : 60S ribosomal protein L19 gene
RT-qPCR : Real-time quantitative polymerase chain reaction
SCAP : Stem cells from the apical papillae
SOCS3 : Suppressor of cytokine signaling-3
SSC : Side scattering
TCA : Trichloroacetic acid
TGF- β : Tumor growth factor-beta
Th(1/2/17): Helper T lymphocyte 1/2/17
TNF- α : Tumor necrosing factor-alpha
Trp : Tryptophan
TSG-6 : Tumor necrosis factor-stimulated gene 6
V/V : Volume-to-volume

I. Introduction

I.1 Stem cells biology

I.1.1 Identity of stem cells

A cell has been defined as the smallest entity dividable in an organism. Even though the exact number of cells inside a human body has not been well computed yet, it has been accepted that that number may exceed 3×10^{13} cells [1]. Those cells derive from the first cell, the zygote, formed by the fusion of the parental gametes.

Four days after, the blastocyst forms. The blastocyst is formed by the arrangement of cells forming the trophoectoderm (forming the future placenta of the fetus) and the inner cell mass. Undergoing multiple mitosis, this inner mass cells will allow the formation of three distinct tissues forming the embryo: the ectoderm, mesoderm and endoderm. Each of these embryonic tissues will form a cluster of organs.

A stem cell has been defined as a cell capable of virtually endless self-renewal and differentiation, thus forming new tissues, allowing organ regeneration. Cell differentiation refers to a process at the end of which a stem cell acquires new phenotypic and functional characteristics, guiding it towards a totally functional form.

The cells inside the organism can then be organized in a four-fold manner:

- According to their embryonic origin : Ectodermic, mesodermic and entodermic;
- According to their division origin : Gametes or somatic cells;
- According to their differentiation potency : Differentiated or undifferentiated.
- According to their organ/tissue origin

I.2 Differentiation mechanism

Stem cells can be classified regarding their potency, capacity directly proportional to the amount of cell types they can differentiate into [2]. As explained above, the zygote and the cells derived from the two first divisions, are capable of forming an entire organism. Therefore, they have been named totipotent¹ as they can form any cell and tissue in the body. Once the blastocyst is formed, the embryonic inner mass cells can be isolated, cultured and exhibit endless division properties and differentiation, thus can be categorized as embryonic stem cells (ESC). ESC are pluripotent² as they can differentiate into the three embryonic tissues but cannot form the extra-embryonic tissues. As the embryo has formed and as the fetus is developing, an isolated cell from fetal tissues, from one specific germ layer, can still show signs of limitless proliferation but is now, theoretically, incapable of dividing into the two other embryonic layers. Therefore, this stem cell of fetal origin is called multipotent³. Oligopotent⁴ stem cells are adult stem cells capable to form several cells in the same lineage. Finally, unipotent⁵ stem cells are stem cells also found in an adult tissue whose purpose is to renew it by differentiating into one type of cells [2]. Stem cell differentiation mechanism is summarized by the figure 1.

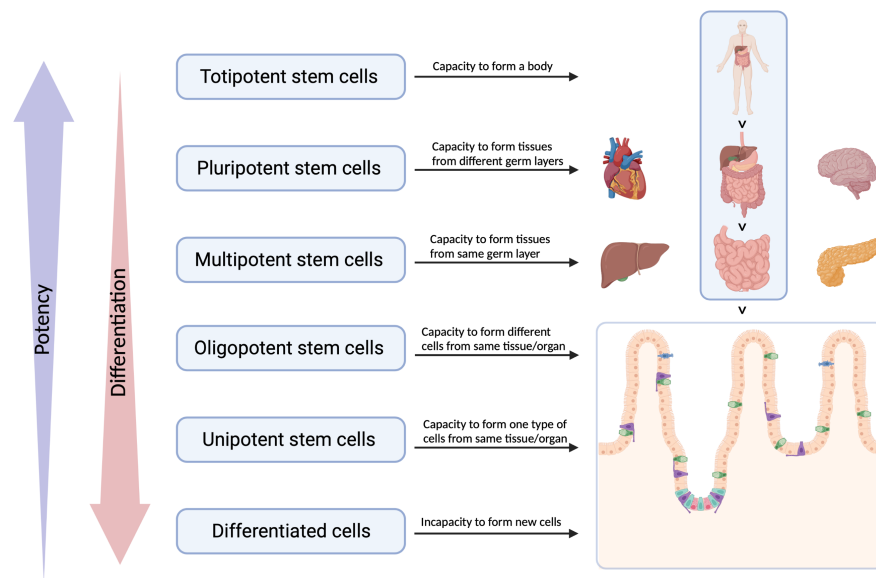


Figure 1. Stem cells (SC) potency and differentiation

¹ Totus + Potens : Whole + potent

² Pluri- + Potens : Several + potent

³ Multi- + Potens : Many + potent

⁴ Oligo- + Potens : Some + potent

⁵ Uni- + Potens : One + potent

Stem cells replicate through asymmetric mitosis resulting in the formation of an identical daughter cell and other cells that can enter differentiation. Through multiple asymmetric divisions, the stem cell pool remains the same but new differentiated cells are created.

Initially, one hypothesis of this potency dissolution was posed by scientists in which through mitosis, cells eventually lose quantitatively a portion of their DNA [3]. This hypothesis has been refuted as the somatic cell nuclear transfer from a somatic cell to a haploid cell (secondary oocyte) resulted in a new cell capable of dividing by mitosis and differentiating thus fulfilling the definition of a stem cells [4]. A possible explanation was that there was a mechanism by which a cell could qualitatively repress or allow expression of genes by using epigenetics. Nowadays, many studies have shown that epigenetics is responsible of the pluripotency dissolution by modifying stemness gene expression.

Scientists subsequently assessed whether the cells could regain morphological and functional characteristics of pluripotent cells without somatic cell nuclear transfer by removing the epigenetic markers of a differentiated cell. Kariagannis et al were the first to attempt to generate human induced PSC (iPSC) from fibroblasts by identifying and integrating four transcriptions factors whose role were to modify stemness-related genes [4]. The success of their experiments confirmed that stemness-related genes are suppressed by epigenetic markers through differentiation.

1.3 Usage of stem cells

In the wake of the excitement surrounding stem cells, paving the way for possible regenerative therapies, the scientific world has attempted to study stem cell biology. However, the usage of embryonic PSC has been closely regulated by European legislation due to ethical reasons. Up to this day, the hESC used in biomedical research originate from two processes [5]:

- Direct in vivo/vitro fecundation of a secondary oocyte with a spermatozoid followed by the isolation of the ICM from the blastocyst.
- Somatic cell nuclear transfer where the cell of an adult somatic cell is put directly into the cytoplasm of an oocyte emptied of its own nucleus.

Owing the ethical problems of hESC usage, their usage has lessened in profit of adult stem cells obtained by biopsy. Indeed, in the following years, the biomedical world has been interested in the properties of adult stem cells. Notwithstanding their lower potency, adult stem cells used in research are multipotent and allow multiple tissues creation. Following interests, adult stem cells of the three germ lineages (ectodermic, mesodermic and endodermic) are used but mesenchymal stem cells (MSC) have, progressively, gained more interest. Moreover, iPSC are a good solution to bypass any ethical problems as generated from somatic cells and exhibits pluripotency.

To conclude, stem cells can be classified in a five-fold manner :

- **From the organism** : Human, mouse, ...
- **From the stage of development** : Embryonic or adult
- **From their potency** : Totipotency, pluripotency, multipotency, oligopotency and unipotency
- **From the embryonic tissue** : Ectodermal, mesenchymal or endodermal
- **From the differentiated tissue** : Bone marrow, adipose tissue, ...

I.4 Mesenchymal stem cells

I.4.1 Definition of mesenchymal stem cells

Friendenstein and his colleagues cultured a sample of mice bone marrow and observed a layer of adhesive cells with fibroblast-like appearance which will be then defined as mesenchymal stem cells [6]. The International Society for Cellular Therapy (ISCT) has issued criteria that regulate the definition of hMSC [7]. In this way, the ISCT has formulated three *minimal criteria* to ensure consensus among scientists using hMSCs. The first criterion is the ability of hMSCs to adhere to plastic surfaces when cultured. The second criterion is a summary of the antigenic profile of hMSCs. Indeed, hMSC express three surface markers which have become a *sine qua non* condition for their isolation: CD105, CD73 and CD90. However, to maximize the purity of hMSC samples, cells must express at most 2% of other markers such as: CD45, CD34, CD79 α , CD11b, CD14, CD19 and HLA-DRII. Finally, the third criterion highlights the multipotency of these cells by objectifying their differentiation into three distinct mesenchymal cell species in predetermined in vitro conditions : adipocytes, chondroblasts and osteoblasts [7].

1.4.2 hMSC potency

hMSC are multipotent, but it is important to note that the sole multipotency of hMSCs mentioned above has recently been shown to be obsolete, as numerous studies have successfully shown that MSCs can differentiate into cell types of other embryonic sheets. Currently, differentiation of MSCs could be shown into cells of the cardiovascular system (cardiomyocytes, endothelium), central/peripheral blood tissue, neuronal cells, cells of the trachea, cells of the gastrointestinal tract (enterocytes, hepatocytes, pancreatic cells), sensory organs (cornea and inner ear) and keratocytes [8]–[12]. The figure 2 summarizes the differentiation potential of the hMSC. Furthermore, it was shown that MSCs are able to transdifferentiate [10]. Transdifferentiation is a process by which hMSCs that have entered or completed cell differentiation can undergo a new differentiation process to another cell type by genetic reprogramming. This property is of great importance as it could overcome some of the disadvantages associated with the use of ESC and iPSC [13].

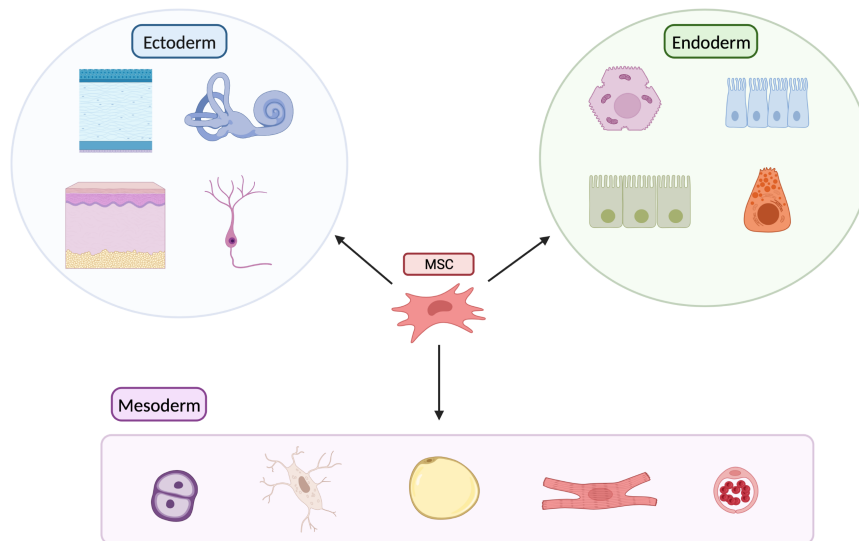


Figure 2. MSC differentiation potency

1.4.3 Types of mesenchymal stem cells

1.4.3.1 Distribution of MSC in the organism

As explained above, initially hMSCs were found in medullar biopsy culture where colony-forming units fibroblastic (CFU-F) were observed [6]. Since then, researches focused on the presence of new MSC in various organs of mesenchymal origin. One interesting discovery is that, in injured tissues, the majority of MSC present seems to be linked with pericytes which

are perivascular MSC [14]. The present hypothesis is that pericytes owing MSC-like properties, pericytes and MSC are thought to be related but the exact nature of this relation is yet to be fully understood [15].

Currently, numerous tissues were found to be sources of hMSCs. The two main sources are bone marrow and adipose tissue providing human bone marrow MSC (BM-MSC) and human adipose-derived MSC (hADSC), respectively [8]. But MSC have been observed in other locations. After BMSC, the adipose tissue-derived MSCs (ADSC) were discovered. Placental annexes (placenta + umbilical cord) are also a rich source of hMSCs, especially Warton's jelly, which can be recovered from umbilical cord donation [16], [17]. hMSCs can also be found in dental tissues and in peripheral blood [18], [19] (Figure 3).

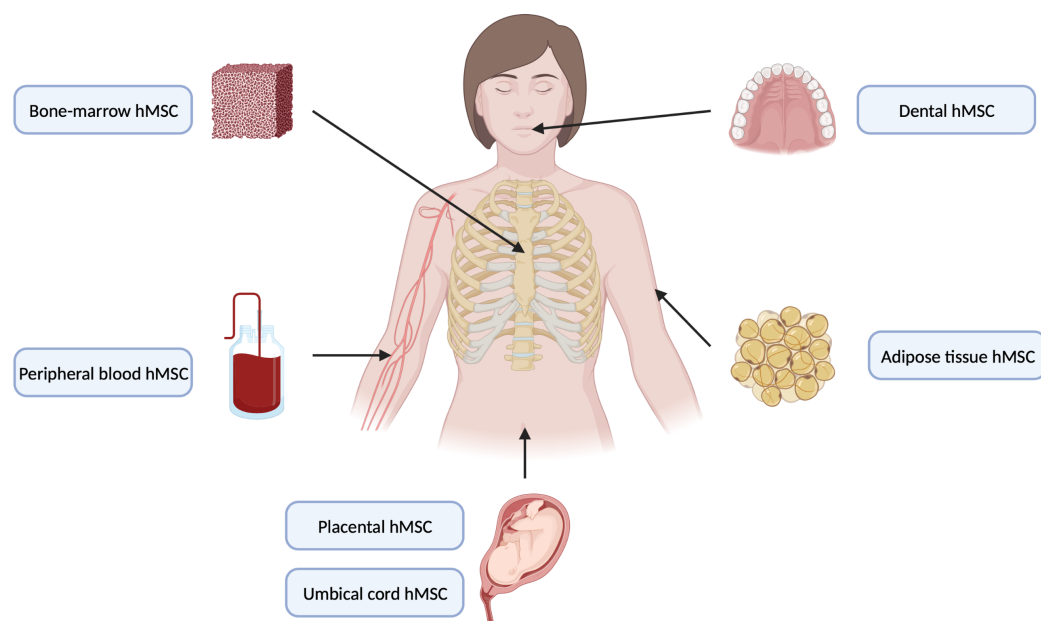


Figure 3. Distribution of hMSC throughout the organism

1.4.3.2 Bone marrow stem cells

In 1996, the first MSC were cultured. Those MSC arose from a bone marrow aspirate and were subsequently named BM-MSC [20]. BMSCs successfully met the minimal criteria issued by the ISCT [21]. These cells are present in several species which allowed scientists to extract them from humans, rabbits, rats, mice and others. In humans, the sources frequently used to obtain hBM-MSCs are axial bones (mostly vertebral body and coxal bone) and the sternum, whereas in rodents, these cells can be obtained from the femur or tibia after euthanasia [20].

Currently, various fields of regeneration research have been investigated. BM-MSCs have been studied in the orthopedic field based on their capacity to differentiate into osteoblasts and chondroblasts [22]. The fields of osteoarthritis and bone tissue regeneration have been also explored [23]. Until now, BMSCs remain the most widely used MSCs in the study of central nervous system regeneration. Thanks to the promising results of preclinical research, the number of clinical trials revolving around BM-MSC have been expansional increasing over the last few decades [24].

1.4.3.3 Adipose tissue stem cells

ADSCs are stem cells present in the perivascular space of the subcutaneous white adipose tissue playing a role in the homeostasis of the adipose tissue by regulating adipogenesis and the caloric balance. These cells have been isolated since 2002 from human abdominal tissue by lipoaspirates but also from other species such as rodents (mouse, rat) and lagomorphs (rabbit) [25], [26].

Currently, the usage of these stem cells has exceed that of BM-MSC in the world of regenerative biomedical research [27]. Moreover, ADSCs have been shown to be able to differentiate into multiple mesenchymal species including, of course, adipocytes, chondrocytes and osteoblasts, but also myocytes (skeletal myocytes, cardiomyocytes and smooth muscle), endotheliocytes and fibroblasts. Their differentiation towards the ectodermal and endodermal embryonic layer is also possible [27]. This has been demonstrated in their differentiation into neurons, Schwann cells, keratinocytes, retinal pigment epithelium cells, hepatocytes and Langerhans β -cells [26].

1.5 Teeth anatomy and histology

1.5.1 Histo-anatomy of the tooth and its annexes

A normal adult possesses 32 teeth fixed in the maxillary bone and mandibula by a dense ligament known as the desmondontum. Anatomically, a tooth is composed of three regions: the dental crown with various morphologies depending on its function, the cemento-enamel junction and the dental root, single or multiple depending on the tooth [28].

Once sliced, the tooth reveals its histological structure. A tooth is mainly composed of mineralized tissues based on hydroxyapatite crystals: dental enamel and dentin. The dentin determines a cavity called the pulp chamber filled with a loose connective tissue where sensory fibers and blood capillaries are mixed. The lateral surfaces of the tooth are covered by another mineralized tissue, the dental cementum, which allows the fixation of the tooth in its alveolar process. Finally, dental vascularization is provided by branches from the external carotid artery via the posterior and inferior alveolar arteries, branches of the homolateral maxillary artery. The sensory nerve fibers present in the pulp come from the fifth cranial nerve, the trigeminal nerve (Figure 4).

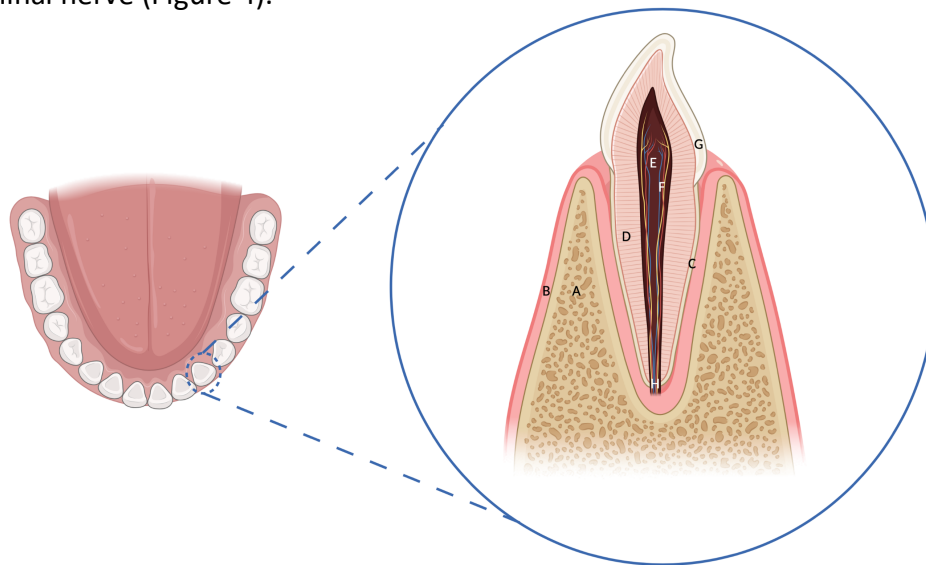


Figure 4. Normal histo-anatomy of the canine tooth and its annexes.

A : Alveolar process of the mandibular bone; B : gingiva; C: cementum; D: Dentin; E : Pulp chamber with pulp tissue; F : Vasculo-nervous elements of pulp tissue; H : Tooth apex; G : Enamel.

1.5.2 Dental stem cells

1.5.2.1 Healthy tooth stem cells

As explained above, the tooth is a complex of tissues conferring a function. Like most tissues of the organism, the tooth also possesses stem cells, therefore referred to as dental stem cells (DSC) but also in its appendages. At present, various cellular niches have been identified through biomedical studies (Figure 5). The ability of the minimal tri-differentiation of those cells have been studied, hence gaining mesenchymal stemness identity [18].

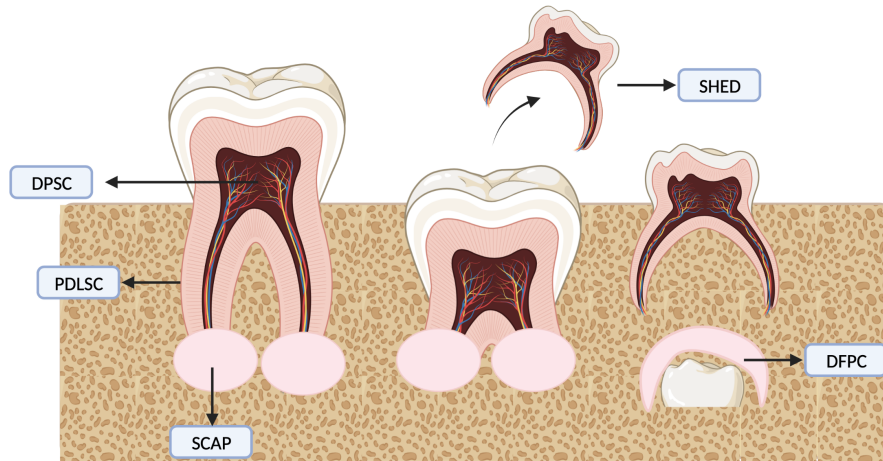


Figure 5. Dental stem cells origin adapted from Zhou et al [29]

As shown in this figure, MSCs can be extracted from several dental niches. MSCs can be obtained from dental follicles and are then called DFSCs. From deciduous teeth, SHEDs can be obtained. Apical papillae, forming future dental roots, are sources of SCAP. Adult teeth also provide DPSCs from pulp tissue and PDLSCs from the desmodontum.

Chronologically, the first population of stem cells were identified by an enzyme digestion of pulp tissue from an extracted third molar in 2002 [18]. Those cells were then called "dental pulp stem cells" (DPSC). Further investigation allowed us to determine that those cells also arise from pulp perivascular tissues and express MSC markers, hence joining the MSC family.

Subsequently, a new population of DMSCs was extracted from the pulp of human decimal teeth in 2003, then called SHED. In 2004, the desmodontum, a dense connective tissue linking cementum to the alveolar process, was also shown to be a source of DSC, so called "periodontal ligament stem cells" (PDLSC). The apical papilla is a cellular-rich ectomesenchymal structure whose role is to form the future tooth roots. In 2005, a team of researchers succeeded in isolating a new population of DMSC from dental follicles (DFSC) [18], [30] In 2006, the richness of MSCs from apical papillae was highlighted by the team of Sonoyama et al. and allowed the isolation of "stem cells from apical papillae" (SCAP) for the first time [31].

Interestingly, these cells harbored better proliferation ability than former discovered DSC of which DPSC. Subsequently, a subculture of SCAP, named RP89 cells, was isolated. These cells have the particularity to maintain an immunophenotypic expression of MSC markers [32].

I.5.3 Granuloma stem cells

In histopathology, a granulomatous formation (or granuloma) describes a protective entity following unresolved inflammation that has progressed to a chronic form. In this chronic context, granulomas can be found in chronic microbial infection (e.g. *Mycobacterium tuberculosis*, *Aspergillus* sp), foreign body or autoinflammatory (Crohn's, Wegener's, ...) inflammation. Granulomas are characterized by the presence of lymphocytes and macrophages, some of which in their activated forms, namely epithelioid cells and multinucleated giant cells (Langhans and Müller giant cells) [33] (Figure 6). Depending on the aetiology, central necrosis in granuloma may be observed as in the case of tubercular granuloma whereas its presence has not been reported in dental granuloma.

In addition to inflammatory elements, multiple mesenchymal elements can be found, including endothelial cells forming capillary structures but also MSCs [34]. Zhang et al, explains in his article that these MSC seem to play a crucial immunomodulatory role in the evolution of the disease [35]. Interestingly, MSCs seem to be able to survive in an inflammatory environment and to interact with adjacent cells hypothetically through paracrine secretion [36].

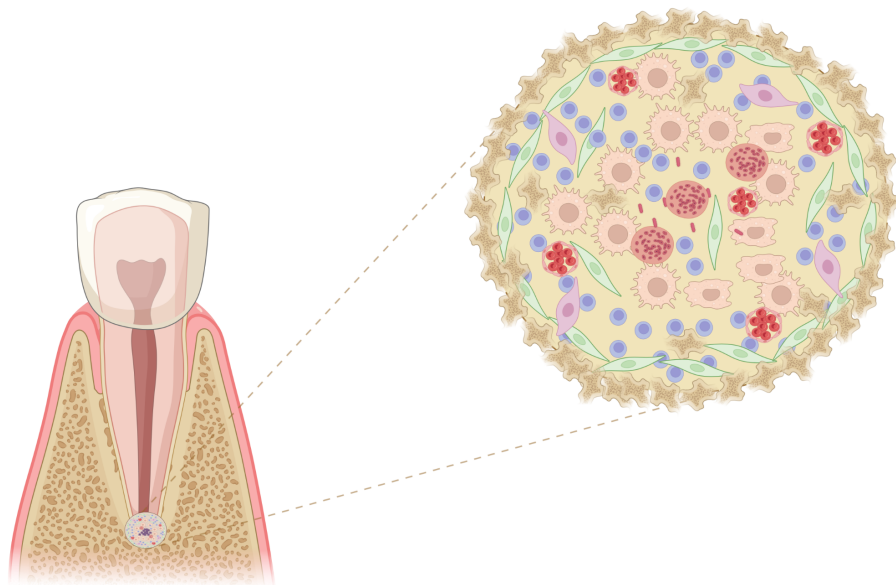


Figure 6. Schematic representation of a bacterial dental granuloma.

Chronic inflammation with multiple activated macrophages including histiocytes and multinucleated cells. Note the presence of lymphocyte infiltration and fibroblastic elements. Several mesenchymal stem cells are present amongst this inflammatory infiltrate.

In the same way, granuloma can occur as a result of microbial colonization of extra-radicular tissue. In 2019, a study by Estrela et al. demonstrated the presence of cells expressing the three immunohistochemical markers of MSCs, as recalled by the ISCT, in pathological periapical tissues, namely acute (abscesses) and chronic (cysts and granulomas) periapical inflammations [34]. As the periapical region is a region rich in MSCs, the precise origin of periapical MSCs found in inflammatory tissues remains to be determined. Several hypotheses have been advanced, including a possible multiple origin from PDLSCs, DPSCs, SCAPs and even odontogenic remnants.

To our knowledge, no study has investigated or compared the properties of MSC from periapical granulation lesions (GDSC) with those of other MSC populations. We could imagine that these cells under chronic inflammatory stress express a different cytokine profile and could reveal a different behavior in regenerative therapy. Remaining the most prevalent periapical lesion, the use of dental granuloma tissue could open the door to new non-invasive sources of MSCs with promising properties yet to be investigated.

I.6 Applications of mesenchymal stem cells

I.6.1 Regenerative therapies

Since their discovery, scientific interest was raised about MSC, whose unique capabilities revived hopes for regenerative medicine. Regenerative medicine refers to a cross-sectional field of research whose aim is to study the mechanisms underlying the homeostasis of regeneration and degeneration in order to promote tissue trophicity overtime.

Indeed, in light of their virtually infinite capacity for division and their ability to differentiate into multiple cell types, a natural application of MSCs was to directly regenerate injured tissue by injecting MSCs into the tissue and allowing them to differentiate. This approach was firstly investigated by H. Lazarus in 1995 [37].

Since then, many studies around the world have tried to exploit the properties of hMSCs in direct regeneration with more than 400 MSC-related clinical trials since 2015 [24]. The impact of MSCs has been mainly studied in chronic degenerative pathologies such as osteoarthritis and polyarthritis in the osteoarticular system, diabetes, neurodegenerative diseases

(including multiple sclerosis, glaucoma, retinitis pigmentosa), but also in acute pathological processes with a poor prognosis, such as myocardial infarction and traumatic spinal cord injury [38].

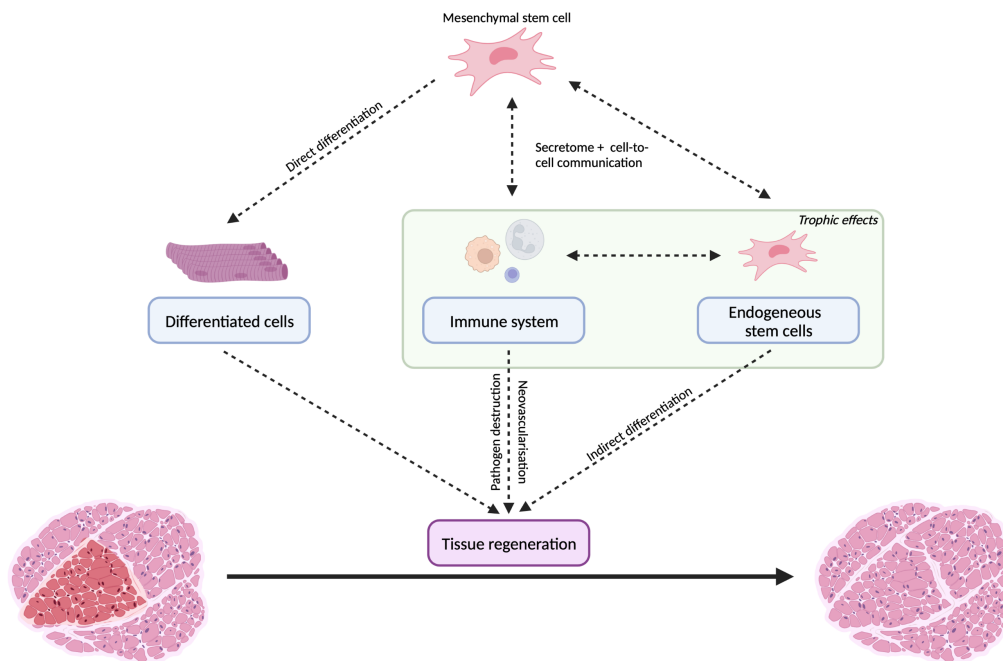


Figure 7. MSC-mediated tissue regeneration

More recently, the indirect effect of MSCs to promote their regeneration of adjacent injured tissue was discovered [39]. This ability is called the "trophic capacity" of MSCs. In this way, it was reported that without differentiation MSCs can regenerate damaged tissues by promoting cell regeneration by cell-to-cell signaling and by paracrine mechanisms as illustrated by figure 7.

Their current applications lie in cosmetic surgery, cell therapy and translational research [26]. The applications of the cells have been demonstrated in the context of central neurologic system pathologies, digestive tract and its annexes, osteoarticular system, skin pathologies and cardiovascular diseases [25].

1.6.2 Cell biology study models

Since their discovery, MSCs have been of interest to scientists trying to understand the mechanisms underlying their properties. Moreover, stem cells are good candidates for studying cell biology because of their remarkable division properties and the possibility to

differentiate into multiple different cell types. Firstly, MSCs offer the possibility to directly study the mechanisms of stemness and differentiation in stem cells. Thus, scientists noticed that the mechanism underlying differentiation was not a gross loss of genetic material but rather a modification of gene expression, which is part of epigenetics. In this way, the understanding of these mechanisms made it possible to create the first iPSCs, pluripotent cells generated from differentiated cells. Secondly, the differentiation properties of stem cells also allow for a range of cell types to be studied in research. In recent years, these 3D cell models have become more complex by combining various cell types and also by creating three-dimensional organoids that more closely represent reality with the diversity of cells present and histological structure [40].

1.7 Immunomodulating properties of mesenchymal stem cells

1.7.1 Immunomodulation of mesenchymal stem cells

1.7.1.1 *Discovery of MSC immunomodulation*

The term "immunomodulation" has been defined by the National Cancer Institute as the ability of an external agent to activate or suppress immunity. This ability of MSCs was discovered by Amelia Bartholomew in 2002 when she and her team brought baboon MSCs into contact with lymphocytes in vitro and found that the proliferation of mixed lymphocyte reactions in co-culture was significantly reduced [41].

Numerous studies have focused on this new ability of MSCs. At present, it is thought that the immunomodulatory properties of MSCs are mediated by intercellular contact mechanisms but also by the secretion into the extracellular environment of a message-carrying molecules, a paracrine signaling [11], [14]. Galipeau et al. even noted the association of MSCs injected into tissues and an improvement in remote conditions, resembling an endocrine mechanism [37].

1.7.1.2 *Immunological properties*

Through multiple studies, MSCs have been shown to inhibit the proliferation of innate immune cells (polymorphonuclear neutrophils, natural killers (NK) cells and dendritic cells) but also acquired immune cells (T and B lymphocytes) [11]. In addition to inhibiting immune

effectors, MSCs have been shown to induce the production of regulatory T cells which will further induce T cell anergy [42], [43].

One of the reasons MSCs seem to be very good candidates at the immune level is thanks to their low MHC class I and do not express MHC class II [42]. However, it has been observed in several studies that the undifferentiated character of MSCs is essential in order not to induce an immune response. Indeed, it seems that by differentiating or when stimulated with IFN- γ , MSCs acquire the MCH II receptor allowing the expression of peptides specific to the immunity cells, thus inducing alloimmunization [37]. Another reason relies on the rich secretome⁶ of MSCs. The main mediators released by these cells are TGF- β , IL-10, nitric oxide (NO), PGE2 and TSG-6 [14]. In addition, intercellular contacts also appear to be important in considering the immunomodulatory mechanisms of these cells.

The identification of such properties has naturally motivated scientists to study the influence of MSC on numerous dysimmune diseases such as allergy, rheumatoid arthritis, gastrointestinal diseases (Crohn's and ulcerative colitis), multiple sclerosis and many others. Numerous positive results seem to suggest that both the MSC conditioned medium and the MSCs, through their trophic effect, can orientate the immunomodulation towards anti-inflammation.

As an illustration, the regenerative and immunomodulation properties of stem cells were for example noted in the study of Navas et al, where intracameral injection of human amniotic membrane-MSC (hAM-MSC) in the context of alkali corneal burns were performed [44]. The injected hAM-MSCs were observed to be differentiated within the corneal stroma and were associated with a significant decrease in corneal opacity and inflammation.

The immunomodulatory properties of DMSCs have also been investigated in recent years. One of the first studies to highlight this was the study by Wada et al. In this pioneering study, Wada et al investigated the ability of three dental cell populations (DPSCs, PDLSCs, and gingival fibroblasts (GFs)) to inhibit an INF- γ -pre-stimulated population of peripheral blood

⁶ Defines the ensemble of molecules secreted by a cell.

mononuclear cells (PBMNC) [18]. The researchers found out that, similarly to other MSC, DMSC seem to possess immunosuppressive properties in vitro [18]. To date, few studies have investigated on their direct properties in vivo by injecting DMSC into the organism on models with dysimmune pathologies and seem to prefer focusing on the use of DMSC-derived products as extracellular vesicles (EV) or conditioned medium (CM).

1.7.2 Emphasis on immunomodulators

1.7.2.1 The Kynurenine pathway

The kynurenine pathway is a metabolic pathway allowing nicotinamide adenine dinucleotide (NAD⁺) formation [45]. Indoleamine 2,3-dioxygenase (IDO) is a class 1 cytoplasmic enzyme according to the Enzyme Commission that catalyzes the first limiting reaction of the kynurenine pathway from tryptophan (Trp). The first reaction of the kynurenine pathway catalyzed by IDO is the oxidation of Trp to form the N-formylated product, N-Formylkynurenine [46]. This first product is then hydrolyzed to form L-kynurenine and formate. Several other catabolites derived from L-kynurenine will then be formed by several oxidase and hydrolase reactions (Figure 8).

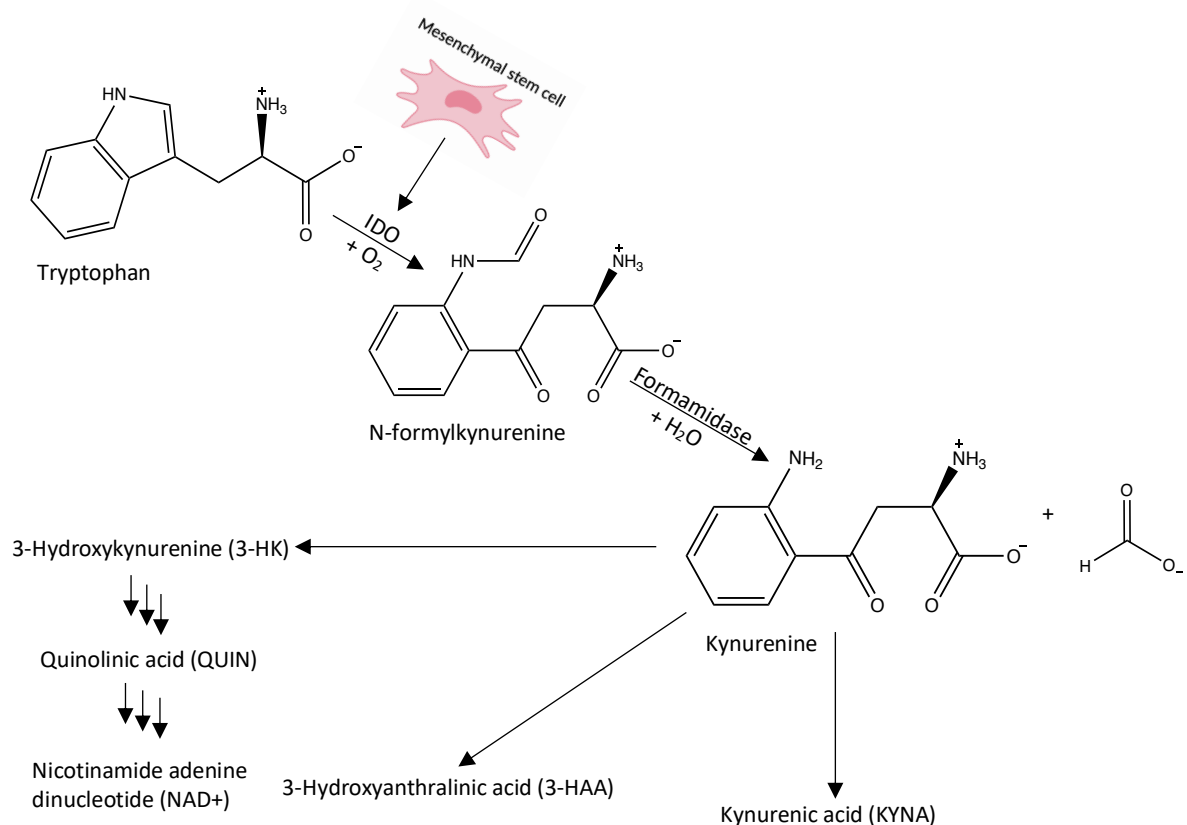


Figure 8. Kynurenine pathway adapted from Hughes et al [45].

The kynurenine pathway has been shown to be stimulated by the upregulated expression of IDO in MSC population. Furthermore, the transcription of the IDO1 isoenzyme was shown to be upregulated by prior stimulation by proinflammatory stimuli including IFN- γ . The kynurenine pathway is one of the immunomodulation mechanisms of MSCs by a dual mechanism. The first mechanism results from the direct consumption of Trp from the extracellular medium. This depletion of Trp results in activation of enzymatic players including general control non-derepressible-2 kinase (GCN2) which will induce a major T cell anergy but also mammalian target of rapamycin (mTOR) which will promote the expression of regulatory T cells [47]. The second mechanism is mediated directly by the production of L-kynurenine derivatives including kynurenic acid (KYNA), 3-hydroxyanthralinic acid (3-HAA), 3-hydroxykynurenine (3-HK) and quinolinic acid (QUIN) [47]. In vitro, these derivatives exhibit several actions on the immune system including induction of apoptosis of T1 helper lymphocytes, limiting lymphocyte proliferation and NK lymphocytes [48].

1.7.2.2 Cytokines

1.7.2.2.1 Interleukine (IL)-10

IL-10 is a 37kDa cytokine belonging to the IL-10 superfamily discovered by Mosmann in 1989 [49]. This cytokine is secreted by many cells including macrophages and T lymphocytes. From an immunological point of view, IL-10 is recognized as an anti-inflammatory cytokine, explaining its former name cytokine synthesis inhibition factor (CSIF), thanks to its capacity to inhibit macrophages, NK and Th1 lymphocytes [50]. However, it should be noted that recent work attempts to nuance this. Indeed, the anergic action of IL-10 on cytotoxic CD8+ lymphocytes (CTL) seems to depend on the cytokine microenvironmental context. In this sense, B lymphocytes even seem to be stimulated by IL-10 [50]. It has been shown that this cytokine is secreted by MSCs including DSC [29].

1.7.2.2.2 Interleukin (IL)-6

IL-6 is a 21-26kDa cytokine secreted by immune cells (dendritic cells, B cells, T cells and macrophages) but also by endothelial cells and fibroblasts following various stimuli such as the presence of tissue injury via damage-associated molecular patterns (DAMPs) or via activation of pathogen-recognition receptors (PRR) [51]. Overall, this cytokine stimulates

inflammation locally but also systemically and is implied in many inflammatory pathologies. IL-6 exerts its effects on inflammation effectors by promoting antibodies production by plasmocytes, CTL proliferation and the polarization of CD4⁺ lymphocytes towards Th17 phenotype [52]. IL-6 has been shown to be secreted by MSC [29].

Interestingly, IL-6 can also exert immunosuppressive effects when TNF- α and IL-1 β promotes the expression of IL-6-induced expression of *suppressor of cytokine signaling (SOCS3)* which inhibits IL-6 pro-inflammatory genes [53]. Finally, IL-6 has immunosuppressive properties by inducing the polarization of helper lymphocytes towards Th2 phenotype via IL-4 upregulation [53].

1.7.2.2.3 Tumor growth factor- β (TGF- β)

TGF- β are a family which comprises 33 distinct cytokines first discovered in 1981 [54]. Firstly, TGF- β 1 plays important role in tissue regeneration as is exocytosed by platelets during platelets activation. Moreover, those regenerative properties have been evaluated in vivo. Secondly, TGF- β possess also interesting immunomodulating properties. Indeed, TGF- β can on one hand inhibit acquired immunity and on the other hand stimulate acquired immunity. TGF- β -mediated immunosuppression can be obtained by induction of Treg cells and by the suppression of Th1, Th2 and CTLs [55], [56]. Conversely, TGF- β -mediated immunity activation is linked with Th9, Th17 and CTL-like cells [55], [56]. Especially in MSC and in DMSC, TGF- β is known to be an important component of their immunosuppressive profile [29].

1.7.2.2.4 Tumor growth factor-inducible protein 6 (TSG-6)

TSG-6 is a small protein, a product of the TNF-stimulated gene-6, of 35 to 38kDa secreted basally by various cell types. TSG-6 is a protein whose action is related to immunoregulation and tissue regeneration via complex interactions with the extracellular matrix. This protein is also secreted by MSCs in response to various inflammatory stimuli (IL-1, TNF- α , ...) [57]. However, it is important to note that the protein is not secreted by two-dimensional cell cultures even if the gene expression is upregulated [57].

At the immunological level, TSG-6 is associated, in a mouse model, with a decrease in the activation of CD4⁺ Th1 lymphocytes and antigen-presenting cells (APCs) and thus with a decrease in acquired immunity. At the regenerative level, in a murine myocardial infarction model where an intravenous injection of hMSCs was performed, the blood concentration of TSG-6 was significantly increased and was associated with myocardial regeneration. Interestingly, this observation was repeated with IV injection of *recombinant human-TSG-6* [58].

II. Objectives

The properties of MSC have been investigated for long. This has historically involved MSC populations derived from BM-MSC and ADSC. However, the invasive nature of a bone marrow or adipose tissue biopsy makes their isolation and use difficult. In contrast, dental stem cell extraction is easily enabled by the third molar removal often required due to the lack of space on the dental arch and is then ethically well accepted worldwide. However, ease of cell isolation is not the only criterion for a cell to be used in cell therapy. Thus, its properties such as regeneration must be carefully studied. In recent decades, immunomodulation, a new property of MSCs has been revealed [14]. This refers to the capacity of MSC to interact with their environment through inflammatory mediators allowing modulation of the tissue regeneration and inflammation homeostasis. This property has been found to be increased by the presence of surrounding inflammation.

To our knowledge, no study has reported a comparison of the immunomodulatory properties of dental stem cells versus another type of mesenchymal stem cells. Moreover, a new population of apical stem cells has been isolated from peri-apical dental tissue with chronic granulomatous inflammation and their properties are still poorly understood. This could open the door to the use of currently undesirable tissues with novel properties.

For this purpose, during this work we focused on the properties of five types of hMSC and we compared the properties of these cells between quiescent and pro-inflammatory culture conditions (TNF- α + IFN- γ):

- Four MSC from dental tissue:
 - Dental pulp stem cells (DPSC)
 - RP89 (characterized monoclonal SCAP cell line)
 - Stem cells from apical papillae (SCAP)
 - Dental apical granuloma-derived stem cells (GDSC)
- One MSC from adipose tissue:
 - Adipose tissue-derived stem cells (ADSC)

The scope of this work was to investigate three main elements:

- a. To study the properties of dental MSC isolated from healthy tissues (RP89, DPSC and SCAP) compared to cells isolated from pathological dental tissues (GDSC).
- b. To compare the properties of dental stem cells to those of ADSC, the new gold standard in MSC cell therapy.
- c. To compare a possible selection effect between a monoclonal population of DMSCs (RP89) and their polyclonal counterpart (SCAP).

III. Materials and methods

III.1 Ethics statement

All human tissues were obtained in accordance with the Declaration of Helsinki. In this regard, free and informed consent was formulated in writing by the patient prior to the extraction of the wisdom teeth by an accredited health professional in stomatology. The properly signed documents are kept in a confidential manner.

III.2 Conservation of human tissues samples

The freshly extracted teeth were stored in a falcon containing 5% Dulbecco's phosphate buffered-saline (DPBS) with antibiotics (penicillin + streptomycin) 1% (volume-to-volume) and

placed in the refrigerator (4°C) overnight. The falcons were transported to the laboratory the next morning.

III.3 SCAP isolation from fresh extracted wisdom teeth

The falcons containing the teeth of the different patients were inspected to objectify any damage or poor preservation following extraction. The teeth were investigated for integrity and the presence of apical papillae under a laminar flux hood. Damaged teeth or teeth without apical papillae were not used. Using previously sterilized surgical instruments, the apical papillae tissues were segregated from tooth roots and placed in a 15 ml falcon containing 2 ml of Gilco's Dulbecco's Modified Eagle Medium (DMEM) (1x) (+) 4.5g/l D-glucose, L-glutamine (-) L-pyruvate (Reference : 41965-039) enriched with 5% fetal bovine serum (FBS) in conjunction with 1% (V/V) L-glutamine and 1% (V/V) penicillin + streptomycin. Tissues were roughly homogenized with a pair of sterile surgical scissors and then disposed in a sterile petri dish. Two ml of culture medium was added to ensure immersion of the tissue in the petri dish. This operation was then repeated for all the teeth of the different patients.

III.4 SCAP incubation and overgrowth

The petri dishes were placed in an incubator at 37°C and partial pressure in carbon dioxide (CO₂) controlled at 5% and 95% humidity. The culture medium mentioned above was renewed every two days. The presence of bacterial or mycotic contaminations as well as the appearance of adherent cell proliferation on the Petri dish were monitored under the microscope when media were changed. Once a homogeneous adherent cell layer covering 90% of the surface of the Petri dish was obtained, the tissue fragments were discarded and a protocol of cell passage was conducted. The cell passage was made using classical trypsinization protocol. The cells were re-suspended in the culture medium and then seeded in two 75 ml culture flasks for further amplification. Two cycles of passage and culture were undertaken.

III.5 Cell freezing protocol

After the third passage, cells from both flasks were resuspended in a falcon at a concentration of 10 cells/ml in full culture medium. At the same time, a sterile solution of dimethylsulfoxide (DMSO) diluted to 10% (V/V) in the medium was prepared. The 10% DMSO solution was carefully poured over the cell suspension using a micropipette and the suspension was immediately placed in labeled cryotubes at -80°C.

III.6 Cell defrosting and culture protocol

Using the same culture medium mentioned above, five cryotubes containing amplified cells from the apical papillae in addition to four previously frozen stem cell species (RP89, DPSC, ADSC and GDSC) were rapidly thawed and then resuspended separately in the culture medium. These suspensions were seeded in their own flasks. The media were discarded and refreshed every two days. Once at 90% confluence, the cells were passaged according to the protocol explained in section 1.5.

III.7 Reagents and cytokines

Murine fluorescein isothiocyanate (FITC) anti-CD31-, CD34, anti-CD45 and anti-human leucocyte antigen (HLA)-DR immunoglobulin (Ig)G1 antibodies were purchased from Biolegend (respective references: 303104, 343504, 304006 and 307604). Murine anti-CD73 Peridinin chlorophyll (Per-CP) IgG1 antibodies were bought from Abcam (Ab106677) while anti-CD90 Allophycocyanin (APC)-vio770 antibodies were bought from Miltenyi (Reference: 130-099-289). Murine IgG1 Phycoerythrin (PE) anti-CD105 and murine IgG2b APC anti-CD326 antibodies were purchased from Biolegend (Reference: 323205 and 324208, respectively). The eFLUOR506 marker was purchased from Thermofischer (Reference: 65-0866-14).

Subsequently, murine IgG1 (κ) FITC, PerCP and PE antibody isotypes were found on Biolegend (respective references: 400107, 400147 and 400113). Then, murine IgG1 (κ) APC-vio770 (Reference: 130-100-098) and IgG2a (κ) FITC (Reference: 400207) antibodies were purchased from Miltenyi and Biolegend respectively.

III.8 Fluorescence activated cell sorting (FACS) protocol

In order to study the immunophenotypic profile of freshly isolated cells and previously frozen cells, we investigated the expression of mesenchymal stem cell markers namely CD73, CD90 and CD105 which we put in perspective against epithelial (CD326) and lymphoid cell markers (CD31, CD34, CD45 and HLA-DR). Mammary carcinoma cells (MCF-7) will be used as a positive control for CD326 while peripheral blood mononucleosis cells (PBMC) will be used as a positive control for lymphoid markers. Finally, cell viability was assessed by the E-Fluor506 marker.

The cultured cells were passaged as described in section 1.5 and then suspended in medium at a $2 \cdot 10^5$ cells/ml concentration. 1 ml of PBS was added to the falcon and it was centrifuged at a rate of 200 rpm/5min. The supernatant was carefully removed and this cycle was repeated twice. eFLUOR506 die labeling was performed by leaving to incubate 30 minutes in the dark and refrigerated a 200µl volume of eFLUOR previously diluted 1000 times in PBS in a falcon containing $2 \cdot 10^5$ cells. Cell labeling by antibodies and their isotypes was allowed by incubating 30 minutes away from light and at 4°C a volume of 50 µl of an antibody solution diluted as listed in the table 1 in the annexes in a buffer solution (PBS, 1% FBS, 1mM EDTA) in a falcon of $2 \cdot 10^5$ cells. After three cycles of rinse-centrifugation, the cell pellet was resuspended in 200µl of buffer. A compensation bead suspension was made by arranging 1 µl of ad hoc antibody in a falcon containing one drop of beads. A 30-minute incubation at 4°C was followed by addition of 2 ml of buffer as well as a 5-minute centrifugation at 500g removed the supernatant to allow final addition of 200 µl of buffer into the Eppendorf.

III.9 Tumor-necrosing factor- α and interferon- γ stimulation protocol

The five stem cell populations were cultured in 75ml flasks containing DMEM FBS 10% + 1% penicillin/streptomycin + 1% L-glycine culture medium until confluence. A passage was performed and cells were seeded in 175ml flasks to increase their number. Once at 90% confluence, the cells were resuspended at a concentration of 10^6 cells/ml in the aforementioned culture medium. The cells were then seeded at a concentration of 10^5 cells/well in 24-well plates according to the experimental plate design shown below (Figure 9). Activated MSC will be referred as “aMSC” and quiescent MSC will be referred as “MSC”.

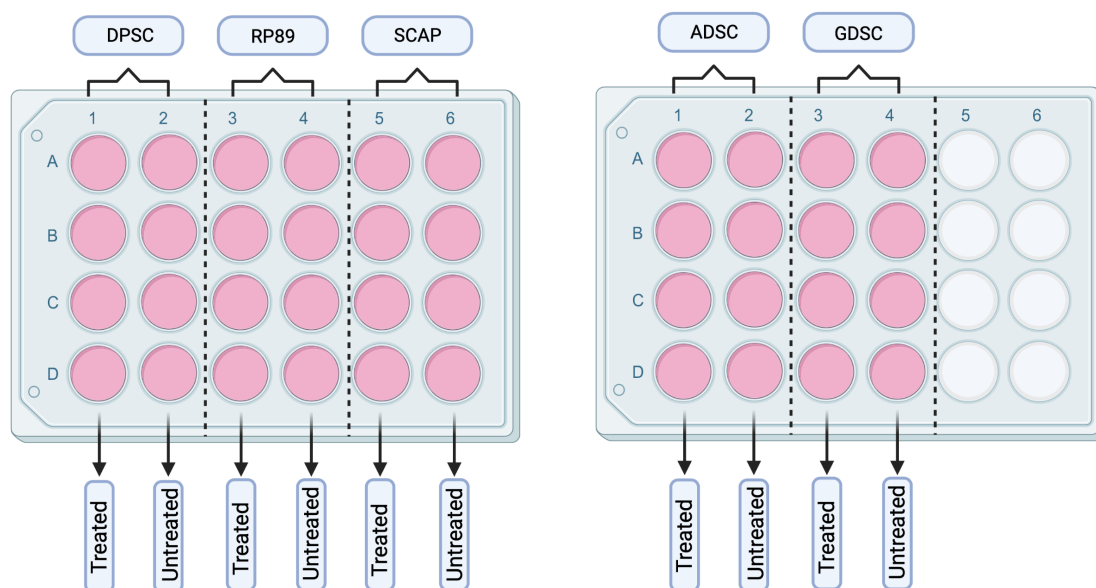


Figure 9. Pro-inflammatory cytokines stimulation plates design

On day +1 of seeding, 1000 ml of the culture medium from each well was sterilely removed and replaced by medium with a 0.5% FBS concentration (Figure 10). Three days later, 1000 ml of the culture medium was renewed with 500 ml of fresh 0.5% DMEM FBS solution. Eight hours later, 1000 ml of the culture medium from each well was again removed and replaced by two distinct solutions of 0% FBS DMEM + 1% penicillin/streptomycin + 1% L-glycine: a test solution (treated) where 20 ng/ml TNF- α and 20 ng/ml IFN- γ were added and a control solution (untreated) without cytokines. On the seventh day, the experiment was completed by sterile harvesting of the supernatant which was then homogenized by vortexing, aliquoted and placed in a refrigerator -80°C. 200 μ l of a Trizol® RNA-free solution was placed on the cellular bottom of each well and stored at -20°C. This experience has been conducted three times (N=3, n= 4).

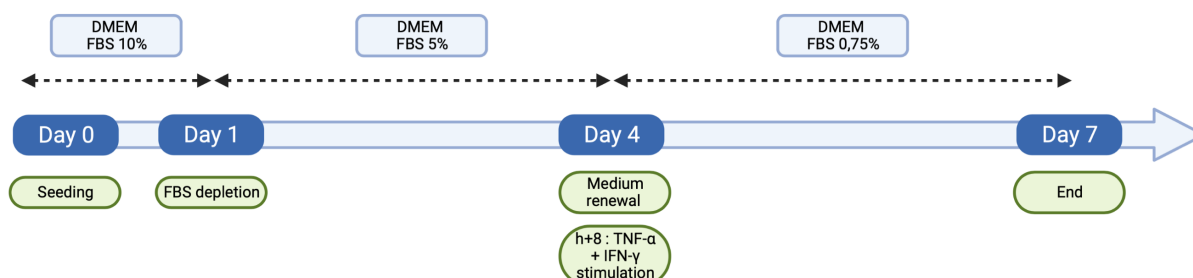


Figure 10 : Cytokine stimulation experiment plan

III.10 Indoleamine 2,3-dioxygenase (IDO) activity assessment

In a 96-well plate, 60 μ l of thawed supernatant from each sample was arranged in a well along the plane of the plate. A standard L-kynurenine solution was created in parallel with a concentration range of 100 μ M to 10 μ M. Distilled water acted as a blank (0 μ M). 30 μ l of a 30% (V/V) trichloroacetic acid (TCA) solution diluted in distilled water was disposed in each previously filled well. The plate was incubated for 30 minutes at 50°C and then centrifuged at 3000 rpm for 10 minutes. 75 μ l of the supernatant was removed and placed in a new plate. Finally, 75 μ l of Ehrlich's solution containing 2% (M/V) 4-dimethylaminobenzaldehyde (DAB) in glacial acetic acid was placed in the wells of the new plate containing a sample or standard. The plate was incubated for 10 minutes at room temperature and the optical density was read with a spectrophotometer at 490 nm.

III.11 Real-time quantitative polymerase chain reaction (RT-qPCR)

An analysis of mRNA expression was conducted by real-time quantitative polymerase chain reaction (RT-qPCR). We focused on the expression of two genes related to inflammation, namely *TSG-6* and *TGF- β* in comparison with the expression of the housekeeping *60S ribosomal protein L19 (RPL19)* gene. Under RNA-free conditions, we performed the three phases necessary to achieve these results, namely: RNA extraction, retrotranscription, and PCR amplification.

First, RNA extraction was conducted by thawing the different cell samples bathed in Trizol previously frozen in a -20°C refrigerator. The different solutions were placed in Eppendorf's where 40 μ l of chloroform and 100 μ l isopropanol were added to allow the precipitation of (deoxy)ribonucleic acids. The samples underwent a centrifugation cycle of 15 minutes at 4°C and 1,2.10⁴g allowing the separation of the DNA/RNA phase by aspiration with a micropipette. The obtained ARN was transferred to a new Eppendorf. The samples underwent three cycles of ethanol rinsing and centrifugation at 4°C and 12000g to be finally suspended in an aqueous buffer solution. The concentration of ribonucleic acid was quantified using the Nanodrop where a control of possible phenol contamination was monitored by a 260/280 ration. Second, retrotranscription was enabled by diluting all RNAs to a concentration of 1 μ g/ μ l using a TE buffer solution. A solution containing reverse transcriptase and triphosphoribonucleotide

acids was prepared and arranged in each Eppendorf. The RNA samples were then arranged in a thermocycler where a pre- and retrotranscription program was undertaken. Once the retrotranscription was completed, the cDNA samples were diluted with TE buffer. Then, 1.6. 1,6 µg of cDNA was collected and placed in a 96-well plate designated for RT-qPCR. Then, 18.4 µl of a solution containing the forward and reverse primers previously diluted in a 10-fold manner, CXR with mix for RT-qPCR (respectively 20µl of CXR in 1ml of mix). The plate was placed in the RT-qPCR machine where the PCR was undertaken. The control chosen was the expression by untreated RP89s which allowed us to calculate the relative expression of the genes by a $\Delta\Delta C_t$ calculation.

III.12 Interleukine-6 (IL-6) and interleukine-10 (IL-10) enzyme-linked immunosorbent assay (ELISA)

An enzyme-linked immune sorbent assay (ELISA) was carried out to evaluate the secretion of cytokines by MSCs stimulated and unstimulated by pro-inflammatory conditions. For this purpose, the secretion of IL-6 and IL-10 was investigated in the supernatants obtained from the stimulation experiment. Following the protocol provided by the supplier, wells of an immunosorbent plate were filled with a capture antibody solution diluted to 0.5 µg/ml for IL-6 and 1 µg/ml for IL-10 in PBS (1x) and incubated overnight. After triple rinsing in wash-buffer solution (PBS (1x) + 0.0005% tween-20), blocking with 1% bovine serum albumin (BSA) solution was undertaken for at least 1h. After rinsing, the samples were incubated for 2h. After further rinsing, detection antibodies diluted in a diluent solution (0.1% BSA in BSA + 0.05% tween-20) were added to each well at a concentration of 0.1 µg/ml for IL-6 and 0.50 µg/ml for IL-10. After washing, streptavidin-HRP solution (0.05 µg/ml) was arranged in each well. A hundred µl of HRP substrate solution was arranged in each well after final washing. A calibration curve was performed using IL-6 (2000 to 0 pg/ml) and IL-10 (3000 to 0 pg/ml) provided by the producer. An optical density reading at 460 nm with correction at 490 nm was performed with a spectrophotometer.

III.13 Statistical analyses

The results generated from these experiments are analyzed by the statistical software Prism 9.5.0 (250). Any outlier detected by the software will not be considered in the statistical analysis. A one-way ANOVA analysis with multiple comparisons between each condition will be conducted if the condition of normal distribution and homoscedasticity is verified respectively by an Agostino and Pearson test and by QQ plot. In case of heteroscedasticity, a non-parametric test will be preferred followed by a Kruskal-Wallis multiple comparisons test. The value of the type 1 error α is set at 0.05 so any value such that $P(x) < 0.05$ will be considered significant.

IV. Results

IV.1 SCAP isolation from fresh extracted wisdom teeth

The teeth of 10 patients (n=10) were collected. Of these ten patients, the teeth of three patients could not be exploited due to non-integrity of the dental tissues. Due to mycotic contamination, the teeth of the remaining seven patients had to be removed. The second sampling allowed us to obtain the teeth of two patients (n=2). The teeth of the twelfth patient were not used due to difficult extraction by the stomatologist, but those of the eleventh patient could be successfully amplified and allowed us to constitute several cryotubes.

IV.2 TNF- α + IFN- γ cell stimulation protocol

Cytokine stimulation was performed as described. No particular event has occurred during the experiment. No morphological differences between activated and quiescent cells has been microscopically noticed.

IV.3 Analysis of MSC markers by FACS

The aim of this experiment was to objectify the expression of MSC markers by dental stem cells freshly extracted from apical papillae. Moreover, we decided to investigate the surface marker profile of the other tested cells to insure the presence proper MSC markers and purity and for comparison. In this manner, we intended to investigate the expression of MSC markers

(i.e. CD73, CD90 and CD105) and to inspect the presence of undesirable cells types namely epithelium cells (CD326), hematopoietic cells (CD31, CD34 and CD45).

IV.3.1 Side scattering (SSC) and forward scattering (FSC)

The side scattering (SSC) and forward scattering (FSC) detectors allowed us to study the dispersion of the cells in regards to their size and the granular character of the cells. Among every hMSC, it was objectified, except for RP89, extracted apical papillae and ADSC, that every other type of cells presented subpopulations of cells dispersing in a two-tails manner: one tail in which cells exhibit lower cell size and lower granular cytoplasm and one tail in which cells exhibit bigger cell size and increased granular cytoplasm.

In RP89, cells seemed to be distributed along a steep axis of higher granulation-to-cell size ratio. In contrast, the SSC/FSC profile of ADSC was of a rather homogenous oblong population of significant cell size. Finally, the analysis conducted on SCAP revealed a lower cellular content (37,5%) than those of the other stem cells. Notwithstanding, a denser population of SCAP seems to be dispersed preferentially with a steep granular to cell size-ratio.

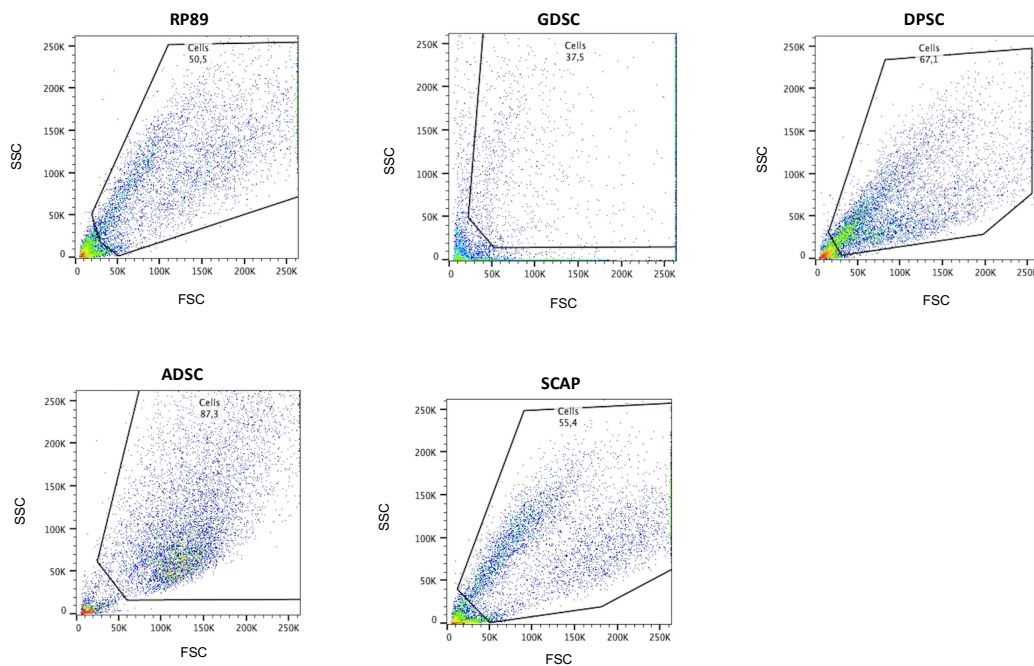


Figure 11. Side scattering and forward scattering profile comparison

SSC/FSC profile of five types of MSC. Note the two-tail distribution of the cells in RP89, GDSC and DPSC in contrast to the oblong distribution of ADSC. SCAP possesses a low cellular content and a glimpse of linear distribution of high SSC-to-FSC ratio.

IV.3.2 Mesenchymal stem cells positive markers

The expression of those three markers (CD73, CD90 and CD105) were sought on our five cell samples. The mesenchymal stemness markers (CD73, CD90) were indeed expressed on the surface of all five stem cell populations (Figure 12). However, the expression of CD105, the third mesenchymal stemness marker, was not found on the surface of the MSCs tested here.

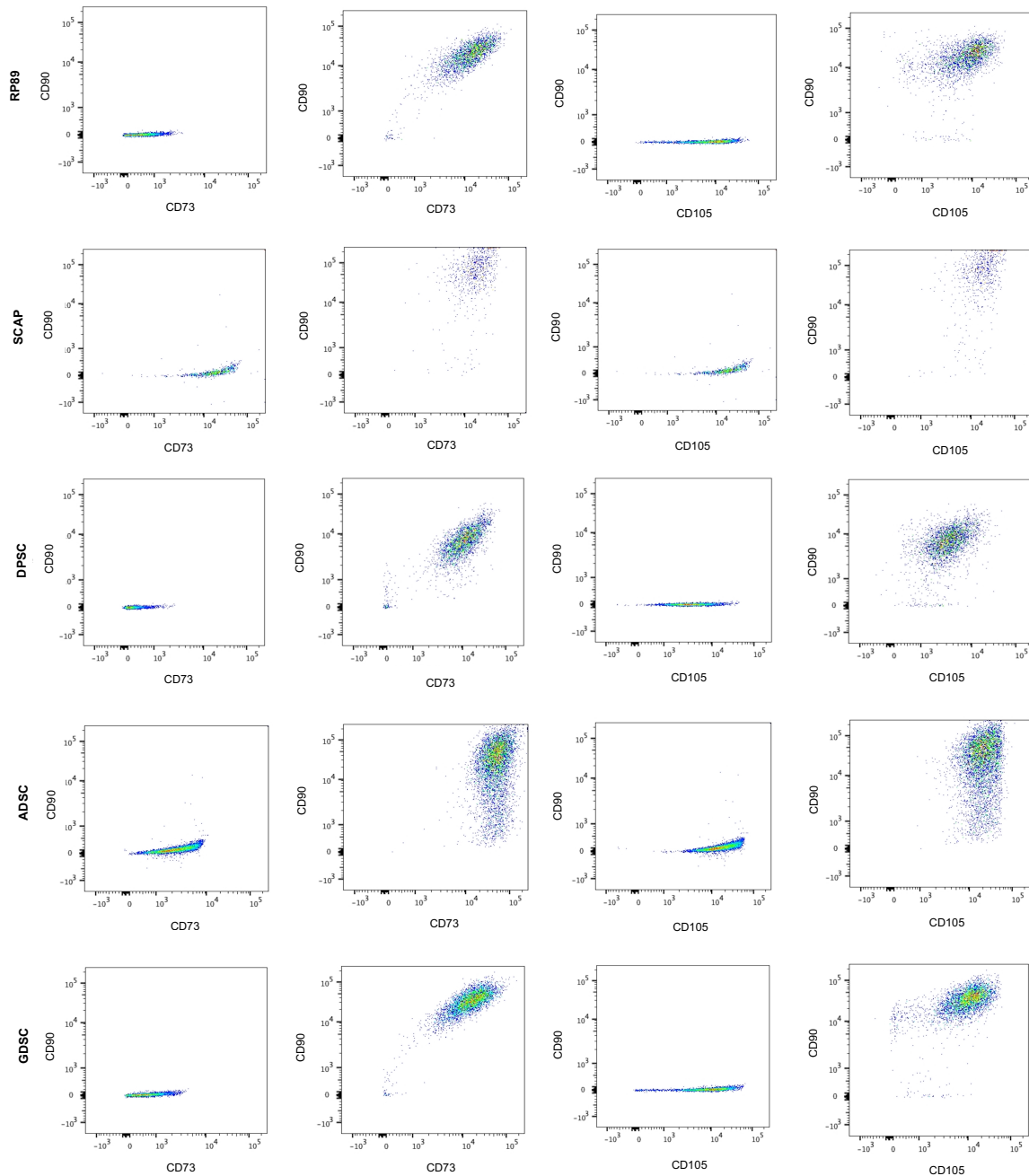


Figure 12. Mesenchymal markers expression by FACS

Study of MSC markers profile (CD73-PerCP, CD90-APC and CD105-PE) of five types of mesenchymal stem cells. Data is shown as a point cloud. Each point represents an event detected by the machine and arranged on a double logarithmic axis graph. The first and third columns represent the events with isotopic antibody immunostaining without variable portion (VF). The second and fourth columns represent the events with full antibody immunostaining.

IV.3.3 Mesenchymal stem cells negative markers

The expression of non-mesenchymal surface markers was investigated by FACS (Figure 13). The focus was directed to the presence of hematopoietic (CD31, 34, 45 and HLA-DR) and epithelial (CD326) markers. It is noteworthy to note that due to an overlap between fluorophore absorbance curves, the investigation of haematopoietic markers could not be conducted for ADSC and SCAP. Nevertheless, the absence of haematopoietic markers can be observed in the other three stem cell populations (RP89, DPSC and GDSC).

The expression of epithelial marker (CD326) was carried out to confirm the absence of stem cells expressing an epithelial phenotype in our samples. Indeed, the expression of the epithelial CD326 marker was not found in any of the samples (Figure 13). Nevertheless, it is important to bring attention to a discreet spreading of the DPSC population in CD326 canal.

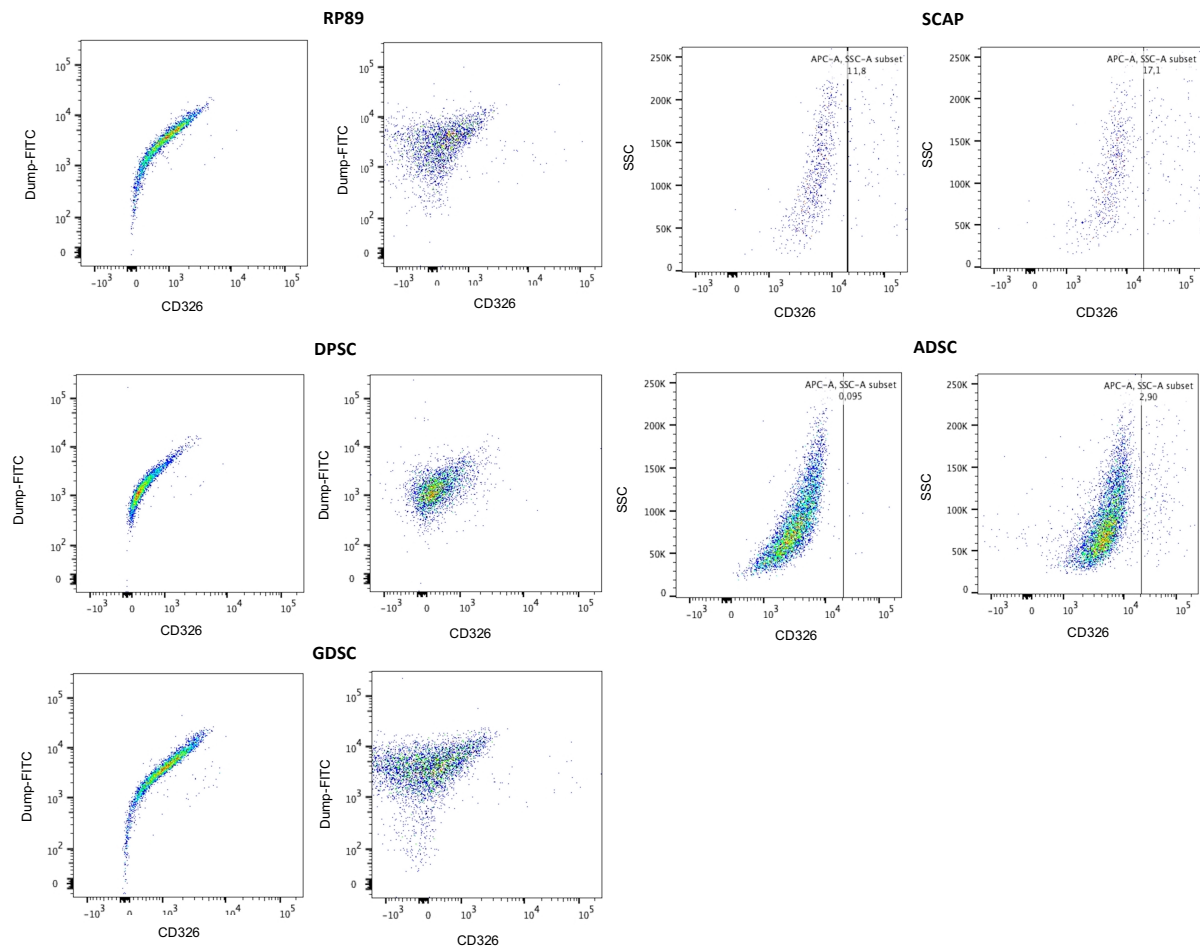


Figure 13. Hematopoietic (CD31, 34, 45 and HLA-DR) and epithelial marker profile (CD326) of five types of mesenchymal stem cells.

Study of hematopoietic (CD31, 34, 45 and HLA-DR) and epithelial markers profile (CD326) of five types of mesenchymal stem cells. Data is shown as a point cloud. Each point represents an event detected by the machine and arranged on a double logarithmic axis graph. Hematopoietic markers are referred to as “dump-FITC”. Note that the evaluation of hematopoietic markers in ADSC and SCAP is absent.

IV.4 Impact of pro-inflammatory stimulation on IDO activity

The objective here was to study the activity of IDO upon pro-inflammatory cytokines (TNF- α and INF- γ) stimulation. Significantly increased IDO activity can be seen in every cell type under inflammatory cytokine conditions (Figure 14). Comparing the different stem cells, no difference has been noticed in their basal state but once activated, RP89 and GDSC seemed to possess the highest IDO activity. More specifically, only RP89 and GDSC differed from ADSCs in L-kynurenine secretion. Simultaneously, RP89 cells had a higher secretion than ADSCs. Finally, the secretion of GDSCs significantly exceeded that of DPSCs, SCAPs and ADSCs.

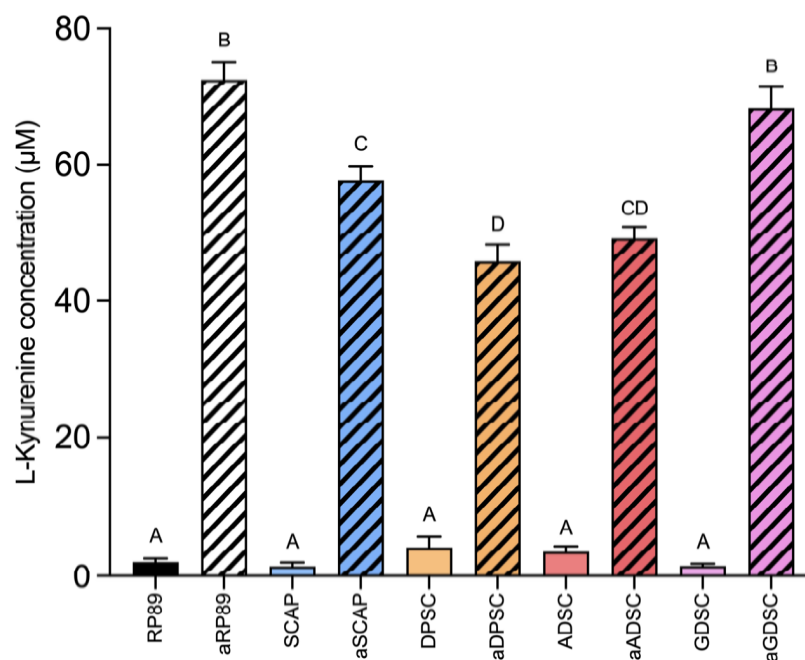


Figure 14. Impact of proinflammatory cytokine stimulation on IDO enzyme activity

Each stem cell species was activated by a treatment of TNF- α (20ng/ml) + IFN- γ (20ng/ml). Data are represented as arithmetic mean + SEM (n=4 and N=3). Each condition was compared with the others by a one-way parametric ANOVA test with multiple comparisons. The α error is set at 0.05. Two conditions with at least one letter in common are not significantly different (p-value $\geq$ 0.05). Conditions not linked by similar letters are significantly different (p-value $<$ 0.05).

Table 1. Impact of proinflammatory stimulation on IDO activity enzyme activity

Condition	Sample size (n)	L-Kynurenine concentration (μM) ± SEM	Fold increase	Statistical significance
RP89	12	2.2 ± 0.5	1,0	
aRP89	12	72.4 ± 2.8	32,5	****
SCAP	12	1.6 ± 0.6	1,0	
aSCAP	12	57.8 ± 2.0	37,1	****
DPSC	12	4.3 ± 1.6	1,0	
aDPSC	12	45.7 ± 2.7	10,5	****
ADSC	12	3.7 ± 0.8	1,0	
aADSC	12	49.2 ± 1.9	13,2	****
GDSC	12	1.6 ± 0.4	1,0	
aGDSC	12	68.0 ± 3.2	41,8	****

Data are shown by their arithmetic mean of L-Kynurenine concentration in solution ± SEM (in μM). Relative concentration has been computed between the concentration of the activated condition and quiescent stem cells. Ordinary one-way ANOVA + multiple comparisons : **** p-value < 0,0001.

IV.5 Impact of pro-inflammatory stimulation on TSG-6 and TGF-β genes expression

The expression of two genes involved in inflammation, TSG-6 and TGF-β, were studied by RT-qPCR.

First, the study of TSG-6 gene expression revealed a statistically significant increase between activated and quiescent conditions (Figure 15A). It should be noted that no difference between activated cells could be detected despite a 2-fold increase in expression between aSCAP and aDPSC. Similarly, no difference in TSG-6 expression between quiescent cells was detected.

The study of TGF-β gene expression revealed no difference in expression between activated and inactivated conditions or between them, respectively (Figure 15B). Importantly, there was a notable trend of upregulated gene expression under inflammatory conditions, particularly in aADSC and aGDSC without being statistically significant.

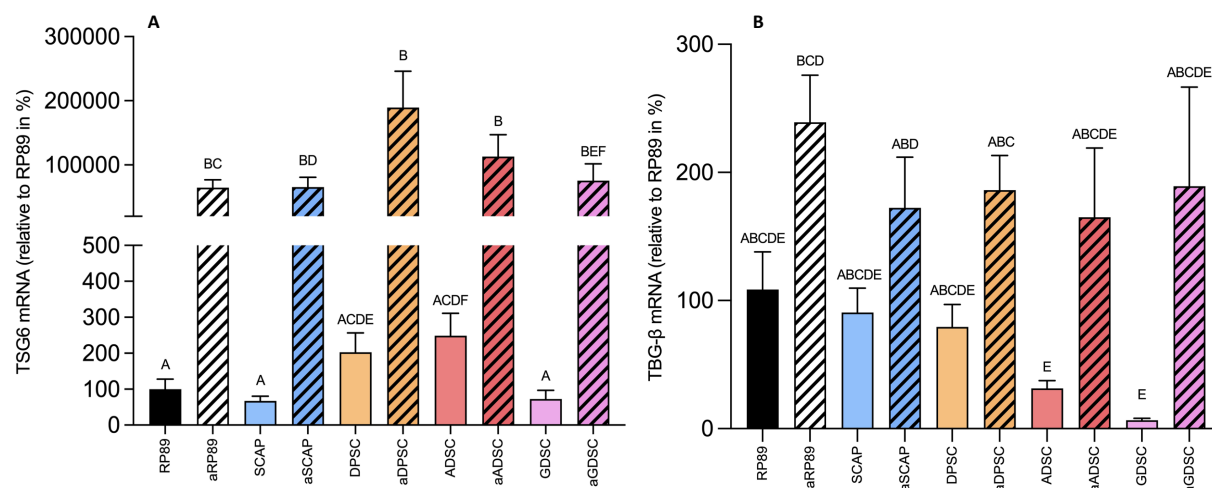


Figure 15. TSG-6 (A) and TGF-β (B) expression in pro-inflammatory conditions

Data are shown by the arithmetic mean ($\pm$ SEM) of the relative expression of mRNA (in %) in comparison to that of RP89. The data obtained by RT-qPCR were computed with a nonparametric ANOVA + Kruskal-Wallis's multiple comparisons test : Two conditions with at least one letter in common are not significantly different (p -value ≥ 0.05). Two conditions with no letters in common are significantly different (p -value < 0.05).

Table 2. TSG-6 mRNA expression in pro-inflammatory conditions relative to that of RP89

Condition	Sample size (n)	TSG-6 relative expression (relative to RP89 in %) $\pm$ SEM	Relative expression to quiescent cells	Statistical significance
RP89	11	100,00 $\pm$ 27,91	1,00	
aRP89	11	64 641,93 $\pm$ 12 177,00	646,42	**
SCAP	9	67,50 $\pm$ 13,43	1,00	
aSCAP	11	65 749,74 $\pm$ 15 256,36	974,09	**
DPSC	7	202,58 $\pm$ 54,41	1,00	
aDPSC	12	189 414,72 $\pm$ 56 830,24	935,01	**
ADSC	10	248,65 $\pm$ 62,20	1,00	
aADSC	10	113 218,10 $\pm$ 34 188,59	455,33	*
GDSC	8	72,76 $\pm$ 24,46	1,00	
aGDSC	11	75 522,02 $\pm$ 26 448,33	1037,94	*

Table of the results of RT-qPCR on TSG-6 in mesenchymal stem cells stimulated by pro-inflammatory cytokines. Data are shown by their arithmetic mean of relative mRNA expression to that of RP89 (in %) $\pm$ SEM. Nonparametric ANOVA + Kruskal-Wallis's multiple comparisons: * p -value $< 0,05$ and ** p -value $< 0,01$.

Table 3. TGF- β mRNA expression in pro-inflammatory conditions relative to that of RP89

Condition	Sample size (n)	TGF-β relative expression (relative to RP89 in %) $\pm$ SEM	Relative expression to quiescent cells	Statistical significance
RP89	10	108,59 $\pm$ 29,36	1,00	
aRP89	11	239,21 $\pm$ 36,59	2,20	ns.
SCAP	10	90,63 $\pm$ 19,00	1,00	
aSCAP	9	172,44 $\pm$ 39,50	1,90	ns.
DPSC	7	79,46 $\pm$ 17,56	1,00	
aDPSC	8	186,29 $\pm$ 26,97	2,34	ns.
ADSC	11	31,40 $\pm$ 6,08	1,00	
aADSC	10	165,02 $\pm$ 53,96	5,26	ns.
GDSC	7	6,59 $\pm$ 1,50	1,00	
aGDSC	8	189,21 $\pm$ 77,36	28,71	ns.

Table of the results of RT-qPCR on TGF- β in mesenchymal stem cells stimulated by pro-inflammatory cytokines. Data are shown by their arithmetic mean of relative mRNA expression to that of RP89 (in %). Nonparametric ANOVA + Kruskal-Wallis's multiple comparisons: ns. p-value $\geq$ 0,05.

IV.6 Impact of pro-inflammatory stimulation on IL-6 and IL-10 secretion

IL-6 and IL-10 secretion analysis were performed by ELISA on supernatants obtained upon ending of TNF- α and IFN- γ stimulation protocol.

IL-6 expression was statistically increased in all activated conditions compared to their quiescent counterparts (Figure 16). Interestingly, among the stimulated conditions, GDSCs were the ones with the highest IL-6 secretion followed by aSCAPs, by aRP89 and aADSCs with no detected difference and finally by aDPSC. Each inactivated condition did not secrete IL-6 except for GDSCs whose basal expression equaled that of aRP89 and aADSCs. IL-10 secretion could not be detected by ELISA technique in both stimulated and quiescent conditions.

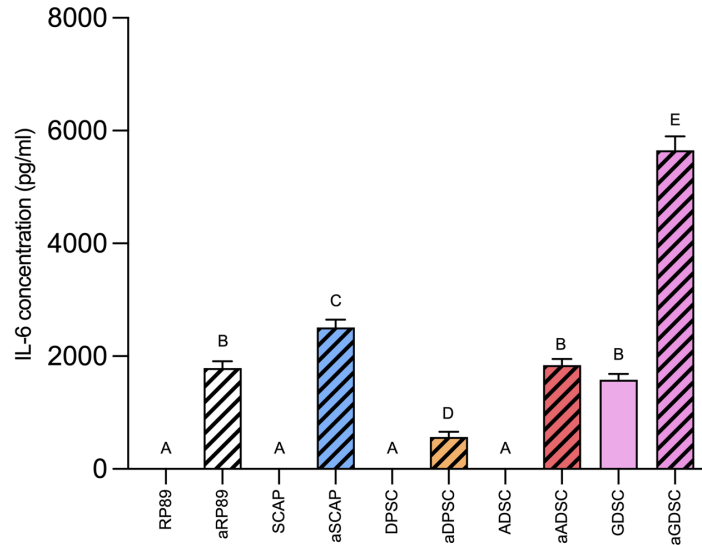


Figure 16. IL-6 expression in pro-inflammatory conditions

Data are shown by the arithmetic mean ($\pm$ SEM) of the IL-6 concentration (in pg/ml) supernatant. The data were computed with an ordinary one-way parametric ANOVA + multiple comparisons test : Two conditions with at least one letter in common are not significantly different (p -value ≥ 0.05). Two conditions with no letters in common are significantly different (p -value < 0.05).

Table 4. IL-6 concentration in pro-inflammatory conditions.

Condition	Sample Size (n)	IL-6 concentration (in pg/ml) $\pm$ SEM	Relative expression to quiescent cells	Statistical significance
RP89	12	0,0 $\pm$ 0,0	1,00	
aRP89	12	1791,0 $\pm$ 120,9	∞	****
SCAP	12	0,0 $\pm$ 0,0	1,00	
aSCAP	12	2511,6 $\pm$ 136,6	∞	****
DPSC	11	0,0 $\pm$ 0,0	1,00	
aDPSC	12	569,3 $\pm$ 88,7	∞	*
ADSC	9	1,0 $\pm$ 1,0	1,00	
aADSC	12	1841,1 $\pm$ 108,8	1840,6	****
GDSC	12	1579,9 $\pm$ 106,6	1,00	
aGDSC	12	5652,7 $\pm$ 246,1	3,6	****

Table of the results of ELISA on IL-6 in mesenchymal stem cells supernatants previously stimulated by pro-inflammatory cytokines. Data are shown by their arithmetic mean of relative IL-6 concentration (pg/ml). Ordinary one-way ANOVA + multiple comparisons: * p -value $< 0,05$, **** p -value $< 0,0001$.

V. Discussion:

V.1 Dental stem cell isolation and characterization

Despite an initial loss of several extracted teeth due to mycotic contamination, self-renewing cells with fibroblast-like phenotype were successfully yielded from the apical papillae of wisdom teeth from the last patient. In order to fulfill recommended criteria emitted by the ISCT for the consensus on hMSC usage, we deliberately decided to fulfill only the two first criteria : 1) the capacity of the cells to adhere to plastic culture surface and 2) the specific cluster of differentiation profile of these cells. Due to the possibility of performing this test *a posteriori*, the time-consuming character of this procedure and the focus driven onto immunomodulating properties of MSC, we decided to bypass the last functional criterion consisting of an objectification of the capacity of triple differentiation of MSC into chondrocytes, osteocytes and adipocytes. Nevertheless, we recognize the importance and limitations of this decision.

The amplification procedure yielded in cells from the apical papillae which have the capability of adhesion to plastic surfaces. Flow cytometry allowed us to view the positive markers on that specific isolated population. Hence, we objectified the expression of MSC markers (CD73 and CD95). It is worth mentioning that every samples did not express the third MSC marker, CD105.

The first hypothesis relates to the high autofluorescence of MSCs. Indeed, it is well described that MSCs have a high autofluorescence and that this can be a source of difficulties when interpreting the results. Compensating with a negative control and compensation beads allows to minimize this deleterious effect as states Bohn et al [59]. However, despite a well performed compensation, the CD105-PE fluorescence profile did not seem to differ from the unlabeled one.

The second hypothesis is that MSCs do not indeed express CD105 for several reasons. Indeed, MSCs being very heterogeneous populations, some subpopulations do not express CD105. This has already been described in the literature with ADSC and umbilical cord-MSC [60].

Furthermore, the absence of CD105 may be due to the altered quality of the tissue of origin and tissue removal, senescence of the MSCs and the state of advancement in cell differentiation. However, the cells extracted from the apical papillae were extracted with integrity and the quality of their preservation and the cells were analyzed after 4 passages. Additionally, an article by Pham et al suggests better immunomodulation by MSCs of CD105 (-) phenotype [60]. To our knowledge, the existence of whole CD105 (-) populations in RP89, SCAP, GDSC and DPSC has not yet been reported.

In addition, the analysis was continued by studying negative markers (hematopoietic and epithelial) of mesenchymal stem cells. Whilst, the absence of epithelial markers is unanimous in each MSC population tested, the acquisition of measurements for haematopoietic markers in ADSC and SCAP was more challenging due to an issue in the fluorophore compensation matrix of the haematopoietic markers. It is worth remembering, that in view of the ISCT criteria, the search for positive markers is of much more importance than the absence of negative markers [7]. Thus, it is impossible to rule out the presence of a haematopoietic subpopulation in the stem cells analyzed. Nevertheless, the presence of cells with a mesenchymal appearance, proliferative profile and exhibiting MSC markers supports the correct isolation of SCAP.

In conclusion, these two experiments yielded in SCAP isolation from fresh extracted wisdom teeth and the confirmation of pure populations with MSC phenotype in those five MSC populations.

V.2 Increased IDO activity upon pro-inflammatory stimulation

The IDO activity assay was straightforward and easily reproducible between different MSC supernatants. Our study demonstrates an increase in IDO activity in all conditions upon stimulation by pro-inflammatory cytokines (TNF- α and IFN- γ) compared to quiescent conditions. Interestingly, no difference was detected between quiescent conditions. However, once activated, MSCs appeared to activate IDO in a statistically significant manner. In this way, DPSCs seem to have the lowest IDO activity relative to the other stem cells. Expressed relative to their quiescent condition, cells with the most increased expression are GDSC (41.7-fold)

followed by SCAP (37.2-fold), RP89 (32.4-fold), ADSC (13.3-fold) and finally DPSC (10.5-fold) with the lowest IDO activity increase.

These results are corroborated by multiple studies demonstrating the upregulation of IDO under pro-inflammatory conditions [61]. However, this test remains only comparative and does not provide any effective character on a potential in vitro and in vivo inhibition of lymphocytes.

Interestingly, in a study conducted by Meisel et al where MSCs were placed in co-culture with mixed lymphocyte reaction (MLR) [62], an inhibition of T-cell proliferation was observed in unstimulated BM-MSC and MLR co-cultures compared to a culture without MSC. A concentration of L-kynurenine in those supernatants was determined to be $17.7 \mu\text{M} \pm 4.9 \mu\text{M}$. The L-kynurenine basal secretion of BM-MSCs is found to be higher than those obtained in our conditions but is largely exceeded once our cells were stimulated with TNF- α and IFN- γ . It might be of interest to repeat the experiment and compare the in vitro effect of prior cytokine stimulation of MSCs on lymphocyte proliferation and lymphocyte gene expression. Finally, it should be noted that IDO activity is possibly dependent on the vitality of the MSC and their progress in cell differentiation.

As aforementioned, IDO is not constitutively expressed in MSCs but is induced by INF- γ . IDO activity appears to possess an activation margin when placed under conditions of increasing IFN- γ concentrations [62]. A new study looking for the effect on these cell populations of further stimulation by IFN- γ on L-kynurenine secretion could be worth considering. This particularity could be at the origin of the increased expression of L-kynurenine by GSDCs. Indeed, these stem cells originate from tissues where a lymphocytic inflammatory response is chronically prevalent. This IFN- γ -rich inflammatory environment, 10-50 U/ml in classical T-cell mediated reactions, could allow for earlier and increased expression of IDO by these cells [62]. However, in their basal state, GDSCs do not appear to retain this increased activity.

V.3 Modulation of TSG-6 and TGF- β genes expression upon pro-inflammatory stimulation

Our study confirms the upregulation of the TSG-6 gene under proinflammatory conditions. This increase was demonstrated in all stimulated conditions with statistical difference. Moreover, no difference was observed between quiescent cells. Interestingly, the expression of aDPSCs tended to be twice that of other cells, although not significant. An interesting study by Wang et al. investigated a possible link between the immunomodulatory action of an IDO metabolites (KYNA) and TSG-6. In their study, they found a link between one of KYNA and the expression as well as the action of TSG-6 [63]. However, only an extrapolation can be made in our study showing the concomitant increase of TSG-6 in conditions with high levels of L-kynurenine expect in aADPSC. In order to assess this in these cell populations, we would need to re-assess the concentration of KYNA and TSG-6 in the solution to further evaluate a causal link.

In contrary, TGF- β expression did not reveal any statistically significant trend between quiescent and activated cells. However, it is important to note that biologically, the expression of the gene was increased by a factor close to 29.00 between stimulated and quiescent GDSC; 5,26 in aADSC; 2,34 in aDSPC; 2,20 in aRP89 and 1,90 in aSCAP. This may already be sufficient to induce trophic effects. It might be of interest as a first step to assess the effective secretion of TGF- β into the medium.

We could pose several hypotheses in view of these results. First, the decrease in statistical power is related to the decrease in the number of samples (n) per condition. Secondly, the undetected samples in PCR are associated with the samples whose mRNA concentration were found to be low during the nucleic acid purification phase. Thirdly, this could be explained by the limitations of our in vitro model. Indeed, the addition of TNF- α and IFN- γ to the culture medium was performed at day 4. In this protocol, the stimulation was stopped after 3 days of stimulation, which could result in insufficient time to allow for increased gene expression. To test this hypothesis, we could repeat this experiment by varying the duration of stimulation and study the transcriptional profile of the gene. Finally, the question of sufficient

proinflammatory cytokine concentration need be posed. New experiments may be interesting to explore this issue.

V.4 Increased IL-6 secretion in activated stem cells supernatants with surprising absence of IL-10

Usually described as a pleiotropic cytokine, the mechanisms underlying IL-6 action are not yet fully elucidated. However, the crucial role of IL-6 in MSC proliferation and MSC-mediated immunomodulation has recently been demonstrated [64]. Our study showed a statistically significant elevation in each inflammatory cytokine-stimulated population. Interestingly, no basal secretion was detected in the samples except for GDSC. Indeed, the concentration of IL-6 secreted by GDSCs was equivalent to that secreted by aRP89 and aADSCs. We could hypothesize a role for TNF- α -mediated induction in inflammatory environments such as the apical granuloma that could be responsible for non-zero basal secretion. Even more surprisingly, the concentration of IL-6 secreted by aGDSCs exceeds twice that of aSCAPs. A parallel study of the transcriptome of the same cells stimulated at different times with a particular emphasis on cytokines such as IL-6 tested here would allow us to investigate a possible difference in the temporality of transcription or secretion. Moreover, this emphasis on the study of IL-6 mediated signaling is crucial as a study by Dorronssoro et al. highlighted the IL-6-mediated intracellular mechanism in hMSCs are still poorly understood [64].

Regarding IL-10, our hypothesis was that, like BM-MSCs and PDLSCs, IL-10 secretion would be increased in response to pro-inflammatory stimuli. Surprisingly, no IL-10 secretion was detected in either quiescent or activated cells. We can postulate various hypotheses for this result. The absence of secretion could be related to the time required to secrete IL-10. Indeed, no study to date has investigated the secretion of IL-10 within 3 days in these cells by ELISA, preferring RT-qPCR technique. This leads to a nuance between these two techniques. One analyses the actual secretion of IL-10 by MSCs while the other focuses on the very early and sensitive transcriptome modification of a cell. In order to investigate this hypothesis, a parallel analysis of the gene expression of IL-10 should be worth considering. Nevertheless, as explained above, IL-10 can also be secreted by immune cells including macrophages. Maggini et al reported that the gross secretion of IL-10 into the extracellular medium can also be

increased by the trophic effect of MSCs on IL-10 secretion by LPS-treated macrophages, thus mediating their indirect immunosuppressive effect [65]. To conclude, these results contrast sharply with the results obtained from many studies that have so far investigated the properties of BM-MSCs and PDLSCs.

VI. Conclusions and perspectives

The discovery of the immunomodulatory properties of mesenchymal stem cells is not recent but the underlying mechanisms and the in vivo effects are still poorly understood. However, it is well known in the literature that the effect of immunity can manifest itself in the modulation of the local inflammatory context but also to act on the homeostasis of regeneration. Moreover, MSCs seem to exhibit enhanced immunomodulatory effects when subjected to inflammatory stimuli. Until now, few studies have taken the time to compare the properties of these different cell populations with each other and with ADSCs frequently used in regenerative therapy. Recently, several populations of MSCs have been extracted from dental tissues, the most recent being MSCs extracted from pathological dental tissues, the periapical granulomas. It is in this perspective that our study is situated.

Our study allowed us to show that under in vitro conditions of stimulation by pro-inflammatory cytokines, all tested MSC populations (RP89, SCAP, DPSC, ADSC and GDSC) show an increased immunomodulatory activity. Through the different in vitro experiments performed, MSCs of dental origin seem to have similar properties to each other and to ADSCs, even if RP89 and GDSC seem more distinct. Indeed, RP89, a monoclonal population of SCAP, seems to present a more marked anti-inflammatory profile. Secondly, the properties of GDSCs also seem interesting because of their similar anti-inflammatory profile to that of RP89 with secretion of inflammatory cytokines (IL-6) perhaps linked to its origin.

This study showed that MSCs of dental origin present in vitro properties frequently sought for a cell therapy model, namely an anti-inflammatory induction. Moreover, we showed that the properties of dental MSCs (SCAP, DPSC) are equivalent to those of ADSCs and that those of RP89 and GDSC surpass ADSCs. This will have to be completed by a study of the cell

differentiation of each type as well as an in vitro co-culture study and their immunomodulatory effects in vivo.

In conclusion, the non-inferiority of dental MSCs, in terms of in vitro immunomodulation, seem to confirm their place in cellular therapies. Dental extractions are routinely performed and are often necessary due to the lack of dental space on the dental arch and are much more accepted than marrow puncture or adipose tissue biopsy. Furthermore, the use of pathological tissues derived-mesenchymal stem cells is novel and opens the field to a novel regenerative and immunomodulatory profile whose in vivo properties are still unknown. Thus, a more detailed understanding of these mechanisms is beneficial for the study of inflammation and regenerative therapies.

VII. References

- [1] E. Bianconi *et al.*, “An estimation of the number of cells in the human body,” *Ann. Hum. Biol.*, vol. 40, no. 6, pp. 463–471, 2013.
- [2] G. Kolios and Y. Moodley, “Introduction to stem cells and regenerative medicine,” *Respiration*, vol. 85, no. 1, pp. 3–10, 2012.
- [3] M. A. M. Aboul-soud, A. J. Alzahrani, and A. Mahmoud, “Therapies , Disease Modelling and Drug Screening,” *Cells*, vol. 10, no. 2319, pp. 1–23, 2021.
- [4] P. Karagiannis *et al.*, “Induced pluripotent stem cells and their use in human models of disease and development,” *Physiol. Rev.*, vol. 99, no. 1, pp. 79–114, 2019.
- [5] A. Golchin, A. Chatziparasidou, P. Ranjbarvan, Z. Niknam, and A. Ardeshirylajimi, “Embryonic Stem Cells in Clinical Trials: Current Overview of Developments and Challenges,” *Adv. Exp. Med. Biol.*, vol. 1312, pp. 19–37, 2021.
- [6] A. J. Friedenstein, J. F. Gorskaja, and N. N. Kulagina, “Fibroblast precursors in normal and irradiated mouse hematopoietic organs,” *Exp. Hematol.*, vol. 4, no. 5, pp. 267–274, 1976, [Online]. Available: <http://europepmc.org/abstract/MED/976387>.
- [7] M. Dominici *et al.*, “Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement,” *Cytotherapy*, vol. 8, no. 4, pp. 315–317, 2006.
- [8] Y. Han, X. Li, Y. Zhang, Y. Han, F. Chang, and J. Ding, “Mesenchymal stem cells for regenerative medicine,” *Cells*, vol. 8, no. 8, 2019.
- [9] S. Kanzaki, M. Toyoda, A. Umezawa, and K. Ogawa, “Application of mesenchymal stem cell therapy and inner ear regeneration for hearing loss: A review,” *Int. J. Mol. Sci.*, vol. 21, no. 16, pp. 1–15, 2020.
- [10] L. Song and R. S. Tuan, “Transdifferentiation potential of human mesenchymal stem cells derived from bone marrow,” *FASEB J.*, vol. 18, no. 9, pp. 980–982, 2004.
- [11] V. K. Mishra *et al.*, “Identifying the Therapeutic Significance of Mesenchymal Stem Cells,” *Cells*, vol. 9, no. 5, pp. 1–30, 2020.
- [12] D. Mushahary, A. Spittler, C. Kasper, V. Weber, and V. Charwat, “Isolation, cultivation, and characterization of human mesenchymal stem cells,” *Cytom. Part A*, vol. 93, no. 1, pp. 19–31, 2018.
- [13] L. Luo, D. H. Hu, J. Q. Yin, and R. X. Xu, “Molecular mechanisms of transdifferentiation

- of adipose-derived stem cells into neural cells: Current status and perspectives," *Stem Cells Int.*, vol. 2018, 2018.
- [14] T. J. Kean, P. Lin, A. I. Caplan, and J. E. Dennis, "MSCs: Delivery routes and engraftment, cell-targeting strategies, and immune modulation," *Stem Cells Int.*, vol. 2013, 2013.
 - [15] N. Caporarello *et al.*, "Pericytes in microvessels: From 'mural' function to brain and retina regeneration," *Int. J. Mol. Sci.*, vol. 20, no. 24, 2019.
 - [16] D. C. Ding, Y. H. Chang, W. C. Shyu, and S. Z. Lin, "Human umbilical cord mesenchymal stem cells: A new era for stem cell therapy," *Cell Transplant.*, vol. 24, no. 3, pp. 339–347, 2015.
 - [17] T. Miki, "Stem cell characteristics and the therapeutic potential of amniotic epithelial cells," *Am. J. Reprod. Immunol.*, vol. 80, no. 4, pp. 1–10, 2018.
 - [18] L. Gan, Y. Liu, D. Cui, Y. Pan, L. Zheng, and M. Wan, "Dental Tissue-Derived Human Mesenchymal Stem Cells and Their Potential in Therapeutic Application," *Stem Cells Int.*, vol. 2020, 2020.
 - [19] R. Berebichez-Fridman and P. R. Montero-Olvera, "Sources and clinical applications of mesenchymal stem cells state-of-the-art review," *Sultan Qaboos Univ. Med. J.*, vol. 18, no. 3, pp. e264–e277, 2018.
 - [20] M. Purwaningrum, N. S. Jamilah, S. D. Purbantoro, C. Sawangmake, and S. Nantavisai, "Comparative characteristic study from bone marrow-derived mesenchymal stem cells," *J. Vet. Sci.*, vol. 22, no. 6, pp. 1–13, 2021.
 - [21] W. Zhou *et al.*, "Single-Cell Profiles and Clinically Useful Properties of Human Mesenchymal Stem Cells of Adipose and Bone Marrow Origin," *Am. J. Sports Med.*, vol. 47, no. 7, pp. 1722–1733, 2019.
 - [22] M. C. Ciuffreda, G. Malpasso, P. Musarò, V. Turco, and M. Gnecci, "Protocols for in vitro differentiation of human mesenchymal stem cells into osteogenic, chondrogenic and adipogenic lineages," *Methods Mol. Biol.*, vol. 1416, pp. 149–158, 2016.
 - [23] Y. Jin *et al.*, "Therapeutic effects of bone marrow mesenchymal stem cells-derived exosomes on osteoarthritis," *J. Cell. Mol. Med.*, vol. 25, no. 19, pp. 9281–9294, 2021.
 - [24] U. Galderisi, G. Peluso, and G. Di Bernardo, "Clinical Trials Based on Mesenchymal Stromal Cells are Exponentially Increasing: Where are We in Recent Years?," *Stem Cell Rev. Reports*, vol. 18, no. 1, pp. 23–36, 2022.

- [25] C. Argentati, F. Morena, M. Bazzucchi, I. Armentano, C. Emiliani, and S. Martino, "Adipose stem cell translational applications: From bench-to-bedside," *Int. J. Mol. Sci.*, vol. 19, no. 11, 2018.
- [26] L. Bacakova *et al.*, "Stem cells: their source, potency and use in regenerative therapies with focus on adipose-derived stem cells – a review," *Biotechnol. Adv.*, vol. 36, no. 4, pp. 1111–1126, 2018.
- [27] L. Mazini, L. Rochette, B. Admou, S. Amal, and G. Malka, "Hopes and limits of adipose-derived stem cells (ADSCs) and mesenchymal stem cells (MSCs) in wound healing," *Int. J. Mol. Sci.*, vol. 21, no. 4, 2020.
- [28] V. M. Zohrabian, C. S. Poon, and J. J. Abrahams, "Embryology and Anatomy of the Jaw and Dentition," *Semin. Ultrasound, CT MRI*, vol. 36, no. 5, pp. 397–406, 2015.
- [29] L. L. Zhou, W. Liu, Y. M. Wu, W. L. Sun, C. E. Dörfer, and K. M. Fawzy El-Sayed, "Oral Mesenchymal Stem/Progenitor Cells: The Immunomodulatory Masters," *Stem Cells Int.*, vol. 2020, 2020.
- [30] G. T. J. Huang, S. Gronthos, and S. Shi, "Critical reviews in oral biology & medicine: Mesenchymal stem cells derived from dental tissues vs. those from other sources: Their biology and role in Regenerative Medicine," *J. Dent. Res.*, vol. 88, no. 9, pp. 792–806, 2009.
- [31] W. Sonoyama *et al.*, "Characterization of the Apical Papilla and Its Residing Stem Cells from Human Immature Permanent Teeth: A Pilot Study," *J. Endod.*, vol. 34, no. 2, pp. 166–171, 2008.
- [32] N. B. Ruparel, J. F. A. De Almeida, M. A. Henry, and A. Diogenes, "Characterization of a stem cell of apical papilla cell line: Effect of passage on cellular phenotype," *J. Endod.*, vol. 39, no. 3, pp. 357–363, 2013.
- [33] S. N. Kumar, T. S. Prasad, P. A. Narayan, and J. Muruganandhan, "Granuloma with langhans giant cells: An overview," *J. Oral Maxillofac. Pathol.*, vol. 17, no. 3, pp. 420–423, 2013.
- [34] C. Estrela *et al.*, "Mesenchymal Stem Cell Marker Expression in Periapical Abscess," *J. Endod.*, vol. 45, no. 6, pp. 716–723, 2019.
- [35] X. Zhang *et al.*, "Mesenchymal Stem Cells and Tuberculosis: Clinical Challenges and Opportunities," *Front. Immunol.*, vol. 12, no. July, pp. 1–14, 2021.
- [36] O. Andrukhov, C. Behm, A. Blufstein, and X. Rausch-Fan, "Immunomodulatory

- properties of dental tissue-derived mesenchymal stem cells: Implication in disease and tissue regeneration," *World J. Stem Cells*, vol. 11, no. 9, pp. 604–617, 2019.
- [37] S. Shantanam and MUELLER, "Mesenchymal stromal cells: clinical challenges and therapeutic opportunities," *Physiol. Behav.*, vol. 176, no. 1, pp. 139–148, 2018.
- [38] Y. Han *et al.*, "The secretion profile of mesenchymal stem cells and potential applications in treating human diseases," *Signal Transduct. Target. Ther.*, vol. 7, no. 1, 2022.
- [39] A. I. CAPLAN, "Adult Mesenchymal Stem Cells for Tissue Engineering Versus Regenerative Medicine," *J. Cell. Physiol.*, vol. 213, no. May, pp. 341–347, 2007.
- [40] G. Rossi, A. Manfrin, and M. P. Lutolf, "Progress and potential in organoid research," *Nat. Rev. Genet.*, vol. 19, no. 11, pp. 671–687, 2018.
- [41] A. Bartholomew *et al.*, "Mesenchymal stem cells suppress lymphocyte proliferation in vitro and prolong skin graft survival in vivo," *Exp. Hematol.*, vol. 30, no. 1, pp. 42–48, 2002.
- [42] E. Zappia *et al.*, "Mesenchymal stem cells ameliorate experimental autoimmune encephalomyelitis inducing T-cell anergy," *Blood*, vol. 106, no. 5, pp. 1755–1761, 2005.
- [43] M. D. P. De la Rosa-Ruiz *et al.*, "Mesenchymal stem/stromal cells derived from dental tissues: A comparative in vitro evaluation of their immunoregulatory properties against t cells," *Cells*, vol. 8, no. 12, pp. 1–22, 2019.
- [44] A. Navas *et al.*, "Anti-Inflammatory and Anti-Fibrotic Effects of Human Amniotic Membrane Mesenchymal Stem Cells and Their Potential in Corneal Repair," *Stem Cells Transl. Med.*, vol. 7, no. 12, pp. 906–917, 2018.
- [45] T. D. Hughes, O. F. Güner, E. C. Iradukunda, R. S. Phillips, and J. P. Bowen, "The Kynurenine Pathway and Kynurenine 3-Monooxygenase Inhibitors," *Molecules*, vol. 27, no. 1, pp. 1–26, 2022.
- [46] A. Muneer, "Kynurenine Pathway of Tryptophan Metabolism in Neuropsychiatric Disorders: Pathophysiologic and Therapeutic Considerations," *Clin. Psychopharmacol. Neurosci.*, vol. 18, no. 4, pp. 507–526, 2020.
- [47] C. O. and U. Grohmann, "Indoleamine 2,3-Dioxygenase and Regulatory Function: Tryptophan Starvation and Beyond," *Methods Mol. Biol.*, vol. 677, pp. 269–280, 2011.
- [48] U. Grohmann, F. Fallarino, and P. Puccetti, "Tolerance, DCs and tryptophan: Much ado

- about IDO,” *Trends Immunol.*, vol. 24, no. 5, pp. 242–248, 2003.
- [49] W. Ouyang, S. Rutz, N. K. Crellin, P. A. Valdez, and S. G. Hymowitz, “Regulation and functions of the IL-10 family of cytokines in inflammation and disease,” *Annu. Rev. Immunol.*, vol. 29, pp. 71–109, 2011.
 - [50] M. H. Mannino, Z. Zhu, H. Xiao, Q. Bai, M. R. Wakefield, and Y. Fang, “The paradoxical role of IL-10 in immunity and cancer,” *Cancer Lett.*, vol. 367, no. 2, pp. 103–107, 2015.
 - [51] T. Tanaka, M. Narazaki, and T. Kishimoto, “IL-6 in Inflammation, Immunity, and Disease,” vol. 6, no. Kishimoto 1989, pp. 1–16, 2014.
 - [52] I. I.- Immunotherapy, T. Tanaka, M. Narazaki, and T. Kishimoto, “Interleukin (IL-6) Immunotherapy,” pp. 1–15, 2018.
 - [53] R. Weber *et al.*, “IL-6 as a major regulator of MDSC activity and possible target for cancer immunotherapy,” *Cell. Immunol.*, vol. 359, no. November 2020, p. 104254, 2021.
 - [54] M. Morikawa, R. Derynck, and K. Miyazono, “TGF- β and the TGF- β Family: Context-Dependent Roles in Cell and Tissue Physiology,” *Cold Spring Harb. Perspect. Biol.*, pp. 1–24, 2016.
 - [55] A. Kimura and T. Kishimoto, “IL-6: Regulator of Treg/Th17 balance,” *Eur. J. Immunol.*, vol. 40, no. 7, pp. 1830–1835, 2010.
 - [56] M. A. T. and D. Sheppard, “TGF- β Activation and Function in Immunity,” *Annu Rev Immunol.*, vol. 32, pp. 51–82, 2014.
 - [57] A. J. Day and C. M. Milner, “TSG-6: A multifunctional protein with anti-inflammatory and tissue-protective properties,” *Matrix Biol.*, vol. 78–79, pp. 60–83, 2019.
 - [58] D. J. Kota, L. L. Wiggins, N. Yoon, and R. H. Lee, “TSG-6 produced by hMSCs delays the onset of autoimmune diabetes by suppressing Th1 development and enhancing tolerogenicity,” *Diabetes*, vol. 62, no. 6, pp. 2048–2058, 2013.
 - [59] A. B. Bohn, B. K. Moller, and M. S. Petersen, “Flow cytometry and compensation of highly autofluorescent cells: the example of mesenchymal stem cells,” *Stem Cell Biol. Res.*, vol. 2, no. 1, p. 4, 2015.
 - [60] L. H. Pham, N. B. Vu, and P. Van Pham, “The subpopulation of CD105 negative mesenchymal stem cells show strong immunomodulation capacity compared to CD105 positive mesenchymal stem cells,” *Biomed. Res. Ther.*, vol. 6, no. 4, pp. 3131–3140, 2019.

- [61] J. Ogbechi, F. I. Clanchy, Y. S. Huang, L. M. Topping, T. W. Stone, and R. O. Williams, "IDO activation, inflammation and musculoskeletal disease," *Exp. Gerontol.*, vol. 131, p. 110820, 2020.
- [62] R. Meisel, A. Zibert, M. Laryea, U. Göbel, W. Däubener, and D. Dilloo, "Human bone marrow stromal cells inhibit allogeneic T-cell responses by indoleamine 2,3-dioxygenase-mediated tryptophan degradation," *Blood*, vol. 103, no. 12, pp. 4619–4621, 2004.
- [63] G. Wang *et al.*, "Kynurenic acid, an IDO metabolite, controls TSG-6-mediated immunosuppression of human mesenchymal stem cells," *Cell Death Differ.*, vol. 25, no. 7, pp. 1209–1223, 2018.
- [64] A. Dorronsoro *et al.*, "Intracellular role of IL-6 in mesenchymal stromal cell immunosuppression and proliferation," *Sci. Rep.*, vol. 10, no. 1, pp. 1–12, 2020.
- [65] J. Maggini *et al.*, "Mouse bone marrow-derived mesenchymal stromal cells turn activated macrophages into a regulatory-like profile," *PLoS One*, vol. 5, no. 2, 2010.

