Universidade de Aveiro **Departamento de Química**

2015

MARTA DUQUE

O POTENCIAL IMUNOSSUPRESSOR DE EXTRATO DE MEMBRANA AMNIÓTICA HUMANA

THE IMMUNOSUPPRESSIVE POTENTIAL OF HUMAN AMNIOTIC MEMBRANE EXTRACT

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, Ramo Bioquímica Clínica, realizada sob a orientação científica do Doutor Artur Augusto Paiva, Técnico Superior de Saúde e Assessor no Instituto Português do Sangue e Transplantação de Coimbra, e Doutora Maria do Rosário Gonçalves dos Reis Domingues, Professora Auxiliar com Agregação do Departamento de Química da Universidade de Aveiro.

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Palavras-chave células mesenquimais do estroma, citocina pro-inflamatória, imunossupressão, linfócitos T, membrana amniótica

Resumo

Tanto as células mesenquimais do estroma (MSCs) como a membrana amniótica humana (hAM) possuem capacidade imunoreguladora, levando a que vários estudos se debrucem sobre a sua aplicação na prevenção e tratamento de doenças imunológicas, e especialmente sobre a sua capacidade de modular células T. No entanto, há pouca informação acerca dos efeitos concretos sobre diferentes fases de ativação e diferenciação de células T. O principal objetivo deste estudo foi determinar se um extrato de hAM (hAME) exerce efeito diferencial sobre diferentes subpopulações de células T (células T $CD4^+$ e $CD8^+$ naïve, memória central, memória efetoras e efetoras). Para esse efeito, células mononucleares do sangue periférico (PBMC) foram cultivadas na presença ou ausência de hAME e estimuladas com acetato miristato de forbol (PMA) mais ionomicina. A proliferação celular foi avaliada por um ensaio de incorporação de timidina e as percentagens de linfócitos T produtores de citocinas pró-inflamatórias foram determinadas por citometria de fluxo. O fenótipo de células derivadas de hAM foi também determinado por citometria de fluxo. Foi ainda estudada a expressão de mRNA em células T $CD4^+$ e $CD8^+$, células T reguladoras (Treg) e células T γδ purificadas. As células derivadas de hAM continham células epiteliais e MSCs. O extrato exibiu um efeito anti-proliferativo e reduziu a frequência de células produtoras de fator de necrose tumoral alfa (TNFα), interferão gama (IFNγ), e interleucina-2 (IL-2) em todas as subpopulações de células T estudadas, assim como a frequência de células T produtoras de IL-17 e IL-9. O padrão de inibição variou entre células T $CD4^+$ e $CD8^+$, entre cada subpopulação celular, e dependendo da citocina em estudo. O hAME provovou também diminuição da expressão de mRNA de granzima B, perforina e recetor de ativação NKG2D em células T $CD8^+$e células T γδ, assim como o aumento de expressão de Foxp3 e IL-10 em células T $CD4^+$, e aumento de expressão IL-10 em células Treg. O hAME regula diferencialmente diferentes subpopulações de células T e, portanto, o efeito do hAME sobre respostas de células T será dependente das subpopulações de células T envolvidas, ainda assim, hAME tem uma ação global anti-inflamatória.

Keywords amniotic membrane, immunosuppression, pro-inflammatory cytokine, mesenchymal stromal cells, T lymphocytes

Abstract Both mesenchymal stromal cells (MSCs) and human amniotic membrane (hAM) possess immunoregulatory potential, driving several studies to focus on their application in the prevention and treatment of immunological disorders, and especially on their ability to modulate T cell responses. However there is little information regarding the concrete effects over different activation and differentiation stages of T cells. The main objective of this study was to determine whether or not a hAM extract (hAME) had a differential effect over different T cell subpopulations ($CD4^+$ and $CD8^+$ T naïve, central memory, effector memory and effector cells). Thus, peripheral blood mononuclear cells (PBMC) were cultured in the presence or absence of hAME and stimulated with phorbol myristate acetate (PMA) plus ionomycin. Cell proliferation was evaluated through a thymidine incorporation assay and the percentages of pro-inflammatory cytokine producing T cells were determined by flow cytometry. The phenotype of hAM-derived cells was also assessed by flow cytometry. Plus, the mRNA expression of selected genes was evaluated in purified $CD4^+$ and $CD8^+$ T cells, regulatory T cells (Treg) and γδ T cells. The hAM-derived cells contained hAM epithelial cells and MSCs. The extract displayed an anti-proliferative effect and reduced the frequency of tumor necrosis factor-alpha (TNFα), interferon gamma (IFNγ), and interleukin-2 (IL-2) producing cells, within all T cell subsets. The hAME also diminished the frequency of IL-17 and IL-9 producing T cells. The pattern of inhibition varied between $CD4^+$ and $CD8^+$ T cells, between T cell subsets, and depending on the cytokine under study. The hAME also produced a decrease in mRNA expression of granzime B, perforin and activating receptor NKG2D by $CD8^+$ T cells, γδ T cells as well as an upregulation of Foxp3 and IL-10 gene expression in $CD4^+$ T cells and an upregulation of IL-10 mRNA expression in Treg cells. These results show that the hAME differentially regulates different T cell subsets and therefore the effect of the hAME over T cells responses will depend on the T cell subpopulations involved. Still, the hAME has an overall anti-inflammatory action.

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activity of immune effcetor cells, stimulating the removal of the infection (or worsening the disease state in case of chronic inflammation). Vigorous inflammation, on the othe hand, licenses MSCs to display an anti-inflammatory and regenerative behavior (MSC2) to prevent tissue damage and maintain the homeostasis. The arrows (→) and end-circles (—●) represent stimulation and inhibition, respectively.

Figure 6. Basic scheme of the methodology adopted for the evaluation of the immunosuppressive potential of hAME and hAM-derived cells.

Figure 7. Phenotypic characterization of hAM-derived cells. Left panel: Bivariate dot plot histogram illustrating the hAM-derived cells, following culture, displaying two distinct cell populations; A and B. Right panel: Phenotypic characterization of the two cell populations identified in hAM-derived cell cultures as the expression level of several phenotypic markers, measured as the mean fluorescence intensity (MFI) (mean ± SD). Results were obtained with four hAM samples. Statistically significant differences were considered when $p<0.05$ for the Mann-Whitney Test: * for the groups indicated in the figure.

Figure 8. Thymidine incorporation assay. Results represent PBMC proliferation, measured as $[H^3]$-thymidine incorporation (mean ± SD) without mitogen stimulation or following PBMC activation by mitogenic stimuli. Results were obtained for three PBMC samples. Statistically significant differences were considered when $p<0.05$ for the Wilcoxon paired-sample test: * for the groups indicated in the figure.

Figure 9. Effect of different doses of hAME over the frequency of TNFα-expressing $CD4^+$ and $CD8^+$ T cells. (A-B) Percentage of TNFα-producing $CD4^+$ (A) and $CD8^+$ T cells (B) (mean ± SD) among four different subsets (naïve, central memory, effector memory and effector) following PBMC stimulation in the absence (PBMC + PMA + Ionomycin) or presence of hAME in the culture medium (PBMC + 50µL hAME + PMA + Ionomycin, PBMC + 100µL hAME + PMA + Ionomycin, PBMC + 200µL hAME + PMA + Ionomycin). (C-D) Percent inhibition (mean ± SD) induced by hAME on the frequency of TNFα producing $CD4^+$ (C) and $CD8^+$ T cells (D). Results were obtained for three PBMC samples. Statistically significant differences were considered when

$p<0.05$ for the Wilcoxon paired-sample test: * versus PBMC + PMA + Ionomycin; ** for the groups indicated in the figure.

Figure 10. Effect of hAME over TNFα expressing $CD4^+$ and $CD8^+$ T cells. (A-B) Percentage of TNFα producing $CD4^+$ (A) and $CD8^+$ T cells (B) (mean ± SD), distributed among the naïve, central memory, effector memory and effector subsets, following PBMC stimulation in the absence (PBMC + PMA + Ionomycin) or presence of hAME in the culture medium (PBMC + 200μL hAME + PMA + Ionomycin). (C-D) Amount of TNFα expressed per $CD4^+$ (C) and $CD8^+$ T cell (D), measured as the MFI (mean ± SD). (E-F) Percent inhibition (mean ± SD) induced by hAME on the frequency of TNFα producing $CD4^+$ and $CD8^+$ T cells (E) and amount of TNFα expressed per cell (F). Results were obtained for eight PBMC samples. Statistically significant differences were considered when $p<0.05$ for the Wilcoxon paired-sample test: * versus PBMC + PMA + Ionomycin; ** for the groups indicated in the figure.

Figure 11. Effect of hAME over IFNγ expressing $CD4^+$ and $CD8^+$ T cells. (A-B) Percentage of IFNγ producing $CD4^+$ (A) and $CD8^+$ T cells (B) (mean ± SD), distributed among the naïve, central memory, effector memory and effector subsets, following PBMC stimulation in the absence (PBMC + PMA + Ionomycin) or presence of hAME in the culture medium (PBMC + 200μL hAME + PMA + Ionomycin). (C-D) Amount of IFNγ expressed per $CD4^+$ (C) and $CD8^+$ T cell (D), measured as the MFI (mean ± SD). (E-F) Percent inhibition (mean ± SD) induced by hAME on the frequency of IFNγ producing $CD4^+$ and $CD8^+$ T cells (E) and amount of IFNγ expressed per cell (F). Results were obtained for eight PBMC samples. Statistically significant differences were considered when $p<0.05$ for the Wilcoxon paired-sample test: * versus PBMC + PMA + Ionomycin; ** for the groups indicated in the figure.

Figure 12. Effect of hAME over IL-2 expressing $CD4^+$ and $CD8^+$ T cells. (A-B) Percentage of IL-2 producing $CD4^+$ (A) and $CD8^+$ T cells (B) (mean ± SD), distributed among the naïve, central memory, effector memory and effector subsets, following PBMC stimulation in the absence (PBMC + PMA + Ionomycin) or presence of hAME in the culture medium (PBMC + 200μL hAME + PMA + Ionomycin). (C-D) Amount of IL-2 expressed per $CD4^+$ (C) and $CD8^+$ T cell (D), measured as the MFI (mean ± SD). (E-F) Percent

inhibition (mean ± SD) induced by hAME on the frequency of IL-2 producing $CD4^+$ and $CD8^+$ T cells (E) and amount of IL-2 expressed per cell (F). Results were obtained for eight PBMC samples. Statistically significant differences were considered when $p<0.05$ for the Wilcoxon paired-sample test: * versus PBMC + PMA + Ionomycin; ** for the groups indicated in the figure.

Figure 13. Effect of hAME over IL-9^+ and IL-9^+IL-2^+ T cells. (A) Percentage of IL-9 producing cells (mean ± SD), within total $CD4^+$ and $CD8^+$ T cells, following PBMC stimulation in the absence (PBMC + PMA + Ionomycin) or presence of hAME in the culture medium (PBMC + 200µL Hame + PMA + Ionomycin). (B) Percentage of IL-9 producing T cells that simultaneously express IL-2 (mean ± SD). (C-D) Amount of protein expressed per cell among IL-9^+ (C) and IL-9^+IL-2^+ T cells (D) (MFI, mean ± SD). (E-F) Percent inhibition (mean ± SD) induced by hAME over the frequency of IL-9^+ and IL-9^+IL-2^+ T cells (E) and amount of cytokine expressed per cell (F). Results were obtained for eight PBMC samples. Statistically significant differences were considered when $p<0.05$ for the Wilcoxon paired-sample test: * versus PBMC + PMA + Ionomycin; ** for the groups indicated in the figure.

Figure 14. Effect of hAME over IL-17^+, IL-17^+TNFα^+, IL-17^+IFNγ^+ and IL-17^+IL-2^+ T cells. (A) Percentage of IL-17 producing cells (mean ± SD), within total $CD4^+$ and $CD8^+$ T cells following PBMC stimulation the absence (PBMC + PMA + Ionomycin) or presence of hAME in the culture medium (PBMC + 200µL hAME + PMA + Ionomycin). (B-D) Percentage of IL-17 producing $CD4^+$ and $CD8^+$ T cells that simultaneously express TNFα (B), IFNγ (C) or IL-2 (D). (E-H) Amount of protein expressed per cell among IL-17^+ (E), IL-17^+TNFα^+ (F), IL-17^+IFNγ^+ (G) and IL-17^+IL-2^+ T cells (H) (MFI, mean ± SD). (I-J) Percent inhibition (mean ± SD) induced by hAME on the frequency of IL-17^+, IL-17^+TNFα^+, IL-17^+IFNγ^+ and IL-17^+IL-2^+ T cells (I) and amount of cytokine expressed per cell (J). Results were obtained for eight PBMC samples. Statistically significant differences were considered when $p<0.05$ for the Wilcoxon paired-sample test: * versus PBMC + PMA + Ionomycin; ** for the groups indicated in the figure.

Figure 15. Effect of hAM-derived cells over the frequency of IL-2 expressing T cells. Percentage of IL-2 producing cells among different $CD4^+$ (A) and $CD8^+$ T cell

(B) subsets (naïve, central memory, effector memory and effector) (mean ± SD) following PBMC stimulation in the absence (PBMC + PMA + Ionomycin) or presence of hAM-derived cells, in a proportion of PBMC-to-hAM-derived cells of either 10:1 (PBMC + 10:1 hAM cells + PMA + Ionomycin) or 2:1 (PBMC + 2:1 hAM cells + PMA + Ionomycin), in the culture medium. Results were obtained for two PBMC samples and two replicates. Statistically significant differences were considered when $p<0.05$ for the Wilcoxon paired-sample test: * versus PBMC + PMA + Ionomycin.

Figure 16. mRNA expression in purified $CD4^+$, $CD8^+$, γδ T and Treg cells. Semi-quantitative analysis of mRNA expression in FACS-purified T cells from two separate culture conditions: PBMC stimulated in the absence (PBMC + PMA + Ion; blue bars) or presence of hAME (PBMC + 200μL hAME + PMA + Ion; orange bars). Results were obtained for five PBMC samples. Statistically significant differences were considered when $p<0.05$ for the Wilcoxon paired-sample test: * for the groups indicated in the figure.

LIST OF ABBREVIATIONS

Ang-1	Angiopoietin-1
ANOVA	Analysis of variance
AnxA1	Annexin A1
APC	Antigen presenting cell
APC	Allophycocyanin
APCH7	Allophycocyanin-hilite 7
CD	Cluster of differentiation
CM	Culture medium
COX	Cyclooxygenase
cpm	Counts per minute
CYC1	Cytochrome c1
DC	Dendritic cell
DMEM	Dulbecco's Modified Eagle's medium
DNA	Deoxyribonucleic acid
EAE	Experimental autoimmune encephalomyelitis
EGF	Epidermal growth factor
EPO	Erythropoietin
FasL	Fas ligand
FBS	Fetal bovine serum
FGF	Fibroblast growth factor

FITC	Flurescein isothiocyanate
FOXP3	Forkhead box P3
GATA3	GATA binding protein 3
GvHD	Graft-versus-host-disease
hAM	Human amniotic membrane (AM)
HBSS	Hank's Balanced Salt Solution
HC-HA	Heavy chain-hyaluronic acid
HGF	Hepatocyte growth factor
HGF-1	Hematopoietic growth factor-1
HLA	Human leukocyte antigen
hMSC	Human mesenchymal stromal cell (MSC)
IDO	Indoleamine 2,3-dioxygenase
IFNγ	Interferon-gamma
IGF-1	Insulin growth factor-1
IL	Interleukin
IL1RA	IL-1 receptor antagonist
Ion	Ionomycin
KGF	Keratinocyte growth factor
mAb	Monoclonal antibody
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex

MIF	Macrophage migration inhibitory factor
MIP-1α	Macrophage inflammatory protein 1 alpha
mRNA	Messenger ribonucleic acid (RNA)
NK	Natural killer cell
NKG2D	Natural-killer group 2, member D
NKT	Natural Killer T cell
NO	Nitric oxide
PacB	Pacific blue
PacO	Pacific orange
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered solution
PDGFRβ	Platelet-derived growth factor receptor-β
PDL-1	Programmed cell death ligand 1
PE	Phycoerythrin
PECγ5	Phycoerythrin-cyanine 5
PECγ7	Phycoerythrin-cyanine 7
PerCPCy5.5	Peridinin chlorophyll protein-cyanine 5.5
PG	Prostaglandin
PGE2	Prostaglandin E2
PHA	Phytohaemagglutinin
PMA	Phorbol 12-myristate 13-acetate

PTX3	Pentraxin 3
PWM	Pokeweed mitogen
RPMI	Roswell Park Memorial Institute
RT	Room temperature
RT-PCR	Real-time polymerase chain reaction
SD	Standard deviation
SDF-1	Stromal cell derived factor 1
SF3A1	Splicing factor 3A subunit 1
SPA	Staphylococcal protein A
STAT6	Signal transducer and activator of transcription 6
Tas	T anergic-suppressor cell
Tc	Cytotoxic T lymphocyte
T_{CM}	Central memory T cell
TCR	T cell receptor
T_{EM}	Effector memory T cell
TGFβ	Transforming growth factor beta
Th	T helper cell
TIMP	Tissue inhibitor of metalloproteinase
TNFα	Tumor necrosis factor α
Tr1	Type 1 regulatory T cells
Treg	Regulatory T cell

V500	Violet 500
VEGF	Vascular endothelial growth factor

1. INTRODUCTION

1.1. INNATE AND ADAPTIVE IMMUNITY

Immune responses are a set of complex and interconnected mechanisms that provide the organism protection against disease. These mechanisms are carried out by a vast collection of cells and molecules, which constitute the immune system.

The innate immune responses are the first protective part of the immune system; they pose a barrier to infection and eliminate infecting microbes through universal mechanisms. The adaptive immune responses do not develop immediately, but represent a specific response to the antigen. These responses can also be elicited against life-saving organ transplants resulting in graft rejection, additionally, because the immense variety of receptors of the adaptive immunity is generated at random, the body can sometimes respond to harmless materials such as allergens (leading to allergies) and self-antigens (resulting in autoimmunity) (*Abbas & Lichtman 2011*).

The innate immunity includes the external defenses, for example, the skin and mucous membranes, which form a barrier to the entry of infectious microbes and secrete natural antimicrobial substances. Other agents of innate immunity include lymphocytes, phagocytes and auxiliary plasma proteins (including the complement system and acute phase proteins) (*Abbas & Lichtman 2011, Chapel et al. 2014, Playfair & Chain 2013*).

Antigen recognition by the innate immune system results in the activation of a set of basic functions with the purpose of removing the microbe from the host and releasing important inflammatory mediators (cytokines), to activate other immune cells, and chemoattractant cytokines (chemokines) that recruit immune cells to the site of inflammation. Neutrophils, macrophages and other phagocytes ingest and destroy microbes (cell-mediated immunity) while the products of complement activation coat the microbes (opsonization) to facilitate phagocytosis. Innate immune responses have also a crucial role in the initiation and enhancement of adaptive immune responses (*Abbas & Lichtman 2011, Playfair & Chain 2013*).

Adaptive immune responses are produced exclusively by the activities and products of lymphocytes. They develop more slowly partially because activation only occurs if the antigen can reach a lymphoid organ, where the lymphocyte populations reside. However

the adaptive immunity possesses many unique features that make it very effective, namely: i) the existence of receptors for virtually every antigen; ii) specificity for each particular antigen; iii) creation of specific memory; iv) adaptation through the increased efficacy and specialization of the response; v) clonal expansion to generate large numbers of lymphocytes directed against the antigen and vi) tight regulation and contraction that maintain homeostasis, allowing responses against 'new' antigens, and avoid autoimmunity (*Abbas & Lichtman 2011*).

1.1.1. Lymphocytes

The only host cells that express antigen-specific receptors are lymphocytes, a group of cells homogeneous in morphology but with great diversity in lineage, functions and phenotype. They derive from cell precursors in the bone marrow and mature in generative lymphoid organs; B cells mature within the bone marrow and T cells mature in the thymus. The resulting mature cells, in turn, reside in peripheral blood organs. Natural killer (NK) cells are lymphocytes which are components of the innate immune system. NK cells can directly kill infected host, but they do not possess the clonally distributed receptors typical of T and B cells (*Abbas & Lichtman 2011, Chapel et al. 2014, Playfair & Chain 2013*).

The naïve lymphocytes, which have not yet encountered antigen, can recognize the antigen but cannot perform the actions to combat infection; instead, recognition drives clonal expansion and differentiation into memory and effector cells. B cells can differentiate into the effector plasma cells that produce large amounts of antibodies, while T lymphocytes generate many different effector phenotypes. The activated cells enter the blood and lymphatic fluid and can enter and leave tissues as needed (*Abbas & Lichtman 2011, Playfair & Chain 2013*).

B and T cells are morphologically similar but only T lymphocytes express the cluster of differentiation 3 (CD3); a T cell co-receptor. CD3 is present at the surface of all mature T cells ($CD3^+$). The main subsets of T lymphocytes are the T cells that express CD4 ($CD4^+$) and T cells that express CD8 ($CD8^+$). CD4 and CD8 are co-receptors involved in the recognition of class II and class I major histocompatibility complex (MHC) molecules, respectively. In general, $CD4^+$ T helper cells (Th) promote antibody production by B cells and enhance the activities of phagocytes. $CD4^+$ or $CD8^+$ regulatory T cells (Treg) have a suppressive function over the immune response and $CD8^+$ T cytotoxic, or cytolytic,

lymphocytes (Tc) directly destroy infected cells to eliminate the microbes that are out of reach to antibodies (*Abbas & Lichtman 2011*). Antibodies opsonize and neutralize extracellular microbes. They are exclusively produced by B lymphocytes and especially by their differentiated form of plasma cells. Differentiation occurs when B cells recognize the antigen, soluble or at the surface of the microbe, through their surface receptors, which are membrane-bound antibodies. B cell receptors can recognize several types of antigenic substances; therefore antibody-mediated (humoral) responses can be elicited against a wide variety of antigens. In contrast, T cell receptors (TCRs) recognize only antigenic proteins, or protein fragments, presented by MHC molecules at the surface of antigen presenting cells (APCs), thus T cell responses can only be elicited against protein antigens of microbes. The resulting response depends on the type of T cell and the class of MHC (the human MHC has the designation of human leukocyte antigen, HLA).

All nucleated cells express MHC class I molecules which are recognized by the $CD8^+$ T cells and NK cells, protecting the cell from destruction by inducing apoptosis in Tc cells and inhibiting NK cell activation. The absence of MHC class I molecules or the presence of foreign MHC class I leads to Tc and/or NK mediated cell death (*Abbas & Lichtman 2011, Playfair & Chain 2013*), a process involved in alloreactive recognition and rejection of transplants (*Li & Raghavan* 2012).

MHC class II molecules are typically expressed at the surface of APCs, such as dendritic cells (DCs), B lymphocytes and macrophages. The main function of MHC class II molecules is to present antigenic peptides to $CD4^+$ T cells; the recognition of the MHC class II-antigen complex requires CD4 receptors as well as the T cell receptor (TCR) and triggers the activation of the $CD4^+$ naïve T cell (*Travers et al. 2001*). Non-protein antigens captured by APCs cannot be recognized by T cells, but they can activate B lymphocytes (*Abbas & Lichtman 2011, Chapel et al. 2014, Playfair & Chain 2013*).

T cells are unable to recognize the antigen without the previous processing by APCs and full activation also requires secretion of cytokines by the APC along with the interaction between a CD28 molecule at the T cell surface and a co-stimulatory molecule (CD80 or CD86) expressed by the APC.

Effector lymphocytes are short-lived cells whose survival, proliferation and function is dependent on stimulation by the antigen, thus, after elimination of the infection, effector cells rapidly decrease in numbers, as the lack of stimuli leads to their death by apoptosis,

and the immune system returns to the resting state. However, memory cells (memory B cells and memory $CD4^+$ or $CD8^+$ T cells), also originated from lymphocyte activation, can last for years. Memory cells exist in higher numbers than the population of naïve cells specific for the same antigen and, in the case of re-exposure to the antigen, memory lymphocytes respond more rapidly and efficiently than the naïve cells (*Abbas & Lichtman 2011*).

1.1.1.1. T Lymphocyte subsets

Upon activation, naïve $CD4^+$ T cells can differentiate into several different subsets of effector $CD4^+$ T cells (Th cells), which act mainly through the release of cytokines. Distinct Th subsets exert different effector functions and can be distinguished according to their cytokine-secreting profiles; the most well known are Th type 1 (Th1), type 2 (Th2) and type 17 (Th17) (Table 1). The precursor naïve $CD4^+$ T cells are often considered to represent the type 0 (Th0) subset, characterized by the release of a vast panel of cytokines, and having an effect somewhere in between those of Th1 and Th2 (*Abbas & Lichtman 2011*).

Table 1. Characteristics of the main subsets of effector (and regulatory) T lymphocytes.

	$CD4^+$ T effector cells (T helper cells)		
	Key transcription factors	**Cytokines**	**Main functions**
Th1	T-bet STAT1 STAT4	IL-2 IFNγ TNFα/β	Promotion of Tc and NK cell development Macrophage and NK cell activation Increase of IgG production Stimulation of antigen presentation
Th2	GATA3 STAT6	IL-4, 5, 6, 9, 10, 13	Mast cell and eosinophil activation Increase of IgE production Increase of extracellular matrix production by macrophages
Th17	RORγt STAT3	IL-17, 21, 22	Neutrophilic, monocytic inflammation Stimulation of cytokine/chemokine secretion by non-immune cells

	CD8$^+$ T effector cells (cytotoxic T cells)		
Tc1	Eomes T-bet	IFNγ TNFα	Destruction of infected cells by release of proteins that form pores in the membrane of the target cells and induce DNA fragmentation
Tc2		IL-4, 5, 10, 13	
Tc17		IL-17	
	CD4$^+$ and CD8$^+$ regulatory T cells		
Treg	FOXP3 STAT5	IL-10 TGFβ	Inhibition of cytokine production Inhibition of APCs, Tc cell, NK cell and monocyte activities Suppression of T cell activation

Eomes, Eomesodermin; GATA3, GATA binding protein 3; IFNγ, Interferon gamma; IL, Interleukin; T-bet, T-box transcription factor TBX21; Th, T helper cell; Tc, cytotoxic T lymphocyte; RORγ, RAR-related orphan receptor gamma; STAT, Signal transducer and activator of transcription; TNFα, Tumor necrosis factor alpha; Treg, regulatory T cell

Naïve CD8$^+$ T cells, on the other hand, differeniate into the effector CD8$^+$ cytotoxic T lymphocytes (Tc), highly effective in the direct destruction of damaged, infected, malignant or foreign cells; they typically secrete cytolytic proteins such as granulysin, granzime A/B and perforin.

Due to the discovery of the ability of CD8$^+$ T cells to produce a wider array of cytokines than initially thought, cytotoxic T cells are sometimes divided into distinct subsets according to the cytokines they release, in a manner similar to their Th counterparts: the more common type 1 subset (Tc1), like Th1 cells, is characterized by high production of IFNγ and TNFα; the type 2 (Tc2) arrises when CD8$^+$ T cells undergo activation in the presence of IL-4, resulting in Tc cells that display the ability to secrete IL-4, IL-5, IL-10 and IL-13 (cytokines usually associated with Th2 cells) and may have decreased production of IFNγ; and IL-17 producing CD8$^+$ T cells are refered to as type 17 (Tc17) (Table 1) (*Dobrzanski et al. 2004, Vukmanovic-Stejic et al. 2000*).

Table 1.1. A summary of other (lesser known) T helper effector cell phenotypes.

Additional $CD4^+$ T effector phenotypes		
Th subset	**Cytokines**	**Possible functions**
Th3	TGFβ IL-10 (low amounts)	Oral tolerance towards nonpathogenic, non-self antigens
Th9	IL-9 IL-10	Immunity against helminthes Involvement in tissue damaged associated with allergic inflammation, tumors and parasitosis
Th22	IL-22 (preferentially)	Protective role in the regulation of skin wound repair and healing
Tfh	IL-21 IL-4	Promotion of the development of antibody producing plasma cells and memory B cells
Tr1	IL-10 TGFβ (low amounts)	Promotion and maintenance of immune tolerance

IL, Interleukin; Th, T helper cell; Tfh, follicular B helper T cell; TGFβ, transforming growth factor β; Tr1, type 1 regulatory T cell

Regulatory T cells are the most important functional pool of suppressive mechanisms of the immune system. Most Treg cells are $CD4^+$, but there also exist $CD8^+$ Treg cells. These cells are $Foxp3^+$ and exhibit a high expression of CD25; the transcription factor Foxp3 is essential for the development of regulatory functions and CD25 is the alpha chain of the IL-2 receptor. Stimulation with IL-2 (a pro-inflammatory cytokine) is required for Treg cell survival and activity, which indicates that their suppressive effects are elicited and/or enhanced by inflammatory stimuli (*Abbas & Lichtman 2011, Chapel et al. 2014, Playfair & Chain 2013*).

Despite of their pivotal role in immune regulation, there exist T cell subsets with regulatory phenotypes other than the naturally occurring $CD4^+CD25^+$ Treg cells (nTreg); for instance, the Th subsets include cells that can inhibit immune responses in a contact-independent manner. These populations (induced $CD4^+$ Treg cells or iTreg) are secondary suppressor cells, thought to originate directly from conventional $CD4^+$ T cells in peripheral lymphoid tissues, under certain stimuli. The iTreg cells can be subdivided into two other types, according to the cytokine that cause their induction; Th3 and Tr1 (TGFβ and IL-10,

respectively). Following generation, each iTreg will preferentially produce the same cytokine that first cause their induction (Table 1.1). The nTreg and iTreg cells share similar phenotypic characteristics, such as Foxp3 expression, and have similar suppressive functions (*Jonuleit & Schmit 2003, Lin et al. 2013*).

A portion of the activated lymphocytes differentiates into long-lived memory cells, forming a dynamic reservoir of 'experienced' T cells. The memory T cell pool can be subdivided into distinct subsets of memory-carrying T cells. The central memory T cells (T_{CM}) are responsible for fast expansion in case of re-exposure and secrete IL-2. In response to antigen recognition T_{CM} cells swiftly differentiate into effector memory T cells (T_{EM}) that possess effector functions (*Abbas & Lichtman 2011, Mackay 1999*).

There also exist populations of 'unconventional', more primitive, T cells; the gamma-delta T cells (γδ T) and Natural Killer T cells (NKT), which are considered to have both innate and adaptive immune functions and can recognize non-protein antigens.

The γδ T cells express a form of antigen receptor distinct from the 'traditional' TCR and normally do not express CD4 or CD8 (*Abbas & Lichtman 2011, Delves et al. 2011*). Activated γδ T cells can secrete IFNγ, IL-17 and IL-22 and have cytotoxic activity, through the relase of granzime A/B and perforin. Tγδ cells are also thought to possess immunosuppressive functions due to their ability to produce IL-10 (*Abbas & Lichtman 2011, Delves et al. 2011*).

NKT cells have morphologies and granule contents intermediate between T and NK cells and express surface markers usually associated with NK cells along with the expression of low levels of TCR. They can be either $CD4^-CD8^-$ or $CD4^+CD8^-$. Activated NKT cells stimulate many immune cells, including DCs, NK cells and B lymphocytes, through the secretion of IL-4 and IFNγ (*Abbas & Lichtman 2011, Delves et al. 2011*).

1.2. THE AMNIOTIC MEMBRANE

The amnion, or amniotic membrane (AM), represents the innermost layer of the placenta; it is a thin (approximately 0.02 to 0.05 cm thick), resistant and highly flexible membrane of fetal origin composed of an epithelial layer, a basement membrane and an avascular stromal matrix. The former can be further subdivided into three layers; the compact layer, the fibroblast layer and the intermediate, or spongy, layer (Figure 1) (*Bourne 1962, Mamede et al. 2012*).

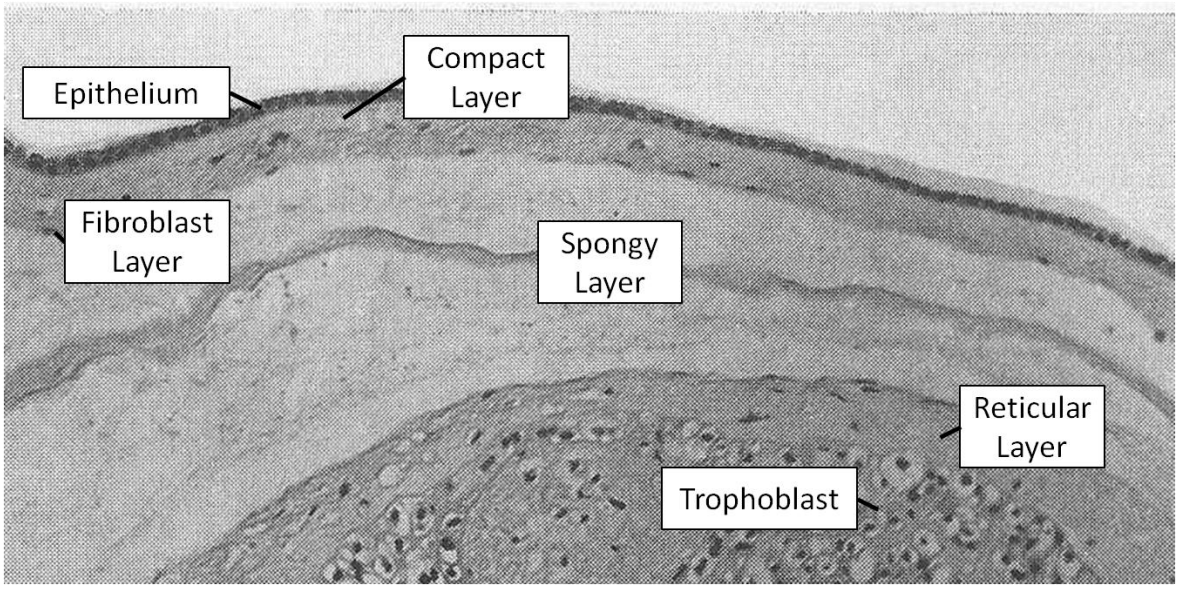

Figure 1. Cross section of the human amnion (above) and chorion (below), showing an epithelial layer, a compact layer, a fibroblast layer, an intermediate (spongy) layer, a reticular layer and the trophoblast. Original magnification ×90; from *Bourne 1962*.

The epithelium of the amniotic membrane consists of a single layer of cuboidal epithelial cells and is in direct contact with the amniotic fluid. The thin basement membrane, tightly adherent to the epithelium, is comprised of reticular connective tissue, as is the underlying compact layer, though the compact layer is much more thicke and dense. The adjacent fibroblast layer is the thickest of the five amniotic layers and, along with the epithelium, the only other which is not devoid of cells; it consists of loosely associated mesenchymal cells (fibroblasts) embedded in a sparse reticular network. The outer spongy layer joins together the amnion and the chorion and is composed of wavy bundles of reticular fibers immersed in mucin. While the chorion is firmly adherent to the underlying maternal

decidua, this intermediate, spongy, layer allows the amniotic membrane to slide smoothly upon the chorion. Additionally, when the amniotic membrane is harvested for medical use, the presence of this layer allows for the easy separation of the amnion from the chorion (*Bourne 1962*) (Figure 2).

Following, approximately, the 18th day of gestation, the fibroblast and spongy layers of the amnion (and occasionally the compact layer) also contain another cell type; the Hofbauer cells. Hofbauer cells are a tye of macrophages of mesenchymal origin that reach high numbers in the placenta, but become rarer as the pregnancy progresses, practically vanishing after the 4th or 5th month of gestation (*Grigoriadis et al. 2013*).

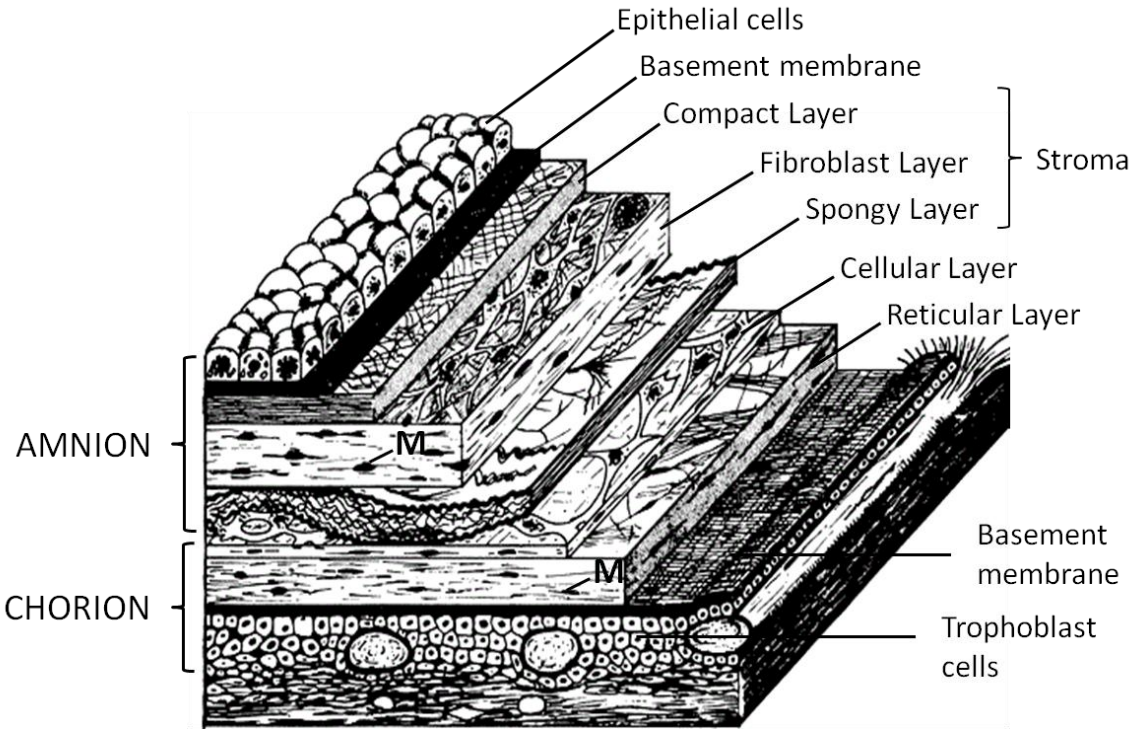

Figure 2. Schematic diagram illustrating the layers that compose the human amnion and chorion. The amniotic membrane is composed of a single layer of epithelial cells, basement membrane, a compact layer and a fibroblast layer. The amnion is separated from the chorion by an intermediate (spongy) layer. The chorion consists of a reticular layer, basement membrane and multi-layered trophoblast cells. The fibroblast (amnion) and reticular layers (chorion) contain mesenchymal cells (M). Adapted from *Bourne 1962*.

Because of its ability to decrease inflammation and fibrosis, promote wound healing and tissue repair, antimicrobial properties and negligible risk of allogeneic rejection, the AM as

long been used in tissue engineering and for therapeutic applications (*Mamede et al. 2012, Davis 1910*).

The immune privilege of AM is due to an active immunosuppressive mechanism, which is thought to be involved in immune tolerance during pregnancy (*Kubo et al. 2001*). Many groups have demonstrated that the AM can inhibit T cell proliferation, Th1/Th2 cytokine production (*Ueta et al. 2002*), reduce infiltration and induce apoptosis of activated neutrophils (*Park & Tseng 2000, Wang e al. 2001*), monocytes, macrophages (*Bauer et al. 2007, Heiligenhaus et al. 2001*) and lymphocytes (*Bauer et al. 2009*), and promote the polarization of M2 macrophages (*Bauer et al. 2012, He et al. 2008*), which have a stimulatory effect on Th2 and Treg cells (*Sica & Mantovani 2012*). These immunoregulatory effects are likely mediated by soluble factors, seeing as they can often be achieved through the use of soluble AM extract or AM culture medium (CM-AM) (*Kubo et al. 2001, Ueta et al. 2002*).

Rossi et al. (*2012*) attempted to identify the factors present in CM-hAM responsible for the suppression of T cell proliferation and reported the involvement of prostaglandins (PGs), which are known to be released by hAM in culture. The group managed to reproduce the inhibitory effect using a mixture of PGs that mimicked those in the CM-hAM, but to a lesser extent. Additionally, the suppression of PG production with cyclooxygenase (COX) inhibitors did not completely abrogate the inhibition of T cell proliferation demonstrating the involvement of other factors (*Rossi et al. 2012*). However, the study focused only on hAM without any type of stimuli and it is thought that the AM can respond to inflammatory stimuli by up-regulating anti-inflammatory factors, possibly enhancing the diversity of AM-derived immunosuppressive factors. Nonetheless, the study does prove that the inhibitory effects of hAM are constitutively expressed and that they rely, at least in part, on PGs. Likewise, it has been demonstrated that hAM produces and releases PGE2, an anti-inflammatory regulator of immune responses (*Ueta et al. 2002*).

Hao et al. (*2000*), in a study with the same objective, reported the expression, by hAM, of several anti-inflammatory proteins, including the cytokine IL10, IL-1 receptor antagonist (IL1RA), all four tissue inhibitors of metalloproteinases (TIMPs) and thrombospondin-1 (TSP-1) (Table 2).

Among the TIMPs, TIMP3 was demonstrated to suppress Th1 cell responses, with a protective effect in autoimmune diseases (*Murthy et al. 2012*). Additionally, TIMP3 is

unique among the TIMP family because it can inhibit not only matrix metalloproteinases, but also the tumor necrosis factor α converting enzyme (TACE) that processes the membrane bound TNFα to its soluble, active form (*Mohammed et al. 2003*). TSP-1, in turn, is capable of regulating T cell responses through several mechanisms; firstly TSP-1 can activate the secreted, inactive, form of TGFβ, bound to the latent TGF-beta binding protein (LTBP) and latency-associated peptide (LAP), by binding to LAP and making it unable to confer latency (*Crawford et al. 1998*). Secondly, the interaction between TSP-1 and the surface T cell receptor CD47 induces the generation of $CD4^+Foxp3^+$ T cells from naïve and memory $CD4^+CD25^-$ T cells. Those cells were named T anergic-suppressor cells (Tas) by *Gimbert at al.* (*2006*) and were similar to the 'normal' Treg cells, but with a CD25 expression level similar to that of Th0 cells, and did not produce granzime B, which means that they did not possess cytolitic activity. Tas cells also produced very low amounts of cytokines (anergic) and were capable of suppressing T cell responses (suppressor) through a mechanism dependent on cell-cell contact, independent from TGFβ and IL-10, and that could not be inhibited by IL-1 or IL-6 (unlike nTreg cells) (*Grimbert et al. 2006, Miller et al. 2013*). Furthermore, the TSP-1/CD47 interaction inhibits hydrogen sulfide (H_2S) signaling, a 'potentiator' of T cell activation, and, simultaneously, hampers the activation-dependent expression of the two enzymes needed for H_2S biosynthesis; cystathionine β-synthase and cystathionine γ-lyase, further disenabling the autocrine actions of H_2S in T cell activation (*Avice et al. 2001*).

Transcriptomic and proteomic analysis of hAM further supported the hypothesis of the involvement of IL1RA, TIMPs and TGFβ (*Hopkinson et al. 2006, Li et al. 2005*), and revealed other proteins with immunoregulatory potential; namely, annexin A1 (AnxA1) (*Hopkinson et al. 2006*), Fas ligand (FasL) (*Hao et al. 2000, Hopkinson et al. 2006*), human leukocyte antigen G (HLA-G) (*Kubo et al. 2001*) and macrophage migration inhibitory factor (MIF) (*Hopkinson et al. 2006*) (Table 2).

FasL expression is typical of for immune-privileged sites (*Griffith et al. 1995*) and has been shown to be involved in the apoptosis of maternal lymphocytes (*Runic et al. 1996*). FasL can induce T cell apoptosis via interaction with its receptor, Fas, expressed by activated T cells. However, it is thought that this regulation is differential between T cells subsets; with $CD4^+$ Th1 cells being more sensitive to Fas-mediated apoptosis than $CD4^+$ Th2 or $CD8^+$ T cells (*Ramsdell et al. 1994, Suzuki & Fink 2000*).

HLA-G is a non-classical HLA class I molecule, typical of fetal-derived placental cells, crucial for maintaining immune tolerance during pregnancy (*Kubo et al. 2001*). While hAM cells display little to no expression of the classical MHC class I molecules (HLA-A, B and C), making them a target for recognition and elimination by cytolytic cells, they do express HLA-G, which can interact with inhibitory receptors of NK cells and Tc cells (*Hunt et al. 2005*). In NK cells, this interaction, induces apoptosis while, in activated T cells, inhibits cell cycle progression (*Bahri et al. 2006, Ketroussi et al. 2011*). Additionally, HLA-G interaction with CD8 in Tc cells can induce apoptosis, independently of activation, through the up-regulation of FasL, which then acts as an autocrine inhibitor (*Contini et al. 2003*). However, this inhibition is thought to be mainly due to the hAM-derived soluble isoforms of HLA-G, particularly HLA-G5, which is known to be involved in the protection of the fetus against aggression by the maternal immune system (*Hunt et al. 2005*), and not for the membrane bound HLA-G (*Bahri et al. 2006, Hunt et al. 2005*).

Heavy chain-hyaluronic acid/pentraxin 3 complex (HC-HA/PTX3), isolated from extracts of hAM, has been demonstrated to induce apoptosis of pro-inflammatory neutrophils and macrophages, reduce macrophage influx to allograft sites, enhance the phagocytosis by macrophages, induce Treg cell expansion and inhibit $CD4^+$ T cell activation, specially towards Th1 cells (*He et al. 2009, He et al. 2014*).

Table 2. Summary of the main hAM-derived factors with immunological properties.

Soluble factor	Effects on immune cells
AnxA1	Promotion of Th1 cell responses (activation dependent) (*D'Aquisto et al. 2007, Gavins & Hickey 2012*)
HLA-G	Induction of apoptosis in NK and Tc cells (*Bahri et al. 2006, Contini et al. 2003, Ketroussi et al. 2011*) Inhibition of T cell proliferation (*Contini et al. 2003, Ketroussi et al. 2011*)
HC-HA/PTX3	Induction of Treg cells; Inhibition of $CD4^+$ T cell activation; Inhibition of Th1 cell responses; Apoptosis of pro-inflammatory macrophages and neutrophils; Decrease in macrophage infiltration; Stimulation of macrophage-mediated phagocytosis (*He et al. 2014*)

TIMP3	Suppression of Th1 cell responses (*Murthy et al. 2012*) Inhibition of TACE activity (*Mohammed et al. 2003*)
IL-10	Inhibition of T cell responses and Th17 differentiation (*Hao et al. 2000*)
IL1RA	Antagonizes the effects of the pro-inflammatory cytokine IL-1 (*Hao et al. 2000*)
FasL	Induction of apoptosis in activated T cells (*Runic et al. 1996*)
MIF	Promotion of Th1 cell responses (*Kithcart et al. 2010, Larson & Horak 2006*) Stimulation of pro-inflammatory cytokine production (*Bacher et al. 1996, Kithcart et al. 2010, Larson & Horak 2006*) Reduction of Treg cell population (*Kithcart et al. 2010*) Increase of PGE2 production (*Kithcart et al. 2010, Leng et al. 2003*)
PGE2	Inhibition of T cell proliferation; Promotion of of Th2 cell responses (*Woorlard et al. 2007*) Inhibition of DC maturation and macrophage activity; Induction of Treg cells (*Baratelli et al. 2005*) Inhibition of Th1 cell responses (*Kalinski 2012*) Suppression of Tc, NK and Th1 cell activation (*Bydlowski et al. 2009*)
TSP-1	TGFβ activation (*Crawford et al. 1998*) Inhibition of T cell activation (*Avice et al. 2001*) Induction of T anergic-suppressor cells (*Grimbert et al. 2006*)

Anx1, Annexin A1; HLA-G, Human leukocyte antigen G; HC-HA/PTX3, Heavy chain-hyaluronic acid/pentraxin 3 complex; TIMP3, Tissue inhibitor of metalloproteinases 3; IL-10, Interleukin 10; IL1RA, IL-1 receptor antagonist; FasL; Fas ligand; MIF, Macrophage migration inhibitory factor; PGE2, Prostaglandin E2; TSP-1, Thrombospondin-1

Contrary to the immunosuppressive effect of the other factors, hAM-derived AnxA1 and MIF seem to have pro-inflammatory actions. AnxA1 favors the generation of Th1 cells by binding to the receptor formyl peptide receptor-like-1 (FPRL-1). FPRL-1 usually exists at very low levels at the surface of T cells but, upon TCR activation, is quickly up-regulated and externalized, along with the increase of AnxA1 secretion (*Gavins & Hickey 2012*). When the FPRL-1/AnxA1 interaction occurs, along with the engagement of TCR, it drives the differentiation towards the Th1 phenotype (*D'Aquisto et al. 2007, Gavins & Hickey 2012*). On the other hand, the FPRL-1/AnxA1 interaction is unable to activate T cells on its

own; therefore this regulatory mechanism may not have a significant effect on T cell responses within an environment that actively inhibits T cell activation. In addition, there have been reports of AnxA1 having a potential role in the suppression of adaptive immune responses in certain contexts (*D'Aquisto et al. 2007*), but the data is still controversial and demands further investigation.

Similarly to AnxA1, MIF is also thought to stimulate Th1 responses (*Larson & Horak 2006*), to promote the secretion of pro-inflammatory cytokines by T lymphocytes (*Bacher et al. 1996, Larson & Horak 2006*) and to reduce the population of Treg cells (*Kithcart et al. 2010*). MIF is bound by CD74 (HLA class II histocompatibility antigen gamma chain) in T cells (*Larson & Horak 2006, Leng et al. 2003*), stimulating the production of TNFα, IFNγ, IL-2, IL-1β, IL-6 and IL-8, and favoring the Th1 phenotype (*Larson & Horak 2006, Kithcart et al. 2010*). However, engagement of CD74 by MIF is also known to up-regulate COX-2 and strongly stimulate PGE2 production (*Kithcart et al. 2010, Leng et al. 2003*), which promotes the generation of $CD4^+$ Treg cells (*Baratelli et al. 2005*), suppresses Tc, NK and Th1 cell activation and promotes the less aggressive Th2 phenotype (*Kalinski 2012*).

Several studies have focused on the clinical efficacy of hAM transplantation in the suppression of inflammation and promotion of wound healing and, in recent years, extracts or homogenates of hAM have been shown to exert the same effects as effectively as hAM transplantation (*Guo et al. 2011, He et al. 2008, He et al. 2009, He et al. 2014*). For instance, *Guo et al.* (*2011*) showed that treatment with an amnion homogenate (with a protein content of 3.6 mg/mL) was as successful as hAM transplantation in the repair of corneal damage, with clear reduction of ocular inflammation, further proving that the cellular factors responsible for the therapeutic effects of hAM are equally present in its extracts, potentially presenting a far simpler alternative to the use of hAM.

hAM extract preparations have become commercially available, although by a very limited number of producers, in the form of a lyophilized powder, prompting investigators to focus their attention on its therapeutically applications, however, the subject is relatively recent and, so far, there have been scarce attempts to examine concrete effects over specific immune cell populations.

1.3. MESENCHYMAL STROMAL CELLS

1.3.1. Nature and function of MSCs

Mesenchymal stromal cells (MSC) are a rare, heterogeneous population of stromal, plastic adherent cells, morphologically similar to fibroblasts, with self-renewal capability and the ability to differentiate into several different lineages (*Bydlowski et al. 2009, Pittenger et al. 1999*).

Although having been originally identified in the stroma of bone marrow (*Friedenstein et al. 1968*), which still constitutes their main source, MSCs can be isolated from virtually every post-natal tissue (*Chong et al. 2012, Hoogduijn et al. 2007, In't et al. 2003, Jo et al. 2007, Patel et al. 2008, Vellasamy et al. 2012, Yang et al. 2013, Zuk et al. 2002*). This wide distribution suggests that, all throughout the lifespan of each individual, MSCs might have a widespread role in tissue repair and regeneration (*Cai et a. 2004*). Because of this, MSCs are considered promising candidates for application in the treatment of several diseases, particularly since they can be activated and recruited to damage sites, where they promote tissue repair. Moreover, MSCs possess several features that further increase the interest in developing MSC-based therapies, namely; i) MSCs are not subjected to ethical issues; ii) they avoid allogeneic rejection; iii) there is very low risk of teratome formation (even if derived from a teratoma-forming human embryonic stem cell line [*Gruenloh et al. 2011*]); iv) they can be isolated from practically any tissue, and v) are effortlessly expanded *in vitro*, from small samples to clinical levels that can be stored for long-periods without significant loss of multipotency (*Madrigal et al. 2014*).

Identification and characterization of MSCs is complicated due to the lack of a unique marker (*Dominici et al. 2006*), still, all MSCs share numerous features and are usually defined by their expression profile based on a panel of several markers (*Bydlowski et al. 2009, Dominici et al. 2006, Qian et al. 2012*) (Table 3).

However, because the expression of surface markers is highly dependent on the cell source and the method of isolation and expansion, definitive identification also requires the assessment of their general biological characteristics, namely; fibroblast-like morphology, ability to adhere to plastic surfaces, self-renewal potential and differentiation into adipocytes, chrondocytes and osteoblasts (*Bydlowski et al. 2009, Dominici et al. 2006*).

Table 3. Positive and negative markers (proposed by the International Society for Cellular Therapy) for MSC identification.

Positive Marker	**Function**
CD105	**Endoglin**; Part of the TGFβ receptor
CD13	**Aminopeptidase N;** Inactivation of bioactive peptides
CD44*	Cell-cell interactions, cell adhesion and migration
CD73	**5' Nucleotidase**; Adenosine monophosphate conversion to adenosine
CD90	**Thy1**; Cell-cell and cell-matrix interactions
Negative Marker	**Excludes**
CD11b and CD14	Monocytes and macrophages
CD34	Hematopoietic and endothelial cells
CD45	Leukocytes
CD79α and CD19α	B cells
HLA class II	APCs and lymphocytes (Stimulated MSCs may express HLA class II)

*CD44 expression is characteristic of MSCs, with the exception of bone-marrow-native MSCs which are CD44-negative, however, when in culture, these cells acquire CD44 expression.

MSC's self-renewal potential allows for repeated passages with negligible changes to their characteristics (*Friedenstein et al. 1974*), though MSCs are usually only capable of expansion for about 25 population doublings (human multipotent adult progenitor cells, for example, share many similarities with MSCs but can be expanded for more than 70 population doublings) (*Bydlowski et al. 2009*). MSCs can be driven to differentiate into specialized cells (Figure 3), through certain physiological/experimental conditions, but, again, to a lesser extent than typical adult stem cells or cord blood stem cells (for example, MSCs display very poor endothelial differentiation) (*Uccelli et al. 2008*). Furthermore, the *in vitro* characteristics of MSCs greatly depend on the culture conditions (*Roobrouck et al. 2011*), which further complicates the elucidation of their *in vivo* characteristics.

MSCs' biological functions and properties within the tissues are not yet completely understood, but it has become increasingly clearer that MSCs are involved in the

maintenance of tissue homeostasis, possibly through the replacement of dead or dysfunctional cells and modulation of the activity of immune cells (*Bydlowski et al. 2009, Pittenger et al. 1999*). Thus, these cells are likely to represent a common component of natural ageing, tissue damage and immunological and inflammatory diseases.

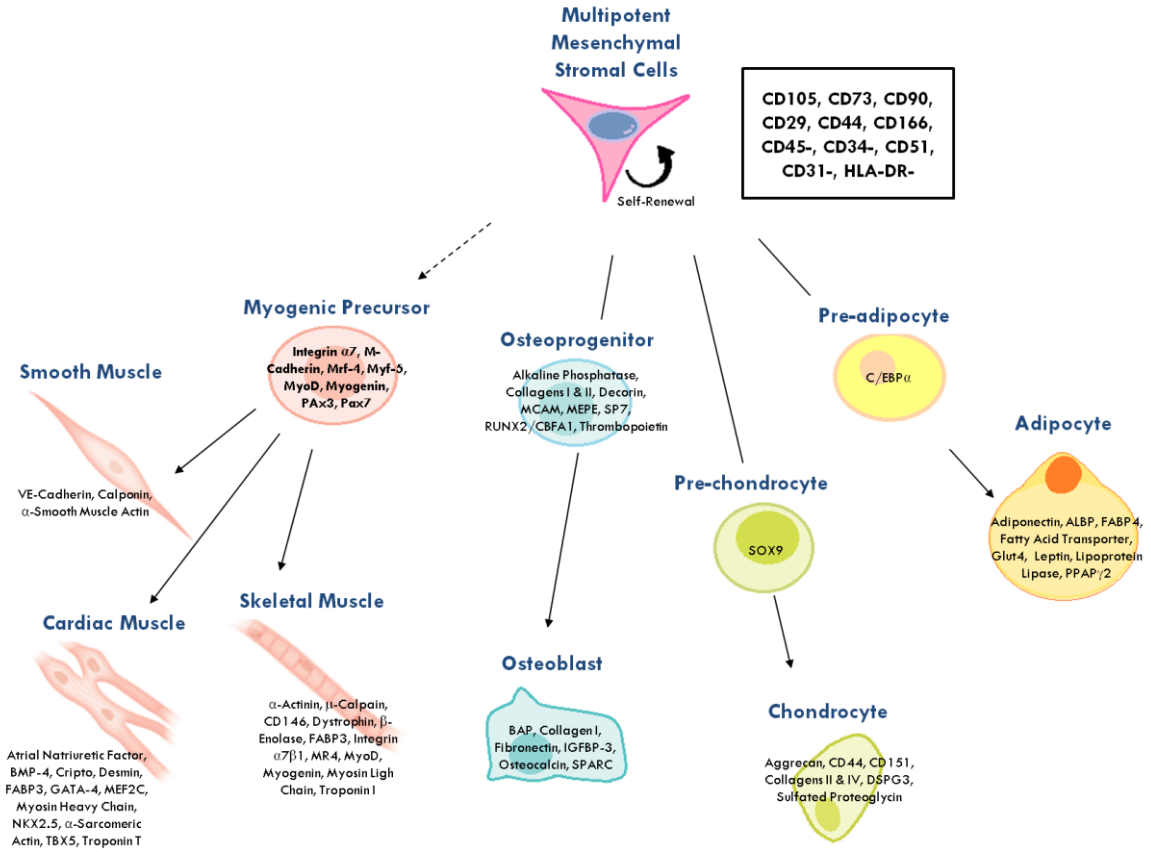

Figure 3. Mesenchymal stromal cell and differentiation markers. Theoretical hierarchy of mesenchymal stem cell differentiation. Myogenic differentiation (- - ►) has been demonstrated *in vitro* but not *in vivo*. From *R&D Systems Tools for Cell Biology Research*^{TM} (*2014*).

The exact anatomical locations of MSC *in situ* are also not clear but, while they can be isolated from interstitial tissues, *in vivo* studies point to them residing mainly near blood vessels (*Corselli et al. 2010, Crisan et al. 2008*), surrounding arteries and veins. Additionally, *Sachetti et al.* (*2007*) isolated, from human bone marrow, MSCs that displayed high expression of CD146 (melanoma-associated cell-adhesion molecule) and represented a subpopulation of pericytes; a cell type present in virtually every organ, enveloping the surface of vascular tubes. *Sachetti*'s group took this to mean that tissue-resident MSCs could be generated from the pericyte fraction in blood vessels, which is in

agreement with the observation that the majority of MSCs can be found in proximity to blood vessels. The hypothesis was further instilled by a study by *Crisan et al*. (*2008*) wherein MSCs were successfully isolated from several tissues based on the expression of surface antigens typical for pericytes, namely, CD146, NG2 and PDGFRβ.

1.3.2. Migration, homing and involvement in tissue repair

Tissue injury always leads to an inflammatory response with activation of immune cells (macrophages, neutrophils, lymphocytes and others), and their mobilization to the injured sites by the spilled contents from apoptotic and necrotic cells, damaged microvasculature and stroma, and secreted inflammatory molecules (cytokines and chemokines) (*Eming et al. 2008, Luster et al. 2005*). Mobilization of MSC to damage sites is already well known for many types of diseases (*Otto & Wright 2011*) and is thought to occur via similar mechanisms. MSCs can respond to some chemoattractants and they express chemokine receptors (*Granero-Molto et al. 2008*), but the identity of the chemokines that regulate their movement is still unknown.

In vitro, MSC differentiation can be driven into a particular lineage by specific regulatory genes, treatment with growth factors, induction chemicals and culture microenvironment. Because they have the same embryonic origin, all MSCs share the ability to differentiate through mesoderm lineages both in culture and *in vivo* (*Dominici et al. 2006*). *In vitro* they are also capable of ectoderm differentiation (*Ding et al. 2011*) and endoderm differentiation, including the differentiation into pancreatic islet β-cells, pancreatic endoderm and pancreatic hormone producing cells (insulin, glucagon and somatostatin) (*Lee et al. 2004*), but these processes have not been confirmed *in vivo*. Furthermore, the MSC-derived cell populations do not fully mimic the biochemical and mechanical properties of the target tissue (*Hwang et al. 2009*), thus the MSC-mediated tissue repair may not be due to the replacement of damaged cells. Additionally, there is still an ongoing debate around MSC differentiation and regenerative abilities *in vivo*. Alternatively, when within the specific injury-related microenvironment, MSCs are stimulated by the typical low oxygen tensions, exogenous toxins and pro-inflammatory cytokines to aid in tissue repair via the release of paracrine factors (Table 4) (*Aguilar et al. 2009, Amo et al. 2014, Bai et al. 2012*).

Table 4. Summary of the hMSC-mediated regenerative effects and the main MSC-derived paracrine factors likely to be responsible.

Growth Factor	Contribution to hMSC-mediated therapeutic effects
Ang-1	Angiogenesis and tissue repair (*Chen et al. 2008*)
EGF	Wound healing, tissue regeneration, neurogenesis (*Kim et al. 2012*)
EPO	Angiogenesis (*Chen et al. 2008*)
FGF	Tissue repair, intrinsic stem cell survival and regeneration (*Arno et al. 2014*)
HGF	Vasculogenesis (*Bai et al. 2012*)
IGF-1	Wound healing (*Chen et al. 2008, Kim et al. 2012*)
KGF	Wound healing (*Aguilar et al. 2009, Chen et al. 2008*)
IL-8, 6	Wound healing (*Kim et al. 2012*)
MIP-1α	Wound healing (*Chen et al. 2008*)
SDF-1	Neuroprotective effect, wound healing (*Chen et al. 2008*)
TGFβ	Wound healing (*Arno et al. 2014*)
VEGF	Angiogenesis and wound healing (*Arno et al. 2014, Chen et al. 2008*)

Ang-1, angiopoietin-1; EGF, epidermal growth factor; EPO, erythropoietin; FGF, fibroblast growth factor; HGF, hepatocyte growth factor; IGF, insulin growth factor-1; KGF, keratinocyte growth factor; MIP-1α, macrophage inflammatory protein 1 alpha; IL, interleukin; SDF-1, stem cell-derived factor-1; TGFβ, transforming growth factor β; VEGF, vascular endothelial growth factor

To further challenge the idea that MSC-mediated tissue repair is achieved through cell differentiation, *Bartholomew et al*. (*2002*), conducted an *in vivo* study on MSC-mediated tissue regeneration and, instead, revealed other surprising therapeutic properties; allogeneic MSCs did not elicit immune recognition and were capable of suppressing immune responses. The latter effect resulted from the inhibition of the activities and proliferation of several different immunocompetent cells, including T and B lymphocytes, NK cells and APCs, regardless of MHC compatibility and source of MSCs (*Aggarwal & Pittenger 2005, Bartholomew et al. 2002, Klyushenkova et al. 2005, Ma et al. 2014, Tse et al. 2003, Zhang et at. 2004*). Thus, besides their possible regenerative properties, MSCs possess strong

immunomodulatory activities, with great potential for application in the field of allogeneic transplantation and in the mitigation of immune disorders.

1.3.3. Immunological profile and immunogenicity

All MSCs, share the weak expression of MHC class I molecules and the lack of expression of MHC class II molecules and co-stimulatory molecules CD40, CD154 (CD40 ligand), CD80 and CD86 (*Le Blanc et al. 2003, Ma et al. 2014, Ryan et al. 2005*).

The absence of the molecules needed to activate T cell alloreactivity is frequently used to explain the apparent immune-privilege of MSCs (*Javazon et al. 2004*), but several studies have shown that the expression of MHC class I and class II molecules can be up-regulated by IFNγ. However, IFNγ stimulation does not enhance MSC immunogenicity or the expression of co-stimulatory molecules (*Klyushenkova et al. 2005*). This would suggest that the lack of co-stimulatory molecules (even with IFNγ) is responsible for the preservation of the immune-privilege but, surprisingly, *Tse et al*. (*2003*) used MSC pre-treated with IFNγ along with direct co-stimulation with anti-CD28 antibodies and still did not achieved a measurable T cell proliferative response. Therefore the reason why MSCs do not elicit T cell responses cannot be fully explained by the absence of co-stimulatory molecules. Nevertheless, IFNγ treatment mimics the inflammatory condition *in vivo* and shows that MSCs can be applied to alleviate inflammation, without loss of immune-privilege, in inflammatory niches (*Ma et al. 2014*).

1.3.4. Immunomodulatory potential

Innate immunity; The MSC or CM-MSC mediated inhibition of monocyte differentiation to mature DCs appears to be irreversible (*Spaggiari et al. 2009, Zhang et al. 2004*) and the resulting immature DCs have strongly reduced ability to stimulate T lymphocytes, up-regulation of anti-inflammatory cytokines, decreased pro-inflammatory cytokine production and the ability to promote the expansion of Treg cells (*Aggarwal & Pittenger 2005, Jiang et al. 2005*).

MSCs express MHC class I molecules, but at low levels, which could make them susceptible to NK cell-mediated killing, but they are not lysed when in contact with resting NK cells. On the other hand, both autologous and allogeneic MSCs can be destroyed by

activated NK cells (*Rasmusson et al. 2003, Spaggiari et al. 2006*). Additionally, MSCs express surface ligands for activating NK cell receptors, but they can be made to successfully escape NK-cell mediated lysis by pre-treatment with IFNγ (*Spaggiari et al. 2006*), which is not surprising seeing as IFNγ can enhance the expression of MHC class I molecules. Despite all of this, MSCs have global suppressive effect over NK cell activities, inhibiting their proliferative activity, cytotoxicity and cytokine production, in a dose dependent manner (*Rasmusson et al. 2003, Spaggiari et al. 2006, Spaggiari et al. 2008*).

Adaptive Immunity; The effect of MSCs, or CM-MSC, leads to the inhibition of proliferation, differentiation into plasma cells (*Asari et al. 2009, Corcione et al. 2006*), expression of chemokine receptors (*Corcione et al. 2006*) and suppression of antibody production by activated B lymphocytes (*Comoli et al. 2008*, *Corcione et al. 2006*). Similarly, MSCs, even when separated from the target immune cells by a semi-permeable membrane, suppress the activation and proliferation of naïve and memory T cells activated by allo-antigens (*Aggarwal & Pittenger 2005, Di Nicola et al. 2002*), mitogens (*Di Nicola et al. 2002, Le Blanc et al. 2004, Otto & Wrigth 2011*) and stimulation with anti-CD3 and anti-CD28 antibodies, in a dose-dependent manner (*Comoli et al. 2008, Krampera et al. 2003*).

Allogeneic MSCs do not elicit T cell activation and both autologous and allogeneic MSCs are equally capable of suppressing T cell responses, suggesting that MSCs' actions are independent from MHC compatibility (*Aggarwal & Pittenger 2005*). The hypothesis was confirmed by *Klyushnenkova et al*. (*2005*) by the demonstration of the inability of MSCs to promote T cell proliferation, even in the presence of cytokines with APC function, demonstrating the existence of an active immunossupressive mechanism. Interestingly, this ability to suppress T cell responses persists even after MSCs enter differentiation through the adipogenic, osteogenic and chondrogenic lineages (*Le Blanc et al. 2003*).

MSCs are usually reported to suppress $CD4^+$ and $CD8^+$ effector T cell activation (*English et al. 2010, Gieseke et al. 2010*), often concomitantly with the stimulation of Tregs expansion. *Gonzalez et al*. (*2009*) observed that MSCs from human adipose tissue (AT-MSCs) (autologous, allogeneic and xenogenic) suppressed the generation Th1 cells and stimulated the expansion of Treg cells, in an animal model for inflammatory bowel

disease. The group concluded that the reduction in Th1 cell numbers was a result of the increase of Treg cells and the exact same conclusion was drawn by an *in vivo* study wherein allogeneic mice MSCs induced normoglicemia in Th1 cell-mediated autoimmune *diabetes mellitus* in non-obese diabetic mice. In both cases, MSCs disfavored Th1 polarization and increased the numbers of both $CD4^+$ and $CD8^+$ Treg cells (*Madec et al. 2009*). And yet, *Ribeiro* and colleagues (*2013*) demonstrated that, when human peripheral blood mononuclear cells were co-cultured with allogeneic human MSCs from bone marrow (BM-MSCs), umbilical cord matrix (UCM-MDCs) or AT-MSCs, there was, indeed, favoring of T cell differentiation towards Treg, but, they also increased T-bet mRNA expression (a key transcription factor in Th1 differentiation) among non-activated, early activated, intermediate activated and later activated T cells, which reveals a polarization towards the Th1 phenotype. While the other mentioned studies assessed Th1-mediated effector responses mainly by evaluating cytokine profiles (mostly IFNγ production), *Ribeiro et al.* (*2013*) used the more direct approach of investigating mRNA expression within different stages of T cell activation and, since, in the same study, MSCs hampered T cell activation, meaning that only a T few cells could undergo activation in the presence of MSCs, the group reasoned that, while the observed skewing towads Th1 cells seems to contradict previous works, the suppression of T cell activation would reduce overall IFNγ production. A gene expression analysis by *Selleri*'s group (*2013*) of T cells stimulated in the presence of MSCs also revealed a Th1 polarization.

Furthermore, MSCs derived from human cord-blood have been shown to induce IL-10 secretion by IFNγ producing Th1 cells, when T cells were stimulated with allogeneic mDCs in their presence, resulting in a concomitant increase of IFNγ and IL-10 production by activated T cells. Interestingly, in the same conditions, activated $CD4^+$ T cells displayed reduced (bur not absent) responsiveness towards IFNγ due to the MSC-mediated suppression of the IFNγ receptor upregulation (a process that usually follows T cell activation), all the while the expression of the IL-10 receptor is not affected (*Selleri et al. 2013*).

There are not many studies focusing on particular T cell activation or differentiation stages, usually focusing on T cells in a broad sense, but the results from the abovementioned studies proved that the investigation of the MSCs' concrete effects over

those stages would geatly contribute to the understanding of the mechanisms through which they affect T cell responses.

The effect of MSCs in diseases with predominant Th2 cell responses has been scarcely studied, but MSC administration into mice with airway inflammation induced by ovalbumin (an allergen, which was given to allergy-prone mice to mimic allergic asthma) was reported to result in the decrease of the Th2 cell population along with the expected induction of Treg cells (*Kavanagh et al. 2011*). On the other hand, co-culture of $CD4^+$ T cells with hMSCs resulted in suppression of the Th1 phenotype and promotion of the Th2 phenotype (along with the induction of Treg cells) (*Batten et al. 2006*). Likewise, *Bai et al*. (*2009*) reported that, in a mouse model for multiple sclerosis, MSCs favored the polarization towards the anti-inflammatory Th2 cell responses, concomitantly with the suppression of Th17 and Th1 cell responses.

Although there isn't many data available, the existing experimental results indicate that MSCs can differentially regulate Th2 cell responses and the contrasting results are likely to be due to different inflammatory conditions, with MSCs having a inhibitory effect over the Th2 subset when the Th2 responses are more prominent (*Kavanagh et al. 2011*) and an enhancing effect when the far more aggressive Th1 and Th17 responses are dominant (*Bai et al. 2009, Batten et al. 2006*). However, the underlying regulatory mechanism is still unknown. Some of the more well accepted hypothesis are; indirect suppression of Th1 responses through the inhibition of APCs (DCs in particular) (*Duffy et al. 2011*), promotion of the Treg phenotype (*Duffy et al. 2011, Castro–Manrreza et al. 2015*), and indirect skewing towards Th2 polarization due to the general reduction in IFNγ levels and increase in IL-10 levels (*Castro–Manrreza et al. 2015*).

The study of MSC-mediated regulation of Th17 cell functions is also relatively recent. *Ghannam et al*. reported, in inflammatory conditions, induction of regulatory behavior in Th17 lymphocytes. These cells became capable of suppressing the proliferation of activated $CD4^+$ T cells (*Ghannam et al. 2010*). Likewise, *Wang et al*. observed suppression of IL-17 production and Th1 cell responses with the stimulation of Th2 cell activities (*Wang et al. 2008*). More recently, MSCs from human bone marrow have been reported to reduce the percentage of T cells that express IL-17 when they were placed in co-culture with mononuclear peripheral blood cells, previous to and during T cell activation, which points to an inhibition, by MSCs, of Th17 differentiation (*Laranjeira et al. 2015*).

Conversely, MSC were reported to promote Th17 differentiation and suppressed Th1 differentiation *in vitro*, but only when added 3 days after T cell activation (*Carrión et al. 2011*), which suggests that the MSC effect depends on the stage of $CD4^+$ T cell activation. The Th17 promotion along with Th1 suppression was also observed for CM-MSC, indicating the involvement of soluble mediators (*Darlington et al. 2010*).

Table 5. Summary of the general actions of MSC over different T cell subsets.

	$CD4^+$			**$CD4^+/CD8^+$**	**$CD8^+$**
	Th2	**Th1** ⇄	**Th17** ⇄	**Treg**	**Tc**
MSC effects	Suppressive effect in Th2-dominant allergic/ autoimmune diseases Stimulating effect in non-Th2-dominant allergic/ autoimmune diseases	Reduced cytokine production Suppressive effect less dependent on activation stage	Usually suppressive effect but promotes Th17 cell responses with late interaction Conversion to a regulatory phenotype Inhibition of cytokine secretion	Increase in $Foxp3^+$ Treg cell numbers Stimulation of IL-10 production Treg induction promotes Th1, Th2 and Th17 suppression	Suppression of Tc cell activation Inhibition of proliferation and Tc cell mediated cytotoxicity

⇄; Th1/Th17 and Th17/Treg interconversion of relevance to MSC immunomodulatory effects (*Duffy et al. 2011*)

MSCs have a consistent stimulatory effect over Treg cells; when $CD4^+$ T cells are co-cultured with MSCs there is an increase of Treg cell numbers (*English et al. 2009*). The same effect has been reported for several *in vivo* disease models including rheumatoid arthritis (*Gonzalez-Rey et al. 2010*) and organ allotransplantation, resulting in protection against rejection and prolonged graft survival (*Casiraghi et al. 2008, Ge et al. 2010, Wang et al. 2009*).

MSCs avoid recognition and destruction by Tc cells and strongly suppress their activities, either directly or indirectly due to the general inhibition of T cell responses, which are required for Tc cell activation. Furthermore, MSCs can hamper both the generation and the activation of Tc lymphocytes, but, like in the case of NK cells, have little to no effect over the cytolytic activity of fully activated Tc cells (*Rasmusson et al. 2003*). Therefore, MSCs

may be ineffective against established $CD8^+$ T cell effector functions; however MSCs are also capable of suppressive effects through the generation of $CD4^+$ and $CD8^+$ Treg cells, which can then amplify the MSC-mediated therapeutic actions. On the other hand, the MSCs may protect tumor cells from destruction due to the increase of Treg cells, Th2 cell responses and, especially, due to the suppression of Tc cells proliferation and activity (*Patel et al. 2010*). Thus, the *in vivo* study of these effects is still necessary to ensure that MSC-based treatments do not have severe side-effects in some patient groups.

While the effect of MSCs over T cell responses has been extensively studied, a much lower number of studies have focused on the specific effect over each different T cell subpopulation individually (Table 5). Additionally, there are very few studies focused on 'unconventional' T cell subsets (γδ T and NKT cells) or in the effects over T memory cells (in comparison with effector cells). Furthermore, there are still many unknown details about the already established effects of MSCs over different T cell subsets, such as; i) considering that MSCs seem to strongly suppress Th1 cell responses (*Bai et al. 2009, Gonzalez et al. 2009, Madec et al. 2009*), while the effect over the Th2 phenotype is much more variable (*Bai et al. 2009, Batten et al. 2006, Kavanagh & Mahon 2011*), there might be an hierarchical influence over the three subsets (possibly Th1 > Th17 > Th2); ii) the apparent different levels of suppression over the Th subsets may be indicative of different regulatory mechanisms; iii) alternatively (or concomitantly) to the differential regulation of T cells, there may be different mechanisms of action among the heterogeneous populations of MSCs; iv) the significance of cell-cell contact in the modulation of T cell responses has not yet been studied in detail; v) the exact duration of the MSC-mediated effects is unknown, and vi) there is not yet a means to accurately determine the immunossupressive potency before MSC administration.

There is multitude of different cell types and subtypes, with diverse functions, and those actions have different weight in different pathological states, therefore, the assessment of MSCs' true therapeutic potential, and possible draw-backs, still requires further investigation regarding their effects over different T cell subpopulations, especially in light of the fact that MSCs have been recently reported to differentially regulate distinct T cell subsets (*Laranjeira et al. 2015*).

1.3.5. Mechanisms of action

MSCs gathered considerable attention in the improvement of wound healing and have been successfully employed in this context by several studies spanning many different diseases. However, the percentage of administrated cells that actually engrafts at the site of damage seems to be fairly small as the majority of MSCs either migrate into the lungs and liver or enter apoptosis, disappearing rapidly after administration. And yet, their short-lived presence still yields therapeutic results (*Askari et al. 2003, Lee et al. 2009, Wang et al. 2012*). It is also quite unclear if differentiation is indeed the main mechanism behind the tissue repair (*Murry et al. 2004*).

Several groups were able to achieve therapeutic results using MSCs or CM-MSC alone (*Gnecchi et al. 2006, Shabbir et al. 2009, Song et al. 2014*) and proposed that the mechanisms responsible for the therapeutic effects of MSC were mainly the result of the secretions of these cells (Figure 4). The hypothesis is supported by the fact that engrafted MSCs have been shown to produce growth factors and cytokines that promote tissue repair (*Amo et al. 2014, Aguilar et al. 2009, Bai et al. 2012*).

Ren et al. (*2008*) reported that MSC can be stimulated to produce high levels of immunosuppressive factors by IFNγ in combination with one of the pro-inflammatory cytokines TNFα, IL-1α or IL-1β. This stimulation also results in the up-regulation of the expression of chemokines and adhesion molecules (*Ren et al. 2008, Ren et al. 2010*) resulting in an accumulation of immune cells in proximity to MSCs, forming a specific localized microenvironment which amplifies the effect of MSC-derived mediators. Because of this, *Aronin & Tuan* (*2010*) proposed that naturally occurring MSCs' main role in tissue injury is to suppress immune responses and to trigger several healing-promoting processes through the secretion of soluble factors (Figure 4).

Studies focused on the identity of the soluble factors responsible for MSC-mediated immunomodulation have pointed to many suitable candidates (Table 6). However, contradictory results are very frequent, although possibly just due to external factors such as the type of responder population and type of stimuli, both of which could influence the immunosuppressive mechanism of MSC.

HGF-1 has been found in high levels in CM-MSC and has been reported to have a crucial role in the protective effects of CM-MSC against experimental autoimmune encephalomyelitis (EAE), the mouse model for multiple sclerosis (*Bai et al. 2012*). TGFβ

is a powerful immunosuppressive molecule involved in immune privilege (*Raghupathy 2001, Smith et al. 2012, Tompkins et al. 1998*) and TGFβ secretion has been demonstrated for MSC from multiple sources (*Zhou et al. 2011, Melief et al. 2013, Yoo et al. 2013*) and, in some cases, anti-TGFβ antibodies or TGFβ silencing can reverse MSC-mediated inhibition of lymphocyte proliferation (*Yoo et al. 2013*). *Di Nicola et al*. (*2009*) reported the involvement of TGFβ and HGF-1 in MSC-mediated immunosuppression and managed to partially rescue activated T cell proliferation with TGFβ and HGF neutralizing antibodies. On the other hand, three different in vitro studies (*Bai et al. 2012, Le Blanc et al. 2004, Plumas et al. 2005*), reported that there was no involvement of TGFβ or HGF-1. However these groups focused on T cells activated by mitogens or by using peripheral blood mononuclear cells as responder cells while *Di Nicola*'s group used T cell activation by allogeneic peripheral blood lymphocytes. Additionally, in *Di Nicola et al*'s study the suppressive effect was transient while a study by *Glennie et al*. (*2005*) verified the arrest of T cells at the G0/G1 phase of the cell cycle, but the group used murine MSCs while *Di Nicola* and colleagues worked with cells of human origin.

These results are consistent with the established notion that the immunosuppressive mechanisms of MSC depend on the source from which they were isolated. Furthermore, the intervenient soluble molecules are not universal, varying from species to species (*Ren et al. 2009*). For example, the mechanism of action of murine MSCs seems to be largely dependent on nitric oxide (NO), an immune suppressor at high concentrations (*Sato et al. 2007*), but for hMSC the role of NO is replaced by indoleamine 2,3-dioxygenase (IDO), an enzyme involved in the degradation of the essential amino acid tryptophan (*Sioud et al. 2011, Xy et al. 2007*). IDO is not constitutively produced by MSCs, but its expression is induced by certain conditions, such as IFNγ stimulation (*Meisel et al. 2004*). Since tryptophan depletion alone cannot fully explain the inhibitory effect of MSC (*Tse et al. 2003*) the IDO-mediated immune suppression is thought to be mainly due to local accumulation of tryptophan metabolites (*Mellor & Munn 2004*).

Several studies support that PGE2 has a crucial role in the modulation of inflammatory responses by MSC, having proven that COX inhibitors can decrease MSCs' therapeutic effects (*Dhingra et al. 2013, Rasmusson et al. 2005, Zhang et al. 2013*). Interestingly, *Rasmusson* and colleagues (*2005*) abrogated MSC-mediated immunosuppression with an inhibitor of PGE2 synthesis, but reported that cell proliferation was only restored for

mitogenic activation and not for a mixed lymphocyte reaction (MLR), thus other inhibitory pathways were involved in the latter condition, which could mean that the mechanisms of suppression of activated T cell proliferation by MSCs differs with the type of activating stimuli. On the other hand, high PGE2 concentrations can have pro-inflammatory effects, partially due to the diversity of PGE2 receptors (*Kalinski 2012*), illustrating how MSCs' activities may have great underlying complexity.

Table 6. Summary of factors possibly involved in hMSC-mediated immunosuppression.

Soluble factor	Effects on immune cells
Galectin-1/-9	Induction of activated T cell apoptosis (*Barondes et al. 1994*)
HLA-G5	Suppression of NK cell activity; Inhibition of T cell proliferation; Promotion of Treg cell generation (*Selmani et al. 2008*)
HGF-1	Inhibition of CD80 and CD86 expression by DCs; Suppression of Th1 cell responses and induction of Treg cells (*Chabannes et al. 2007, Gu et al. 2013*)
HO-1	Inhibition of T cell activation; Promotion of Treg cell generation; Promotion of IL-10 and TGFβ release by Treg cells (*Lee et al. 2002*)
IDO	Inhibition of T cell responses (*Meisel et al. 2004, Mellor & Munn 2004, Ren et al. 2009, Spaggiari et al. 2008*) Enhancement of PGE2 inhibitory effects (and vice-versa) (*Spaggiari et al. 2008*).
PGE2	Inhibition of T cell proliferation; Promotion of of Th2 cell responses (*Woorlard et al. 2007*) Induction of Treg cells (*Baraelli et al. 2005*) Inhibition of Th1 cell responses (*Dohadwala et al. 2005,* Snijdewint et al. 1993) Suppression of Tc, NK and Th1 cell activation (*Dohadwala et al. 2005, Spaggiari et al. 2008*) Inhibition of DC maturation and macrophage activity (*Baraelli et al. 2005, Lee et al. 2002, Spaggiari et al. 2009*) Enhancement of IDO inhibitory effects (and vice-versa) (*Spaggiari et al. 2008*)

PDL-1	Suppression of Th17 cell responses; Inhibition of T cell proliferation (Snijdewint et al. 1993)
TGFβ	Inhibition of DC maturation (*Gandhi et al. 2008*) Induction of Treg cells (*Kushwah et al. 2010*) Suppression of Th17 cell generation (*Romagnani 2008*) Generation and maintenance of IL-10 producing Th1 cells (*Huss et al. 2011*)
TSG-6	Counteracts the effects of TNFα and IL-1 (*Wisniewski & Vilcek 2004*)
Exosomes and microvesicles	Transfer of miRNAs, KGF, anti-inflammatory proteins and mitochondria (*Bruno et al. 2009, Gatti et al. 2012, Islam et al. 2012, Li et al. 2013, Raymond et al. 2009, Zhu et al. 2014*)
Cell-Cell contact	Inhibition of T cell activation (*Krampera et al. 2003*)

HLA-G5, Human leukocyte antigen G5; HGF-1, Hematopoietic growth factor-1; HO-1, hemeoxygenase-1; IL-6, Interleukin 6; IDO, Indoleamine 2,3-dioxygenase; miRNA, micro-RNA, PDL-1, Programmed cell death ligand 1; PGE2, Prostaglandin E2; TGFβ, transforming growth factor β; TSG-6, tumor necrosis factor-stimulated gene-6

MSCs are thought to be capable of IL-10 production, but the involvement of this anti-inflammatory cytokine in MSC-mediated regulation of immune responses is still controversial and a few studies have reported that neither MSCs alone nor LPS/IL-3 stimulated MSCs secrete IL-10 (*Groh et al. 2005, Rasmusson et al. 2005*). Moreover, blocking IL-10 does not seem to affect the immunosuppressive effect of MSC over B and T lymphocytes (*Xu et al. 2007*) so the increase in IL-10 levels in the presence of a population of MSCs and immune cells, might solely be a product of the latter.

Human MSCs can produce TSG6, particularly if stimulated by TNFα, and TSG-6 depletion results in the diminished ability to suppress immune responses (*Lee et al. 2009*). Recently galectins have also been included to the group of regulators likely to be responsible for MSCs' immunosuppressive potential (*Sioud et al. 2011*) and both galectin-1 and 9 knockdown MSCs have their inhibitory effect over $CD4^+$ and $CD8^+$ T cells dampened (*Gieseke et al. 2010, Gieseke et al. 2012*). Soluble HLA-G5, also involved in AM-mediated immunosuppression, is produced and released by MSCs following contact with alloreactive T lymphocytes (*Ryan et al. 2005, Selmani et al. 2008*).

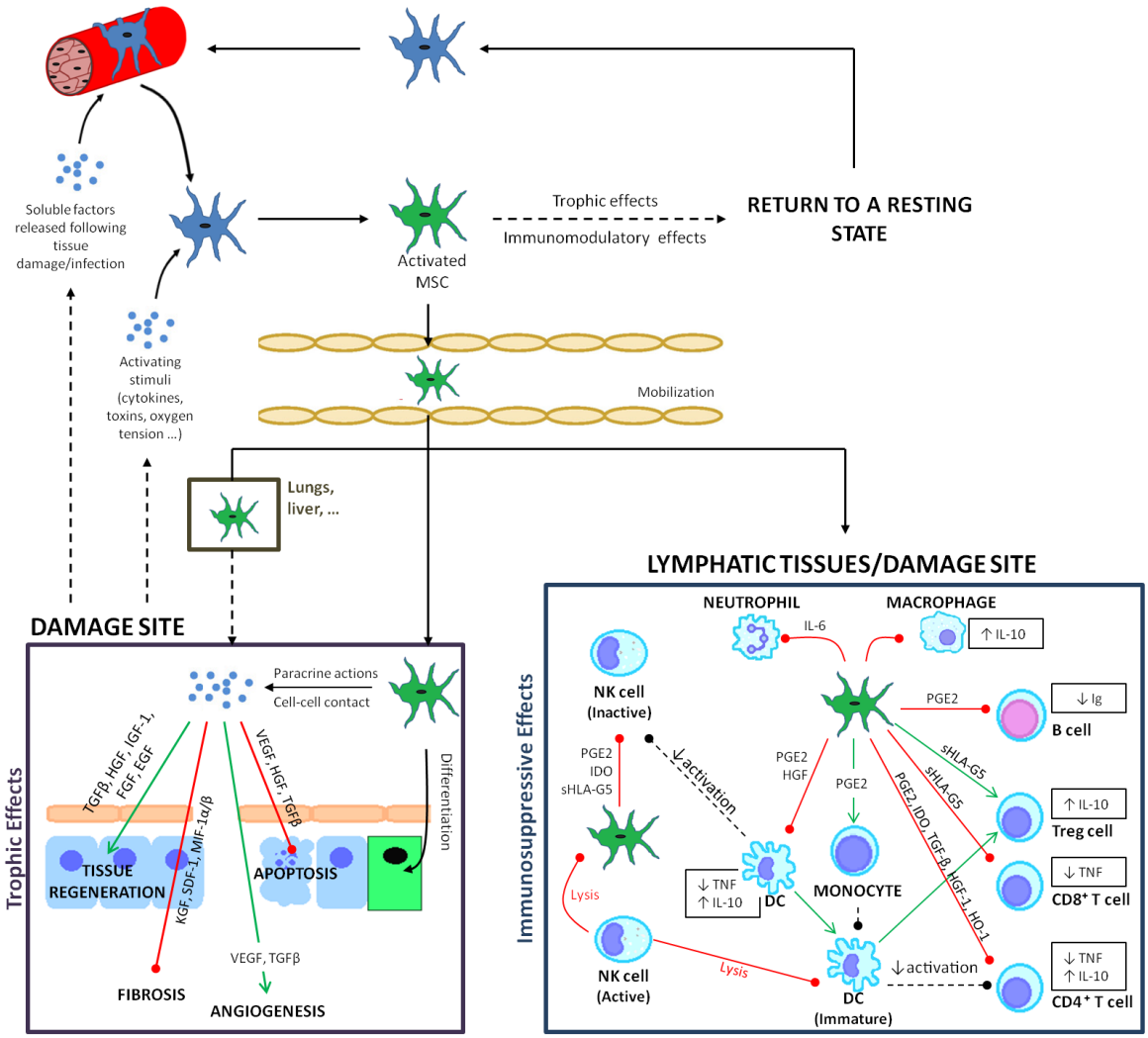

Figure 4. Proposed model for biological functions and mechanisms of action of MSCs in response to tissue damage and/or infection. Transdifferentiation may occur *in vivo* but is not the main mechanism underlying MSC-mediated therapeutic effects, instead, the endocrine/paracrine secretion of regenerative and immunossupressive factors, independently of the anatomical location, promotes tissue repair and protects the host against inflammation damage. No one mechanism of immunomodulation is prevalent, instead, MSC possess a redundant panel of numerous pathways, some of which act in synergy. Solid (⎯) and dashed lines (.....) represent direct and indirect effects, respectively, the arrows (→) represent stimulation and the end-circles (—●) inhibition.

Lastly, cells can communicate through the release of exosomes and/or microvesicles that transport high concentrations of proteins and other molecules within them. They are small portions of cytoplasm formed by budding of the cell membrane into small vesicles; 50 nm to 1000 µm in size for microvesicles and 40 to 100 nm for exosomes. MSCs are capable of

bidirectional communication between injured tissue and cells with regenerative potential, through shuttling of microvesicles containing various bioactive molecules along with coding and non-coding RNAs and even mitochondria (*Bruno et al. 2009, Gatti et al. 2012, Zhu et al. 2014*). hMSC-derived microvesicles have been reported to be associated with protection against immunological disorders (*Bruno et al. 2009, Gatti et al. 2012, Zhu et al. 2014*). Likewise, some groups have implicated the involvement of exosomes in MSC-mediated therapeutic effects (*Li et al. 2013, Raymond et al. 2009*). However the role of MSC-derived exosomes is a new topic of investigation and is not yet established.

Several other soluble factors have been suggested to have some importance in MSC-mediated immunoregulatory activity but, in contrast, *Krampera et al*. (*2003*) reported that the suppression of T cell responses relied on direct cell-cell contact, through which MSCs physically blocked the interaction between inactive, naïve or memory, T cells and APCs thus preventing T cell activation. However it is important to note that these results did not include the effects of MSCs over activated effector T cells.

1.3.6. Modulation of MSC behavior

The exact properties and effects of MSCs over immune cells change drastically depending on culture conditions, leading to initial studies often resulting in contradictory results. Thus, it has become clear that the modulation of immune responses by MSC may not be constitutive but, instead, context-dependent (*Dazzi & Krampera 2011*).

To highlight this aspect; frequently, different studies focusing on the same disease report MSCs to have divergent effects, with one study indicating improvement while another observes the aggravation of the pathological condition (*Augello et al. 2007, Djouad et al. 2005*), especially when there is a significant difference in the timing of MSC administration (since the inflammatory environment changes along the development of the disease). Thus, the patient's response to MSC-based treatment could, in theory be assessed by examining the pathologic microenvironment before treatment. Because of the great impact this has on the clinical applications of MSC it becomes necessary to understand the mechanisms that regulate immune modulation by MSC.

In general, conditions associated with tissue damage, such as low oxygen tensions and high pro-inflammatory cytokine levels, seem to strongly enhance the regenerative and immunosuppressive activities of MSCs (Table 7), but many other culture conditions can

also affect the therapeutic potential of MSCs, for instance, comparatively to the standard monolayer culture, MSCs in micro-emboli of myocardial infracted mice or in a hanging drop system, produce higher levels of TGS-6 (*Bartosh et al. 2010, Lee et al. 2009*) and the CM-MSC or MSCs of spheroid cultures have their anti-inflammatory effect significantly enhanced (*Bartosh et al. 2013, Ylöstalo et al. 2012*) (Table 7). This type of self-stimulation has also been shown to occur naturally *in vivo* as a result of the MSCs' tendency to self-aggregate (*Bartosh et al. 2013, Lee et al. 2009*).

Table 7. Summary of the effects of hypoxia, pro-inflammatory stimuli and 3-dimentional cultures conditions over the paracrine actions of MSCs.

Condition	Modulation of MSC behavior	Up-regulated paracrine factors
Hypoxia (1-2% O_2)	Stimulation of the production and release of paracrine factors (*Chang et al. 2013, Crisostomo et al. 2008, Efimenko et al. 2011, Iida et al. 2010, Rasmussen et al. 2011*); Promotion and maintenance of stem cell potency (*Hawkins et al. 2013, Iida et al. 2010*)	FGF, VEGF, IGF, HGF, IDO (*Chang et al. 2013, Crisostomo et al. 2008, Efimenko et al. 2011, Iida et al. 2010, Rasmussen et al. 2011*)
Inflammatory stimuli (IFNγ, TNFα, LPS)	Up-regulation of the expression of soluble factors (*Crisostomo et al. 2008, Croitoru-Lamoury et al. 2011, Gieseke et al. 2013*)	VEGF, HGF, IDO, TGFβ, PGE2, Galectin-9, TSG-6, microvesicles/exosomes (*Crisostomo et al. 2008, Croitoru-Lamoury et al. 2011*)
3D culture configuration	Stimulation of the production and release of trophic factors (*Bartosh et al. 2010, Lee et al. 2009, Ylöstalo et al. 2012*)	PGE2, TSG-6 (*Bartosh et al. 2013, Lee et al. 2009, Ylöstalo et al. 2012*)

The effectiveness of MSC administration will also depend on other factors; first of all, the method of MSC delivery result is known to affect the therapy outcome, both in terms of engraftment and therapeutic efficiency (*Castelo-Branco et al. 2012, Freyman et al. 2006*), consequently, the selection of the appropriate route of MSC administration for the treatment of a given pathological condition is vital for the success of the treatment. Secondly, an insufficient dose of MSC will not yield significant results, but above a certain dosage the therapeutic effect cannot be improved (*Wu et al. 2008*). On the other hand, too

early of a delivery, at an early stage of an inflammatory disease, reduces the therapeutic effects of the immunosuppressive activities of MSC, but, when it comes to MSC engraftment, injection at early stages of ischemia or EAE, produces greater therapeutic results than if MSC are administrated at the relapse stage (*Constantin et al. 2009, Rafei et al. 2009*).

Therefore, in order to assess the true potential of MSC as therapies, pre-clinical experiments and clinical trials need to take into consideration multiple factors that affect their behavior including, not only the source of MSC, number of administrated cells and the correct timing of cell delivery, but also several aspects of the culture conditions previous to administration.

1.3.7. MSC licensing and the balance between pro- and anti-inflammatory behavior

Although MSCs habitually display immunossupressive effects it is thought that, *in vivo*, in their inactive state (without stimuli), MSCs mainly have an anti-apoptotic and supportive effect over many different cell types, including hematopoietic stem cells (*Dazzi et al. 2006*), immune cells (*Benvenuto et al. 2007*) and neoplastic cells (*Kamoub et al. 2007*). Additionally, certain conditions can prevent the achievement of MSC immunosuppressive effects or lead to an MSC-mediated enhancement of the immune response. Remarkably, MSCs can even display antigen-presenting abilities when induced by low IFNγ levels (the exact concentration value varies greatly from study to study) (*Chan et al. 2006, Romieu-Mourez et al. 2009*). Because of this inducible antigen presenting ability, *Romieu-Mourez et al*. (*2009*) pointed out that MSC could be candidates for the treatment of cancer and infectious diseases through the activation of antigen-specific cytotoxic $CD8^+$ T cells, although the subject is considerably new and still under study. The failure to obtain immunossupressive effect, whether due to wrong timing of administration *in vivo* or inadequate stimuli *in vitro*/*in vivo*, is, therefore, explained by the absence of successful MCS licensing (*Auletta et al. 2012, Chinnadurai et al. 2014*).

Since the inhibitory effect of MSCs is not constitutively expressed, the attainment of an anti-inflammatory behavior depends on a licensing step, which is a complex, multi-step process that ultimately results in the functional maturation of MSCs. The maturation towards an immunossupressive phenotype will depend on the activation, via pro-inflammatory stimuli, and the prevalence of those stimuli over other signals that may

hamper the inhibitory effect of MSCs. It is likely that the MSC licensing involves the induction of specific transcription factors by molecular signals within the microenvironment, in a manner similar the signaling pathways that drive T naïve cells to differentiate into different T cell subsets. Additionally the switch between supportive, anti- and pro-inflammatory effects probably occurs via the modification of intracellular pathways by certain cytokines and other factors (*Auletta et al. 2012*).

Table 8. Possible progression of MSC activities during infection.

Immune system	MSC main functions
0. Resting state	Supportive role over hematopoietic stem cells and immune cells
1. Pathogen/damage detection	Recruitment to site of infection
2. Activation of immune responses	Enhancement of anti-microbial and pro-inflammatory functions of immune effector cells
3. Pathogen Elimination	Enhancement of anti-microbial and pro-inflammatory functions of immune effector cells → Production of immunosuppressive and regenerative soluble factors
4. Return to resting state	Modulation of inflammation Promotion of wound healing, tissue repair and re-vascularization

The functional plasticity of MSCs is thought to be crucial for the maintenance of homeostasis. The local levels of cytokines, chemokines, oxygen radicals and low oxygen tensions (pro-inflammatory stimuli) vary depending on many factors, mainly; pathogen burden, inflammatory soluble factors and longevity of the response. In that sense, MSCs may work as 'sensors' to detect changes in the microenvironment and react accordingly (through the modulation of innate/adaptive immune responses and tissue repair), for instance, MSCs would, theoretically, react to the initial stages of the immune response to infection by stimulating immune effector cells, thus decreasing the pathogen burden (Table 8) (*Auletta et al. 2012, Singer & Caplan 2011, Waterman et al.2010*).

Regarding the mechanisms behind the stimulation of immune responses; MSCs have been shown to produce, under certain conditions, IL-6 and IL-8, both of which contribute for T cell activation (*Waterman et al. 2010*). Additionally, MSCs at low concentrations of

IFNγ are capable of activating Tc cells by antigen presentation in complex with a MHC class I molecule, instead of class II; a property typical of DCs known as antigen cross-presentation (*Romieu-Mourez et al. 2009*).

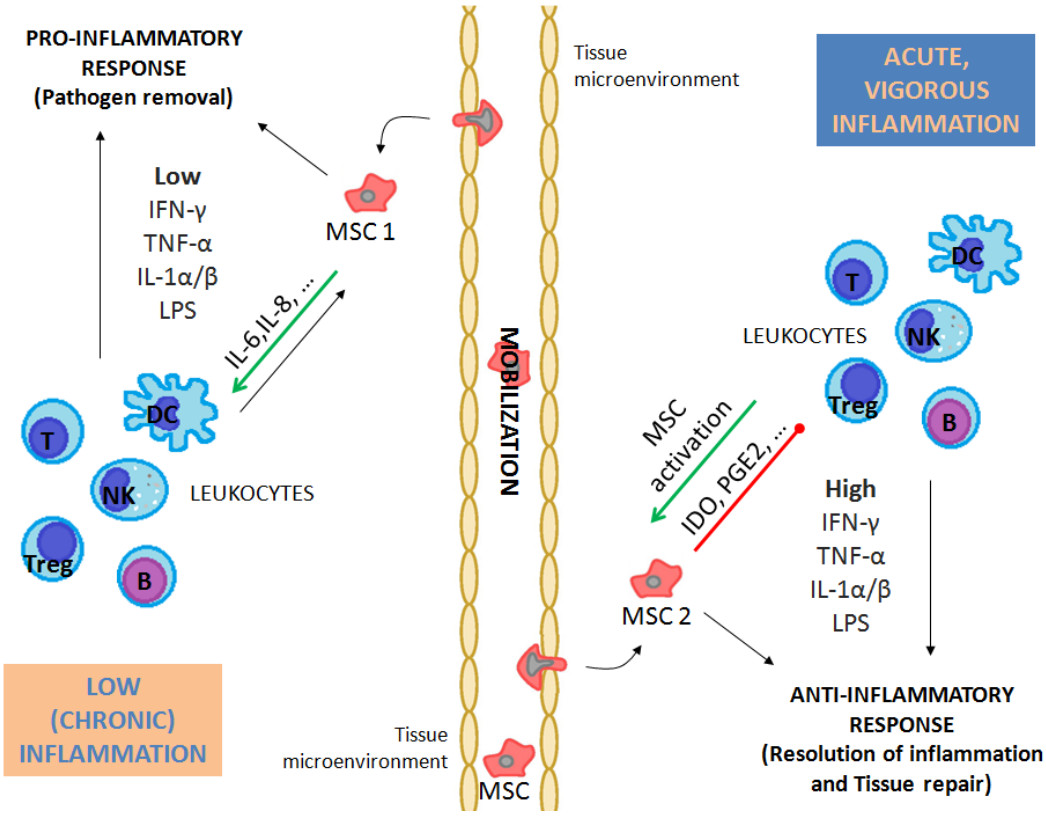

Figure 5. A shematic representation of the functional plasticity of MSCs. During the first stages of the response to infection/injury (or in cases of chronic inflammation), the low levels of inflammation (insufficient inflammatory cytokines) lead MSCs to display a pro-inflammatory behavior (MSC1) that increases the activity of immune effcetor cells, stimulating the removal of the infection (or worsening the disease state in case of chronic inflammation). Vigorous inflammation, on the othe hand, licenses MSCs to display an anti-inflammatory and regenerative behavior (MSC2) to prevent tissue damage and maintain the homeostasis. The arrows (→) and end-circles (—●) represent stimulation and inhibition, respectively.

After the initial pro-inflammatory effect, as the inflammation becomes more vigorous and persistent, in order to maintain the host's integrity, MSCs can then exhibit an immunossupressive behavior to attenuate systemic inflammation and to promote tissue repair (Table 8) (*Auletta et al. 2012, Singer & Caplan 2011, Waterman et al.2010*).

Thus, MSCs are involved in the host protection in more ways than one, supporting hematopoiesis, mobilizing and stimulating immune effector cells and modulating inflammatory processes to prevent tissue damage (Figure 5). However the information regarding exact activating and inhibitory signal or signals, along with the level of inflammatory stimuli at which the MSC switch from pro- to anti-inflammatory behavior, is still considerable new and lacking.

1.3.8. Some other frequent concerns

A few other issues still need addressing before the successful clinical application of MSCs can be guaranteed. One of these concerns is that MSC-based therapies may lead to the loss of the normal protective function of the immune system. However, given that the inhibitory effects of MSCs are only present if licensed by acute, vigorous, persistent inflammation, and that MSCs can modulate their behavior according to physiological needs (*Auletta et al. 2012, Singer & Caplan 2011, Waterman et al.2010*), MSC-based therapies have an advantage over other immunossupressive therapies because they are less likely to increase susceptibility to infection. Additionally, results from clinical trials focusing on the treatment of graft-versus-host-disease (GvHD) have shown that normal, and effective, anti-viral immune responses remain at large in spite of the therapy (*Ball et al. 2007, Le Blanc et al. 2008*).

Also in the treatment of GvHD, MSC-mediated immune inhibition may affect the graft-versus-leukemia effect (GvL), and effect of MSCs has been reported by a few studies wherein MSCs administration ameliorated the GvHD condition but also seemed to increase the frequency of disease relapse in malignant hematological patients after allogeneic hematopoietic stem cell transplantation (*Ning et al. 2008*). On the other hand, *Selleri et al.* (*2013*), in light of their findingds (detailed in section **1.3.4**), have hypothesized that MSCs can treat GvHD without inhibiting the beneficial GvL effect since T cell responsiveness to IFNγ, demonstrated to facilitate the GvL effect (*Yang & Sykes 2002*), is reduced, but not abolished, and Treg cells, whose differentiation and expansion is promoted by MSCs, can suppress GvHD without decreasing the GvL effect. Aditionally, it is always important to consider that the outcome of MSCs-based therapies will greatly depend on multiple factors, such as the point in the disease progression at which they are administred.

There is also the question of whether MSCs can be affected by previous or concurrent administration of immunosuppressive drugs, or other therapies. This issue has been raised by several groups, and has produced many clashing results (*Buron et al. 2009, Eggenhofer et al. 2011, Inoue et al. 2006*), but it is possible that those differences may simply derive from other factors that influence MSCs' behavior. More recently, *Girdlestone et al.* (*2015*) demonstrated that MSC could be made up to 5-fold more proficient in the inhibition of T cell proliferation if pre-treated with one of four different immunosuppressant drugs, namely; rapamycin and everolimus, which are inhibitors of the mTOR pathway (mechanistic target of rapamycin, or mTOR, is a protein with a fundamental in the regulation of cell cycle progression), and the calcineurin inhibitors (calcineurin is involved in the induction of transcripton of IL-2 and other cytokines) tacrolimus and cyclosporine A. The group also demonstrated that this effect was likely due to the ability of MSCs to absorb the drug, during pre-treatment, and then act as a drug delivery system. Interestingly, cyclosporine A required higher doses to improve MSC-mediated immunosuppression than the other drugs, and, for all immunosuppressants, this effect could not be enhanced above a certain dose (*Girdlestone et al. 2015*). Thus, MSC-based therapies could greatly benefit by the definition of appropriate drug combinations.

On the whole, in spite of these gaps and others that may not have been yet revealed, clinical studies regard MSC-based therapies as feasible and safe. Nevertheless, before the implementation of MSC-based immunotherapies, it is important to understand how they are influenced by external factors, which immune cells their act on and how these cells are affected. Additionally, while short-term safety seems to be a certainty, data concerning long-term safety is still lacking along with the establishment of the suitable dosage.

2. THE HUMAN AMNIOTIC MEMBRANE AS A SOURCE OF MESENCHYMAL STROMAL CELLS

The hAM is composed of only two cell types (excluding fetal-derived macrophages [*Bourne 1962, Casey & MacDonald 1996, Grigoriadis et al. 2013*]); amniotic epithelial cells, derived from the embryonic ectoderm, and mesenchymal cells, derived from the embryonic mesoderm, dispersed among the collagenous stromal layer that underlies the amniotic epithelium (*Alviano et al. 2007, Lindenmair et al. 2012*).

At the earlier stages of embryogenesis the amniotic epithelial layer is immediately adjacent to the mesenchymal layer, however, as collagen is deposited between the epithelial and mesenchymal cells, the two become separated by an acellular, collagenous compact layer (*Casey & MacDonald 1996*), therefore, while hAM-derived MSCs can be isolated from first-, second-, and third-trimestre amnion mesoderm, pure fractions are more easily obtained from human term placenta, after mechanic separation of the hAM from the chorion, by enzymatic digestion with collagenase (*Parolini et al. 2008, Lindenmair et al. 2012*). But, while the expansion rate of epithelial cells can maintain a continuous epithelial layer throughout the growth of the amniotic sac, mesenchymal cells have a lower growth rate and thus become dispersed within a loose network of connective tissue. Generally, each gram of amnion tissue can yield roughly 1 to 2 × 10^6 MSCs, approximately 10-fold less than the number of amniotic epithelial cells (*Casey & MacDonald 1996, Alviano et al. 2007*).

The bone marrow is still the most well-known source of MSCs, however, the isolation process is highly invasive and this population represents less than 0.1% of the nucleated cells and tends to decrease with age (*Alviano et al. 2007, Bieback & Brinkmann 2010, Alikarami et al. 2015*). On the other hand, perinatal tissues, such as the placenta and its membranes, are considerably richer sources of MSCs than most somatic tissues (*Alviano et al. 2007, In 't Anker et al. 2004, Miki 2011*). For instance, *Alviano* and colleagues (*2007*) found that, comparativey to the human bone marrow, a greater number of MSCs could be obtained when hAM was used as the source tissue, additionally, only a very small area of amniotic membrane, less than 1/300 of the total area, was needed to achieve significant MSC quantities. In the same study, it was also reported that the hAM-derived MSCs exhibited higher proliferation capacity in comparison to the bone marrow-derived adult

MSCs, a property that had already been demonstrated for human placenta-derived fetal MSCs (*In 't Anker et al. 2004*). This superior expansion potential had a great contribution for the final cellular yield; the group observed that, at each point in time during culture, the number of bone marrow-derived MSCs was lower than the number of hAM-derived MSCs, and the latter increased, approximately, 300-fold during 21 days of culture (*Alviano et al. 2007*).

Another noteworthy advantage of postnatal gestational tissues is the easy access through completely non-inasive methods, since they are generally discarded at birth, consequentely, cell harvesting presents no risk to the donor, these tissues are, in theory, in unlimited supply and are not associated with any major ethical issues (*Bieback & Brinkmann 2010, Hass et al. 2011*).

Furthermore, given that the supporting connective tissue of the AM completely lacks vasculature, smooth muscle, lymphatic tissue and nervous tissue, the amnion is the only source of MSCs without contamination from non-fibroblastoid cells (*Alviano et al. 2007, Casey & MacDonald 1996*). Conversely, culture of placenta or umbilical cord-derived MSCs is often associated with contamination from hematopoietic and endothelial cells (*Alviano et al. 2007*).

Several studies have suggested that MSCs derived from birth-associated tissues possess superior biological properties in comparison with MSCs originated from adult tissues; the most important of which is that MSCs originated from neonatal tissues display less environmental and age-related DNA damage (*Miki 2011, Steindler & Pincus 2002*). Additionally, the aforementioned study by *Alviano et al.* (*2007*) reported that hAM-derived cells had higher expression levels of octamer-binding transcription factor 4 (Oct-4, a transcription factor involved in the self-renewal of totipotent embryonic cells) than bone marrow-derived MSCs.

Finally, although MSCs derived from the bone marrow, and other commonly studied sources, only display immunosuppressive properties when cultered with activating stimuli, such as pro-inflammatory cytokines (*Chinnadurai et al. 2014*), recently, it has been shown that immunomodulation by hAM-derived MSCs does not require previous stimulation; for instante, *Pianta et al.* (*2015*) reported that, even without stimulating culture conditions, conditioned medium derived from MSCs isolated from the mesenchymal layer of the hAM

suppressed $CD4^+$ and $CD8^+$ T lymphocyte proliferation induced by allogeneic target cells or through T-cell receptor engagement.

The intrinsic immunosuppressive properties of hAM-derived MSCs, along with their distribution throughout the amniotic membrane, lead to the speculation that these cells are involved in the maternal immune tolerance to the fetus (*Magatti et al. 2008*) and, the discovery of the possible involvement of MSCs in scarless fetal wound healing seems to support this hypothesis (*Hu et al. 2014*). Scarless fetal wound healing is a process intrinsic to the fetus dring early gestation which allows regeneration of fetal skin wounds absent of inflammation and scarring, through a not yet fully understood mechanism (*Hu et al. 2014*). However, the contribution of MSCs for these processes is only speculative and the hypothesis is still under study.

Overall, term hAM appears to be an ideal source of MSCs; it is redily available from each delivery, ethically acceptable, the isolation procedure is relatively easy with high recovery efficiency and hAM-derived MSCs may have superior biological properties, such as higher expansion potential and less DNA damage. However, it still remains to identify what immune cells are affected by hAM-derived MSCs, how they are affected and what are the key underlying mechanisms.

3. OBJECTIVES

The aim of this work was to evaluate the modulatory effect of a hAM extract (hAME), an enriched mixture of hAM-derived factors, over specific $CD4^+$ and $CD8^+$ T cells subsets in order to determine wether or not the different functional compartments were differentially regulated. Hence, the following defined objectives were established;

i) Phenotypic characterization of hAM-derived cells.

ii) Assessment of the effect of hAME over lymphocyte proliferation.

iii) Evaluation of the effects of hAM-derived cells over the frequency of $CD4^+$ and $CD8^+$ T cells that produce inflammatory cytokines (TNFα, IFNγ, IL-2, IL-17 and IL-9) among different functional compartments (naïve, central memory, effector memory and effector).

iv) Evaluation of the effects of hAME over the frequency of $CD4^+$ and $CD8^+$ T cells that produce inflammatory cytokines among the same T lymphocyte subpopulations.

v) Analysis of the effects of hAME over mRNA expression in purified $CD4^+$ T cells, $CD8^+$ T cells, Treg cells and Tγδ cells.

4. MATERIALS AND METHODS

4.1. Source of the hAM extract

The hAM-derived cells and hAM extract (hAME) used in the experiments were received, ready-for-use, from the Faculty of Medicine of the University of Coimbra (Coimbra, Portugal); the basic methodology of hAM cells isolation and extract preparation is described below.

4.1.1. hAM reception

hAM were obtained at the Coimbra Hospital and University Centre (Coimbra, Portugal) from 6 healthy women after elective caesarean sections with informed consent according to the guidelines of the Ethical Committee of the Coimbra Hospital and University Centre. hAM was mechanically peeled from the chorion and washed with phosphate buffered saline (PBS) with 2% antibiotic-antimycotic (Sigma-Aldrich, St. Louis, Missouri, USA). One portion of hAM was used to prepare the hAM extract (hAME), and other portion was used to isolate hAM-derived cells.

4.1.2. hAME preparation

hAM was briefly placed on PBS and then minced, homogenized, sonicated and centrifuged (14000 x g for 15 minutes) on ice. The supernatant was collected and stored at -80ºC until protein quantification in NanoDrop (ND-1000 Spectrophotometer, Wilmington, Delaware, USA). No detergents were used for hAME preparation to avoid any interference in the *in vitro* results.

The final protein concentration of hAME was approximately 3.7 ± 0.2 mg per mL.

4.1.3. hAM-derived cells isolation, culture and characterization

hAM were incubated for 7 minutes at 37^{o}C under stirring in 50% dispase (Stem Cell Technologies, Vancouver, Canada), and 50% PBS. hAM was then subjected to a resting period of 8 minutes at room temperature (RT, 18^{o}C) in Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich) pH 7.4, with 10% heat-inactivated fetal bovine serum (FBS) (Sigma-Aldrich), 0.25% sodium pyruvate (Gibco, Life Technolgies, Paisley, UK) and 2mM of L-Glutamine (Sigma-Aldrich). Afterwards, hAM was totally digested at 37^{o}C

under stirring in an enzyme cocktail composed by 0.75mg/mL collagenase IV (Gibco), 20µg/mL DNAse I (Sigma-Aldrich), 3mM calcium chloride (Sigma-Aldrich) and 0.25% trypsin (Sigma-Aldrich) in PBS. After total digestion, cells were collected by centrifugation (471 x g for 5 minutes) and then cultured in DMEM, pH 7.4, with 10% heat-inactivated FBS, 0.25% sodium pyruvate and 1% antibiotic/antimycotic. Cells were expanded until 3 passages and maintained under culture at 37°C with 5% CO_2.

4.2. Isolation and culture of mononuclear cells from peripheral blood samples

A total of 8 healthy volunteers (4 male and 4 female with an average of 27.1 ± 6.5 years of age, ranging from 21 to 38 years of age) donated peripheral blood samples, which were collected, in heparin (Becton Dickinson Biosciences, San Jose, CA, USA), at the Blood and Transplantation Center of Coimbra (Coimbra, Portugal). All participants had previously given written informed consent regarding their participation in the study.

Isolation of the peripheral blood mononuclear cells (PBMC) was carried out through Lymphoprep™ (Axis-Shield Diagnotics, Oslo, Norway) gradient density centrifugation, within one hour of collection. Afterwards, the cells were washed twice in Hanks' balanced salt solution (HBSS) (Gibco) by 5 minutes centrifugation at 540 x g. The resulting PBMC pellet was resuspended in Roswell Park Memorial Institute (RPMI) 1640 with GlutaMax medium (Invitrogen, LifeTechnologies, Waltham, Massachusetts, USA) containing 0.1 % antibiotic-antimycotic (Gibco), to a final concentration of 10^6 cells per mL.

PBMC were then cultured in 24-well TPP tissue culture plates (TPP Techno Plastic Products AG, Zollstrasse, Trasadingen, Switzerland) and incubated, in a sterile environment, at 37°C, in a humidified atmosphere with 5% CO_2, under the following experimental conditions: PBMC (10^6 cells) in 1 mL of RPMI-1640 medium only (negative control); PBMC (10^6 cells) in 1 mL RPMI-1640 medium with the addition of hAME to the culture medium (50, 100 or 200 µL); PBMC (10^6 cells) in 1 mL RPMI-1640 medium in co-culture with hAM-derived cells (either $0.5x10^6$ or $0.1x10^6$ cells, for a ratio PBMC-to-hAM cells of 2:1 and 10:1, respectively).

The incubation period was distinct for the different sets of experiments and is detailed below.

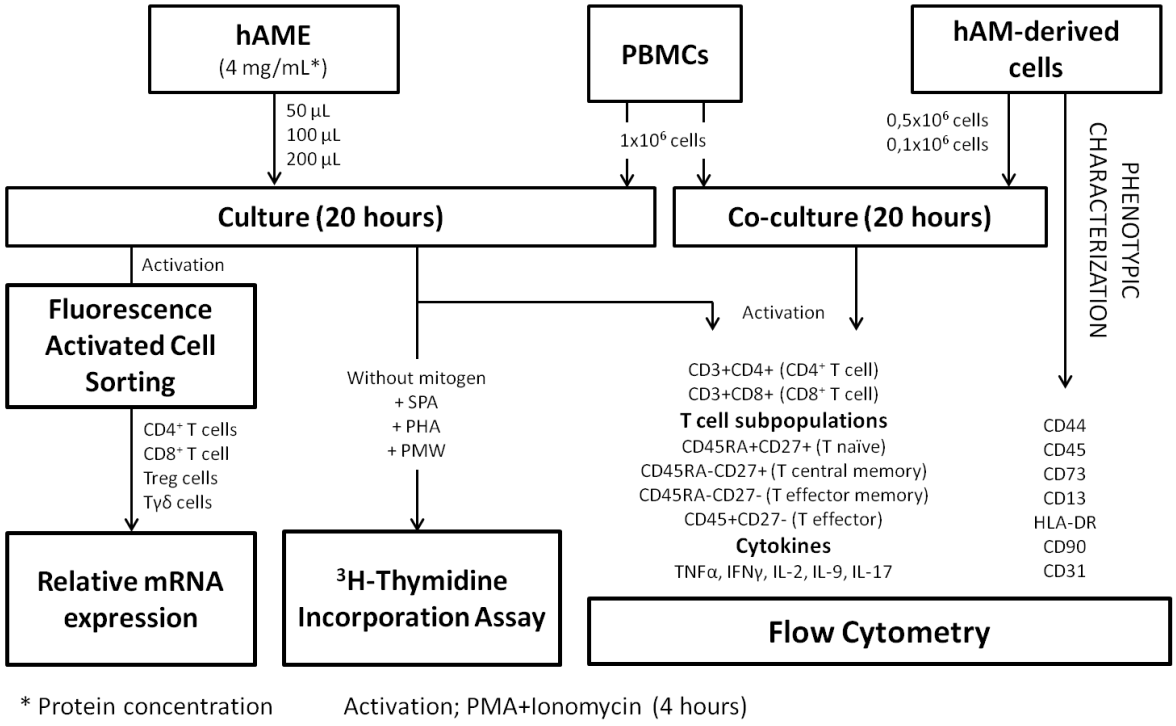

Figure 6. Basic scheme of the methodology adopted for the evaluation of the immunosuppressive potential of hAME and hAM-derived cells.

4.3. Measurement of T lymphocyte proliferation

PBMC differentiated under the abovementioned culture conditions, with or without addition of 200 µL of hAME to the cuture medium, were used for the assessment of cell proliferation after 5 days of culture (3 samples), with or without the addition of mitogen; either phytohemagglutinin (PHA), Staphylococcal protein-A (SPA) or pokeweed mitogen (PWM). Proliferation was evaluated through a thymidine incorporation assay, by adding [^{3}H]-thymidine (0.67 Ci/well, Perkin Elmer, Life Sciences, Zaventem, Belgium) to the cells, and then allowing the cells to be labeled during 18 additional hours of incubation. Thymidine incorporation was measured following incubation, after harvesting the cells with a Filtermate Harvester (Perkin Elmer), with a microplate scintillation and luminescence counter (Top Count NXT, Perkin Elmer).

Data were expressed as mean and standard deviation (SD), and differences between the two different cell cultures were considered significant when the p-value for the Wilcoxon signed-rank test for paired samples was lower than 0.05.

4.4. Flow cytometry analysis of immunophenotype

T lymphocytes can be identified by their expression of CD3, T helper and T cytotoxic cells differ from each other in the expression of CD4 and CD8 ($CD4^+CD8^-$ and $CD4^-CD8^+$, respectively) and the naïve, memory and effector T cell subsets are phenotypically distinguished by the expression of isoforms of the CD45 antigen (or protein tyrosine phosphatase receptor type C), an important regulator of antigen receptor signaling in T and B lymphocytes. In the $CD4^+$ and $CD8^+$ T cell populations the CD45RA isoform is expressed by naïve and effector T cells ($CD45RA^+$) but not by memory cells ($CD45RA^-$), which, instead, express the CD45RO isoform. The difference arises because, after antigen experience, expression of CD45RA is lost while CD45RO expression takes its place (*Mackay 1999*). Central memory (T_{CM}) and effector memory (T_{EM}) T cells, in turn, can be distinguished by using one of several possible second markers, each associated to a slightly different stage of differentiation from the central to the effector memory phenotype. The chemokine receptor CCR7 is commonly used, as is the homing receptor CD26L, both of which are expressed by naïve T ($CD45RA^+CCR7^+CD26L^+$) and T_{CM} cells ($CD45RA^-CCR7^+CD26L^+$). However, CCR7 is expressed at low levels and detection requires very bright antibody staining while CD26L expression is absent following certain protocols such as density gradient centrifugation, cryopreservation and T cell activation with phorbol myristate acetate (PMA) plus ionomycin. On the other hand CD27 and CD28, a tumor necrosis factor receptor and a co-stimulatory molecule, respectively, are expressed at higher levels by naïve T ($CD45RA^+CD27^+CD28^+$) and T_{CM} cells ($CD45RA^-CD27^+CD28^+$) (*Okada et al. 2008*), but only one of those markers would be necessary to distinguish them, thus, for the purpose of identification of the different T cell subsets, the selected panel of markers was; CD3, CD4, CD8, CD45RA and CD27; naïve T cells were identified as $CD45RA^+CD27^+$ T cells, effector T cells as $CD45RA^+CD27^-$, T_{CM} cells as $CD45RA^-CD27^+$ and T_{EM} cells as $CD45RA^-CD27^-$. The panel also included the neural cell adhesion molecule CD56 to exclude NK cells (identified as $CD3^-CD56^+$ lymphocytes) (Table 9).

The panel of markers selected for the characterization of hAM-derived cells included the profile suggested by the International Society of Cellular Therapy (*Horwitz et al. 2005*) for the identification of MSCs which other authors had reported to be applicable to hAM-derived MSCs (*Insausti et al. 2010, Lindenmair et al. 2012, Miki 2011*); namely, HLA-DR, CD13, CD31, CD44, CD45, CD73 and CD90 (Table 9).

Table 9. Panel of mAb reagents (with clones and commercial sources) used for immunophenotypic characterization and fluorescence-activated cell sorting (FACS).

		Fluorochrome							
		PB	**V500 or KO**	**FITC**	**PE**	**PECγ5 or PerCPCγ 5.5**	**PECγ7**	**APC**	**APCH7**
T cells	**TNFα / IL-17**	**CD3** UCHT1 BD Biosciences	**CD8** RPA-T8 BD Biosciences	**cyTNFα** Mab11 BD Biosciences	**cyIL-17** SCPL1362 BD Biosciences	**CD27** 1A4-CD27 Beckman Coulter	**CD56** N901 Beckman Coulter	**CD45RA** HI100 BD Biosciences	**CD4** SK3 BD Biosciences
	IFNγ / IL-17	**CD3** UCHT1 BD Biosciences	**CD8** RPA-T8 BD Biosciences	**cyIFNγ** 4S.B3 BD Biosciences	**cyIL-17** SCPL1362 BD Biosciences	**CD27** 1A4-CD27 Beckman Coulter	**CD56** N901 Beckman Coulter	**CD45RA** HI100 BD Biosciences	**CD4** SK3 BD Biosciences
	IL-2 / IL-17	**CD3** UCHT1 BD Biosciences	**CD8** RPA-T8 BD Biosciences	**cyIL-2** MQ-17H2 BD Biosciences	**cyIL-17** SCPL1362 BD Biosciences	**CD27** 1A4-CD27 Beckman Coulter	**CD56** N901 Beckman Coulter	**CD45RA** HI100 BD Biosciences	**CD4** SK3 BD Biosciences
	IL-2 / IL-9	**CD3** UCHT1 BD Biosciences	**CD8** RPA-T8 BD Biosciences	**cyIL-2** MQ-17H2 BD Biosciences	**cyIL-9** MH9A3 BD Biosciences	**CD27** 1A4-CD27 Beckman Coulter	**CD56** N901 Beckman Coulter	**CD45RA** HI100 BD Biosciences	**CD4** SK3 BD Biosciences
hAM-erived cells		**CD44** Clone IM7 Biolegend	**CD45** J.33 Beckman Coulter	**CD31** WM59 BD Pharmingen	**CD73** AD2 BD Pharmingen	**-**	**CD13** L138 BD Bio-sciences	**CD90** 5E10 BD Pharmingen	**CD44** Clone IM7 Biolegend
Cell Sorting		**CD8** RPA-T8 BD Biosciences	**-**	**CD25** M-A251 BD Biosciences	**CD127** IM1980U Beckman Coulter	**CD4** 13B8.2 BD Bio-sciences	**TCRγδ** R9.12 BD Bio-sciences	**CD56** IM2474 Beckman Coulter	**CD3** SK7 BD Biosciences

APC, allophycocyanin; APCH7, allophycocyanin-hilite 7; FITC, flurescein isothiocyanate; KO, khrome orange; mAb, monoclonal antibody; PB, pacific blue; PE, phycoerythrin; PECγ5, phycoerythrin-cyanine 5; PerCPCy5.5, peridinin chlorophyll protein-cyanine 5.5; PECγ7, phycoerythrin-cyanine 7; V500, Violet 500. Commercial Sources: BD Biosciences (Becton Dickinson Biosciences, San Jose, CA, USA); Beckman Coulter (Miami, Florida, USA); Biolegend (San Diego, CA, USA)

Flow cytometry analysis of T cells and hAM-derived cells was performed in BD FACSCanto^{TM} II flow cytometer (BD Biosciences, Franklin Lakes, New Jersey, USA) and

data analysis was carried out through resort to the Infinicyt (version 1.6) software (Cytonos SL, Salamanca, Spain).

4.4.1. Immunophenotypic study of peripheral blood T lymphocytes

After 20 hours of PBMC incubation with hAME or hAME-derived cells (8 samples), the stimulants PMA (1 ng/mL, Sigma-Aldrich) and ionomycin (0.5 mg/mL, Sigma-Aldrich) were added to the wells, along with brefeldin A (5 mg/mL, Sigma-Aldrich) to prevent the release of *de novo* produced cytokines. The plates were then incubated for 4 more hours, in a sterile environment, at 37^{o}C, in a humidified atmosphere with 5% CO_2, after which analysis was performed by flow cytometry.

Prior to cell staining, the cells were concentrated, by 5 minutes of centrifugation at 540 x g, and the supernatants discarded. All cells were stained with the same monoclonal antibodies (mAb) for surface proteins antigens CD3, CD4, CD8, CD56, CD27 and CD45RA (Table 9). The subsequent permeabilization step was performed with the permeabilization kit Intraprep™ (Beckman Coulter, Brea, California, USA) according to manufacturer instructions: after 10 minutes of incubation in the dark at RT, with the mAb, cells were washed in PBS (5 minutes at 540 x g) and treated with fixing solution for 10 more minutes in the dark at RT. Cells were then washed one more time with PBS (5 minutes at 540 x g) and the supernatants discarded. Lysis and permeabilization solution was applied for lysis of red blood cells and permeabilization of the leukocytes' membranes. Cells were subsequently stained with the mAb for intracellular TNFα along with IL-17, IFNγ with IL-17, IL-2 with IL-17, or IL-2 with IL-9 (combinations depicted in Table 9). After 10 minutes of incubation in the dark at RT, samples were washed twice with PBS (5 minutes at 540 x g); the cell pellet was resuspended in 250 µL PBS and immediately acquired.

As previously mentioned, the subpopulations of $CD4^+$ and $CD8^+$ T cells (phenotypically characterized as $CD3^+CD4^+CD8^-$ and $CD3^+CD4^-CD8^+$, respectively) were identified as falling into the naïve, effector, central memory and effector memory functional compartments according to their expression profile of the surface markers CD45RA and CD27, as follows; $CD45RA^+CD27^+$ were identified as being naïve T cells, $CD45RA^-CD27^+$ as T_{CM} cells, $CD45RA^-CD27^-$ expression was attributed to T_{EM} cells and $CD45RA^+CD27^-$ to effector T cells.

Data were expressed as mean ± SD and differences between different culture conditions were considered significant when the p-value for the Wilcoxon signed-rank test for paired samples was lower than 0.05.

4.4.2. Immunophenotypic characterization of hAM-derived cells

The hAM-derived cells, from 4 different samples, were concentrated (5 minutes at 540 x g) and the supernatants discarded. Cells were then labeled with the mAb for surface markers HLA-DR, CD13, CD31, CD44, CD45, CD73 and CD90 (Table 9) in a 10 minutes incubation, in the dark at RT. Cells were then washed with PBS (5 minutes at 540 x g), the cell pellet was resuspended in 250 µL PBS and immediately acquired.

Differences between identified cell populations were considered significant when the p-value for the Mann-Whitney test was lower than 0.05.

4.5. Purification of T cell subtypes by fluorescence-activated cell-sorting

Using a 7-color mAb combination (depicted in Table 9), the $CD4^+$ and $CD8^+$ T cells in culture (from 5 samples) were purified by fluorescence-activated cell-sorting (FACS) according to their characteristic phenotype, using a FACSAria^{TM} II flow cytometer (BD Biosciences).

The cells were concentrated by 5 minutes of centrifugation at 540 x g and the supernatants were discarded. Subsequently, cells were stained with the mAb for surface proteins antigens CD8, CD25, CD127, CD4, CD56, CD3 and TCRγδ (Table 9). $CD4^+$ T cells were identified as $CD3^+CD4^+CD8^-$ and $CD8^+$ T cells as $CD3^+CD4^-CD8^+$, the remainder immunophenotypic markers were used to identify regulatory T cells ($CD25^+CD127^+$) and γδ T cells ($CD3^+TCR\gamma\delta^+$). The purified T cell populations were subsequently used for the relative quantification of mRNA expression.

4.6. Relative mRNA expression by purified $CD4^+$, $CD8^+$, regulatory and gamma-delta T cells.

Purified T cell populations were transferred to a 1.5 mL eppendorf tube, centrifuged 5 minutes at 300 x g and the pellet resuspended in 350 µL of RNeasy Lysis Buffer (RTL, Qiagen, Hilden, Germany). Total RNA was extracted with the RNeasy Micro kit (Qiagen), according to supplier's instructions. Total RNA was eluted onto a 20 µL volume of RNase-

free water. Reverse transcription was then performed with iScript™ Reverse Transcription Supermix for RT-PCR (Bio-Rad, Hercules, California, USA), according to manufacturer's instructions. Relative quantification of gene expression by real-time polymerase chain reaction (RT-PCR) was performed in the LightCycler™ 480 II (Roche Diagnostics, Rotkreuz, Switzerland). RT-PCR reactions were performed using 1xQuantiTect SYBR Green PCR Master Mix (Qiagen), 1xQuantiTect Primer Assay (GATA3: QT00095501; STAT6: QT00097426; IL-4: QT00012565; FOXP3: QT00048286; IL-10: QT00041685; TGFβ1: QT00000728; PRF1: QT00199955; GZMB: QT01004875; KLRK1: QT00197183; EOMES: QT00026495) (Qiagen), in a final volume of 10 μL. The reactions were performed using the following thermal profile: 15 minutes at 95°C, 50 cycles of 15 seconds at 94°C, 30 seconds at 55°C and 30 seconds at 72°C, 1 cycle of 15 seconds at 95°C, 15 seconds at 60°C and continuo at 95°C and 1 cycle of 30 seconds at 37°C. All samples were run in duplicate and melting point analysis was done to ensure the amplification of the desired product. RT-PCR results were analyzed with the LightCycler™ software (Roche Diagnostics).

Two reference genes for data normalization were selected using the GeNorm software (PrimerDesign Ltd., Southampton, England), for all T cell populations the more stable reference genes were; cytochrome c1 (CYC1) and splicing factor 3a subunit 1 (SF3A1). The normalized expression levels of the genes of interest were calculated using the delta-Ct method.

Relative mRNA expression data was expressed through their quartiles with non-parametric box-plots. Differences between the two culture conditions were considered significant when the p-value for the Wilcoxon paired-sample test was lower than 0.05.

4.7. Statistical analysis

Statistical analysis was performed by the non-parametric Wilcoxon paired-sample test or Mann-Whitney Test. Statistical significance was considered when p-value was lower than 0.05 (significance level of 5%). The data analysis was generated using the *Real Statistics Resource Pack* software (Release 3.8) (Copyright 2013 – 2015, Charles Zaiontz).

5. RESUTS

5.1. hAM-derived cells characterization

The cells isolated from hAM had demonstrated plastic culture adherence and reached 80 to 90% confluence (proportion of plastic surface covered) after a few days of culture. The cellular population was easily expanded in vitro until 3 passages without any visible morphological alterations. Two types of cells were present in the total population: the epithelial cells, with a polygonal morphology and an irregular, large and homogeneous nucleus, and the mesenchymal cells, with typical fusiform/fibroblast-like morphology. The hAM-derived cells used in all co-culture experiments had a maximum of 2 passages.

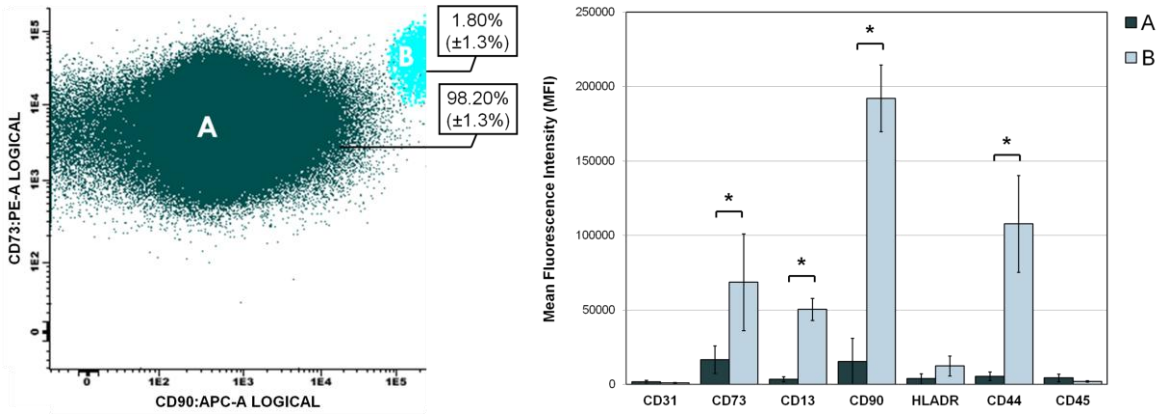

Figure 7. Phenotypic characterization of hAM-derived cells. Left panel: Bivariate dot plot histogram illustrating the hAM-derived cells, following culture, displaying two distinct cell populations; A and B. Right panel: Phenotypic characterization of the two cell populations identified in hAM-derived cell cultures as the expression level of several phenotypic markers, measured as the mean fluorescence intensity (MFI) (mean ± SD). Results were obtained with four hAM samples. Statistically significant differences were considered when $p<0.05$ for the Mann-Whitney Test: * for the groups indicated in the figure.

The phenotypic characterization of 4 samples of hAM-derived cells revealed two major cell populations (Figure 7), one more abundant than the other (98.2% ± 1.3% and 1.8% ± 1.3%, respectively), which differed from each other, mainly, in the expression of the phenotypic markers CD73, CD13, CD90 and CD44, with the scarcer population having a significantly higher expression level for all aforementioned markers, particularly CD90 ($CD73^{+}CD13^{+}CD44^{+}CD90^{high}$).

5.2. Evaluation of the anti-proliferative effect of hAME

The presence of hAME in the culture medium did not cause significant changes in basal cell proliferation in the absence of mitogenic stimulation, but, there was a decrease in [H^3]-thymidine incorporation when PBMC were activated with either PHA or PWM (Figure 8). Additionally, in the cases where hAME produced an inhibitory effect, there was no significant difference between 50 µL, 100 µL and 200 µL of hAME.

These results demonstrate that hAME can inhibit lymphocyte proliferation induced by mitogenic stimuli.

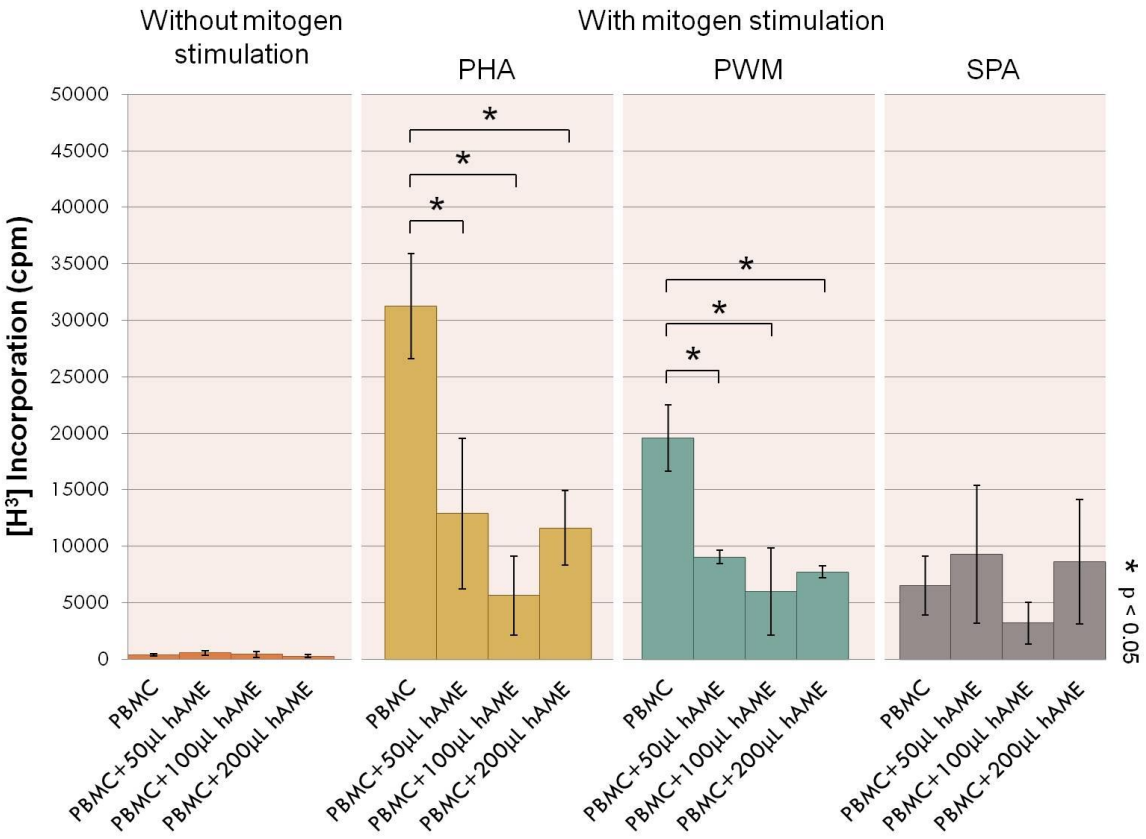

Figure 8. Thymidine incorporation assay. Results represent PBMC proliferation, measured as [H^3]-thymidine incorporation (mean ± SD) without mitogen stimulation or following PBMC activation by mitogenic stimuli. Results were obtained for three PBMC samples. Statistically significant differences were considered when $p<0.05$ for the Wilcoxon paired-sample test: * for the groups indicated in the figure.

5.3. Influence of the dosage over hAME-mediated anti-inflammatory effects

PBMC culture with hAME resulted in a decrease of the frequency of T cells expressing TNFα, following stimulation with PMA plus ionomycin (Figure 9).

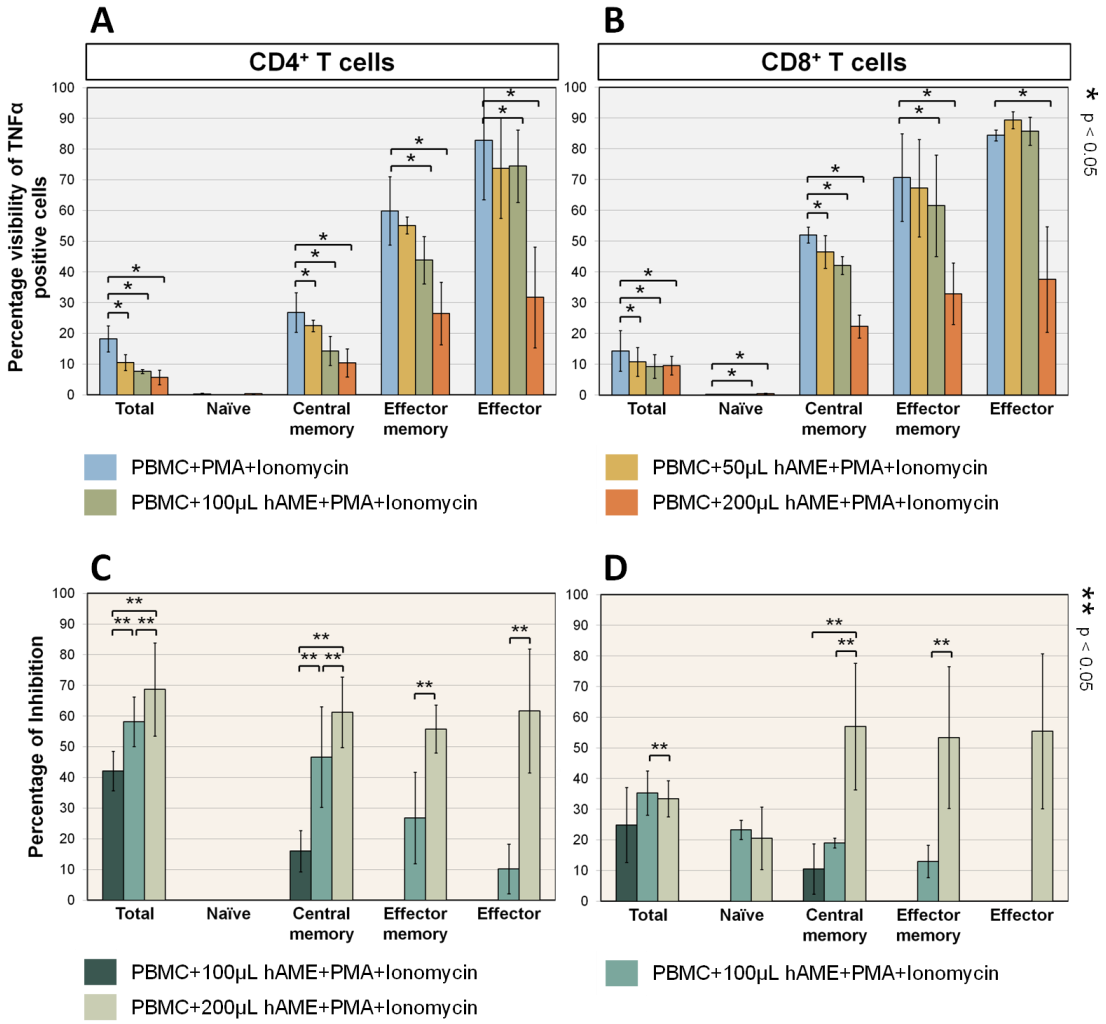

Figure 9. Effect of different doses of hAME over the frequency of TNFα-expressing $CD4^+$ and $CD8^+$ T cells. (A-B) Percentage of TNFα-producing $CD4^+$ (A) and $CD8^+$ T cells (B) (mean ± SD) among four different subsets (naïve, central memory, effector memory and effector) following PBMC stimulation in the absence (PBMC+PMA+Ionomycin) or presence of hAME in the culture medium (PBMC+50µL hAME+PMA+Ionomycin, PBMC+100µL hAME+PMA+Ionomycin, PBMC+200µL hAME+PMA+Ionomycin). (C-D) Percent inhibition (mean ± SD) induced by hAME on the frequency of TNFα producing $CD4^+$ (C) and $CD8^+$ T cells (D). Results were obtained for three PBMC samples. Statistically significant differences were considered when $p<0.05$ for the Wilcoxon paired-sample test: * versus PBMC+PMA+Ionomycin; ** for the groups indicated in the figure.

Unlike the observed inhibitory effect over cell proliferation, this effect was dependent on the amount of hAME added to the culture medium, in most cases only being significant for the highest dose of hAME.

Additionally, $CD4^+$ and $CD8^+$ T cells, and the different T cell subsets amongst them, displayed different susceptibility to inhibition. For instance, the decrease in the frequency of total $CD4^+TNF\alpha^+$ T cells was more pronounced than that of total $CD8^+TNF\alpha^+$ T cells. The effector subsets, among both $CD4^+TNF\alpha^+$ and $CD8^+TNF\alpha^+$ T cells, were only affected by the highest dose of hAME, on the other hand, T_{CM} cells seemed the most susceptible to the inhibitory effect of hAME, being the only subset among $CD4^+TNF\alpha^+$ and $CD8^+TNF\alpha^+$ T cells which was affected by the lowest dose of hAME.

Thus, hAME decreases the frequency of TNFα producing T cells, in a dose-dependent manner, with different susceptibility to inhibition among different T cell subsets.

In all subsequent experiments only the highest dose of hAME (200 μL) was added the culture medium.

5.4. Frequency of TNFα, IFNγ and IL-2 producing T cells

The addition of hAME to the PMA+Ionomycin-stimulated PBMC culture medium decreased the frequency of $CD4^+$ and $CD8^+$ TNFα, IFNγ and IL-2 expressing T cells, with different susceptibility to inhibition among different T cell subsets.

The presence of hAME reduced the percentage of $CD8^+TNF\alpha^+$ T cells and $CD4^+TNF\alpha^+$ T cells among all T cell subsets and, as previously observed, the overall inhibitory effect was more preeminent among $CD4^+$ T cells comparatively with $CD8^+$ T cells (Figure 10). The difference arose mainly from a more pronounced effect over $CD4^+TNF\alpha^+$ T_{CM} cells, comparatively to $CD8^+TNF\alpha^+$ T_{CM} cells, while no significant differences were observed between the two groups among the other subsets.

Regarding $IFN\gamma^+$ T cells, the frequency of cytokine producing cells was significantly reduced in nearly all T cell subsets, with the exception of $CD8^+IFN\gamma^+$ effector T cells (Figure 11). Once again the overall inhibition appears to be greater for $CD4^+$ T cells in comparison with $CD8^+$ T cells. The same discrepancy was not observed among $IL\text{-}2^+$ T cells; in this case, there was a decrease of IL-2 producing cells in all T cell subsets without major differences between $CD4^+IL\text{-}2^+$ and $CD8^+IL\text{-}2^+$ T cells, or between different T cell subsets (Figure 12).

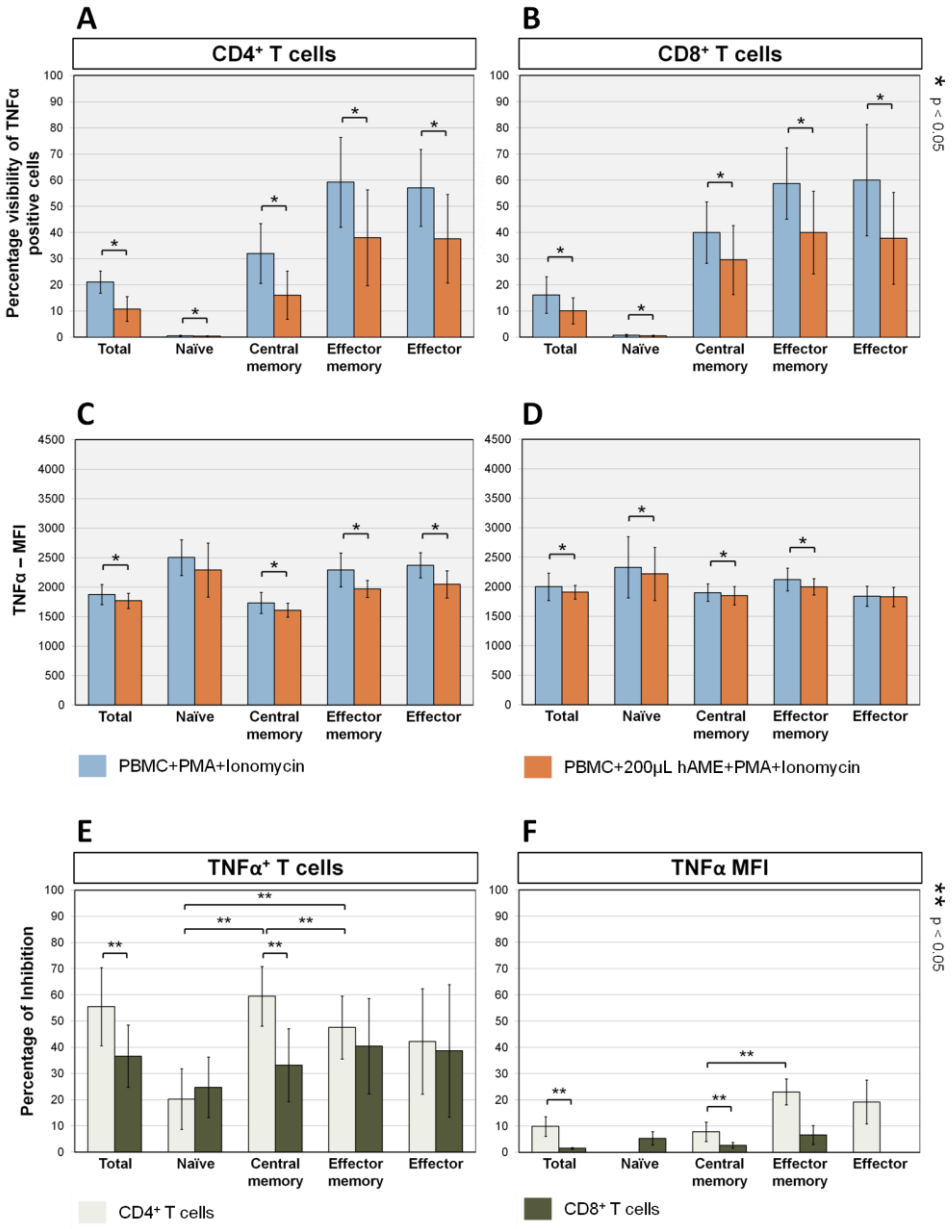

Figure 10. Effect of hAME over TNFα expressing $CD4^+$ and $CD8^+$ T cells. (A-B) Percentage of TNFα producing $CD4^+$ (A) and $CD8^+$ T cells (B) (mean ± SD), distributed among the naïve, central memory, effector memory and effector subsets, following PBMC stimulation in the absence (PBMC+PMA+Ionomycin) or presence of hAME in the culture medium (PBMC+200µL hAME+PMA+Ionomycin). (C-D) Amount of TNFα expressed per $CD4^+$ (C) and $CD8^+$ T cell (D), measured as the MFI (mean ± SD). (E-F) Percent inhibition (mean ± SD) induced by hAME on the frequency of TNFα producing $CD4^+$ and $CD8^+$ T cells (E) and amount of TNFα expressed per cell (F). Results were obtained for eight PBMC samples. Statistically significant differences were considered when $p<0.05$ for the Wilcoxon paired-sample test: * versus PBMC+PMA+Ionomycin; ** for the groups indicated in the figure.

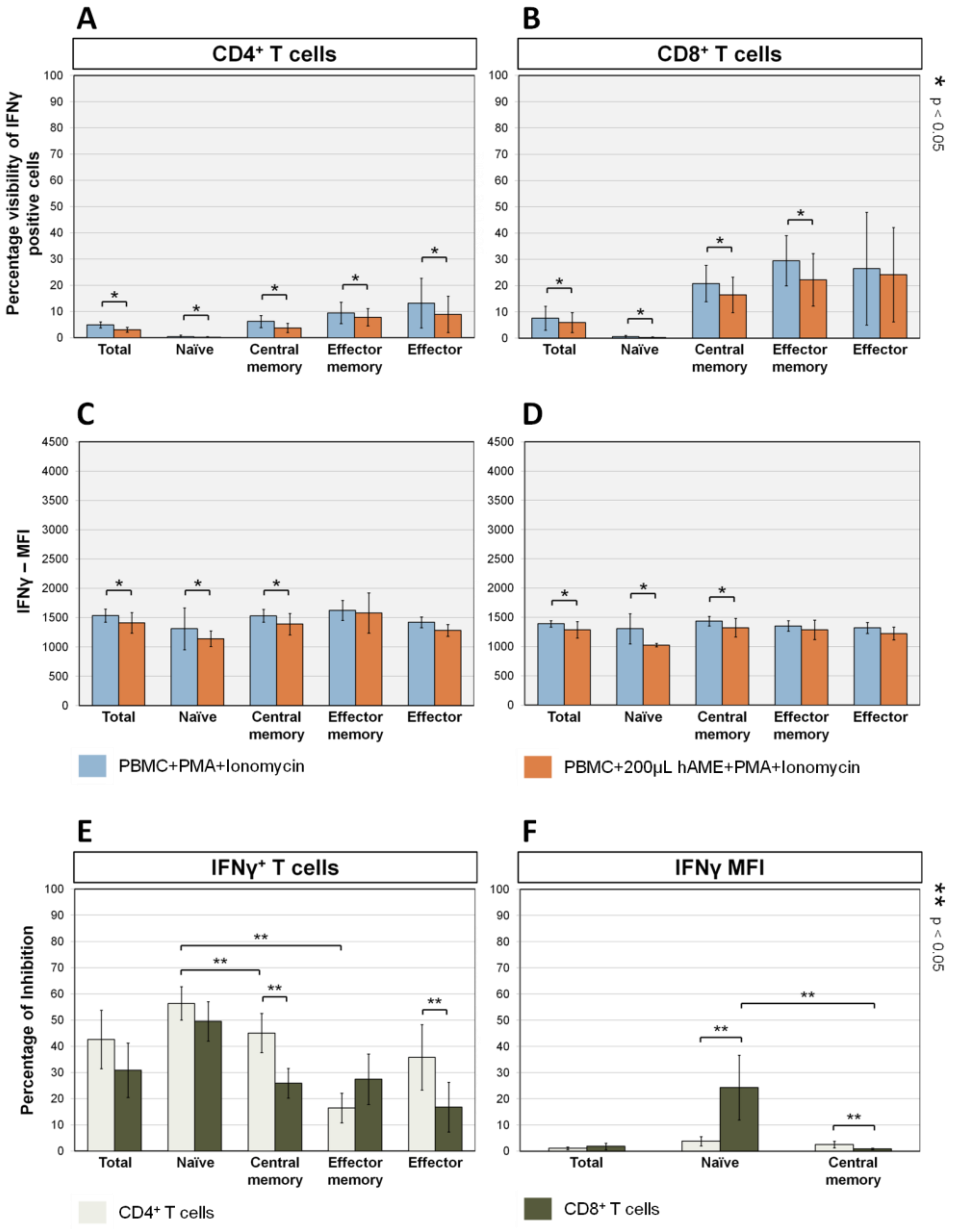

Figure 11. Effect of hAME over IFNγ expressing $CD4^+$ and $CD8^+$ T cells. (A-B) Percentage of IFNγ producing $CD4^+$ (A) and $CD8^+$ T cells (B) (mean ± SD), distributed among the naïve, central memory, effector memory and effector subsets, following PBMC stimulation in the absence (PBMC+PMA+Ionomycin) or presence of hAME in the culture medium (PBMC+200µL hAME+PMA+Ionomycin). (C-D) Amount of IFNγ expressed per $CD4^+$ (C) and $CD8^+$ T cell (D), measured as the MFI (mean ± SD). (E-F) Percent inhibition (mean ± SD) induced by hAME on the frequency of IFNγ producing $CD4^+$ and $CD8^+$ T cells (E) and amount of IFNγ expressed per cell (F). Results were obtained for eight PBMC samples. Statistically significant differences were considered when $p<0.05$ for the Wilcoxon paired-sample test: * versus PBMC+PMA+Ionomycin; ** for the groups indicated in the figure.

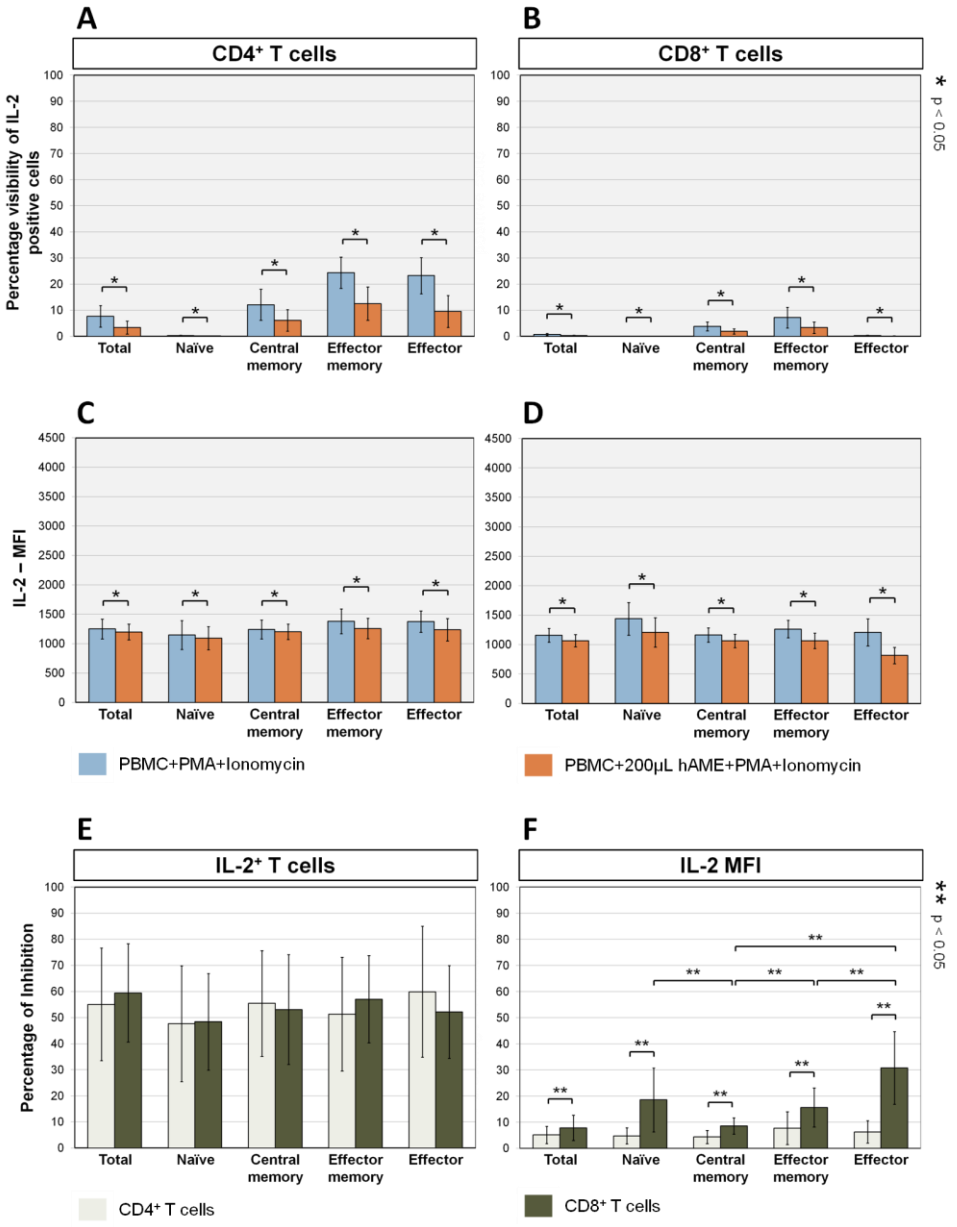

Figure 12. Effect of hAME over IL-2 expressing $CD4^+$ and $CD8^+$ T cells. (A-B) Percentage of IL-2 producing $CD4^+$ (A) and $CD8^+$ T cells (B) (mean ± SD), distributed among the naïve, central memory, effector memory and effector subsets, following PBMC stimulation in the absence (PBMC+PMA+Ionomycin) or presence of hAME in the culture medium (PBMC+200µL hAME+PMA+Ionomycin). (C-D) Amount of IL-2 expressed per $CD4^+$ (C) and $CD8^+$ T cell (D), measured as the MFI (mean ± SD). (E-F) Percent inhibition (mean ± SD) induced by hAME on the frequency of IL-2 producing $CD4^+$ and $CD8^+$ T cells (E) and amount of IL-2 expressed per cell (F). Results were obtained for eight PBMC samples. Statistically significant differences were considered when $p<0.05$ for the Wilcoxon paired-sample test: * versus PBMC+PMA+Ionomycin; ** for the groups indicated in the figure.

The inhibitory effect of hAME over the frequency of cytokine producing cells seemed to differ not only between $CD4^+$ and $CD8^+$ T cells, and between different subsets, but also according to the cytokine under study. Interestingly, among $CD4^+TNF\alpha^+$ T cells, the naïve T cells were less significantly affected than $CD4^+TNF\alpha^+$ T_{CM} or T_{EM} cells ($CD4^+TNF\alpha^+$ T_{CM} cells seemingly being more strongly inhibited than the rest), while the opposite result was observed for $CD4^+IFN\gamma^+$ naïve T cells, which yielded greater percent inhibition than $CD4^+IFN\gamma^+$ T_{CM} or T_{EM} cells. On the other hand, there was no major difference in the effect over the frequency of $IL\text{-}2^+$ T cells between different T cell populations.

In addition to the clear reduction in the frequency of cytokine producing cells, there was also a slight decrease in the quantity of cytokine produced per cell, expressed by the diminishing mean fluorescence intensity (MFI), in most $CD4^+$ and $CD8^+$ T cell subsets, the exceptions being $CD4^+TNF\alpha^+$ naïve, $CD8^+TNF\alpha^+$ effector, $CD4^+IFN\gamma^+$ T_{EM}, $CD8^+IFN\gamma^+$ T_{EM}, $CD4^+IFN\gamma^+$ effector and $CD8^+IFN\gamma^+$ effector T cells (Figures 10-12). However, the decrease in MFI, albeit consistent throughout the data, is much less pronounced than the reduction of cytokine producing cells: in the presence of hAME there was an overall decrease in the frequency of $TNF\alpha^+$ T cells in 56% and 37% (for $CD4^+$ and $CD8^+$ T cells, respectively), while the TNFα-MFI decreased only in 5% and 4%. Similarly, the overall frequencies of $IFN\gamma^+$ and $IL\text{-}2^+$ T cells decreased in 43% and 31% (for $CD4^+IFN\gamma^+$ and $CD8^+IFN\gamma^+$ T cells), and 55% and 60% (for $CD4^+IL\text{-}2^+$ and $CD8^+IL\text{-}2^+$ T cells) while the respective percent inhibition of MFI was 8% (for both $CD4^+IFN\gamma^+$ and $CD8^+IFN\gamma^+$ T cells), and 4% and 8% (for $CD4^+IL\text{-}2^+$ and $CD8^+IL\text{-}2^+$ T cells).

5.5. Frequency of IL-17 and IL-9 producing T cells

$CD4^+$ and $CD8^+$ T cells activated in the presence of hAME also displayed lower frequencies of IL-9 and IL-17 producing cells, in comparison with the negative controls. However, for both cytokines under study, no significant difference was observed between $CD4^+$ and $CD8^+$ T cells (Figures 13 and 14).

The hAME also decreased the frequency of $CD4^+$ and $CD8^+$ T cells that simultaneously express IL-9 and IL-2 (Figure 13), and IL-17 with TNFα, IFNγ or IL-2 (Figure 14). Although, in the case of $IL\text{-}17^+IFN\gamma^+$ T cells, there was only a significant decrease in percentage among $CD4^+$ T cells and not within $CD8^+$ T cells (Figure 14).

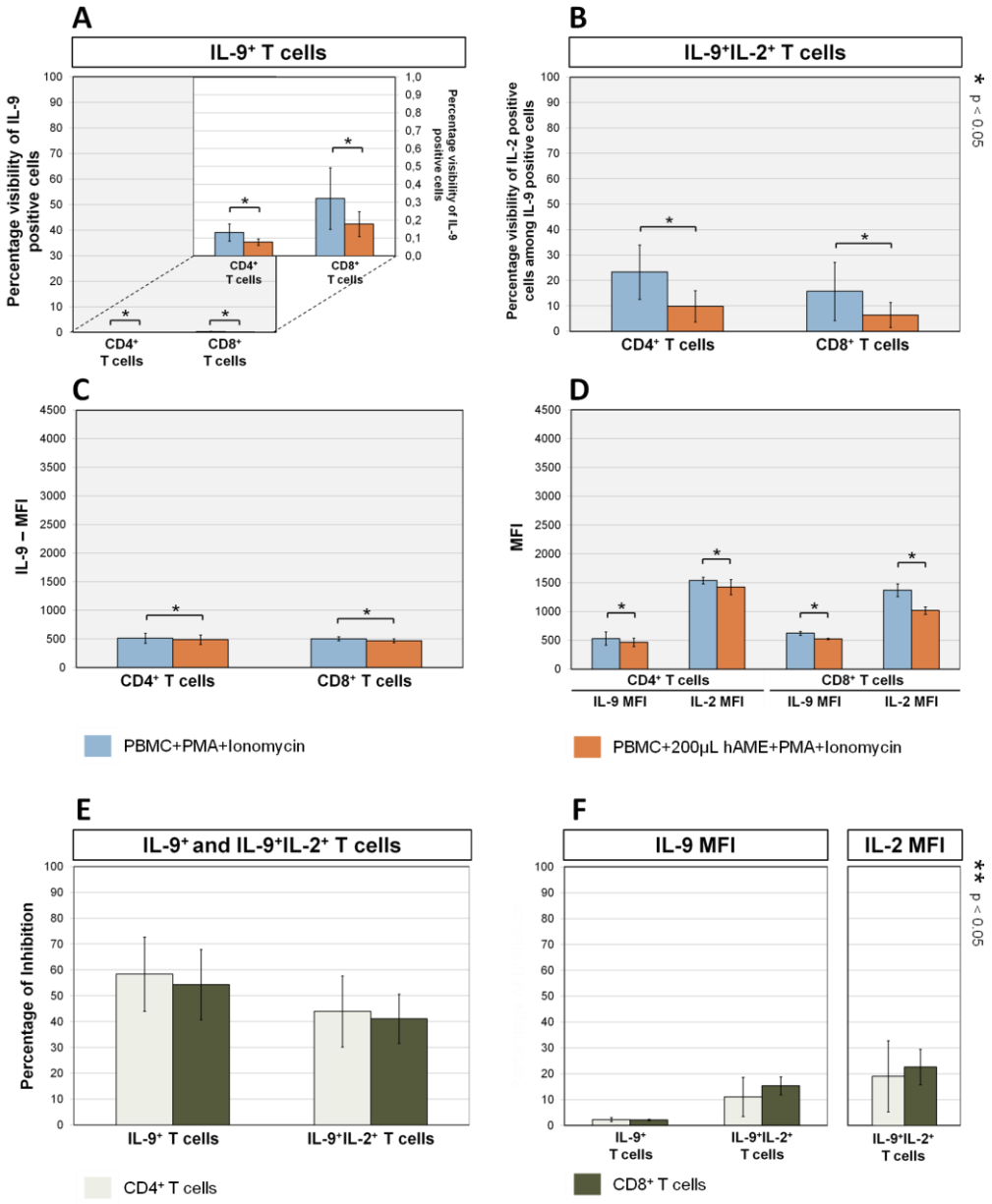

Figure 13. Effect of hAME over IL-9$^+$ and IL-9$^+$IL-2$^+$ T cells. (A) Percentage of IL-9 producing cells (mean ± SD), within total CD4$^+$ and CD8$^+$ T cells, following PBMC stimulation in the absence (PBMC+PMA+Ionomycin) or presence of hAME in the culture medium (PBMC+200µL hAME+PMA+Ionomycin). (B) Percentage of IL-9 producing T cells that simultaneously express IL-2 (mean ± SD). (C-D) Amount of protein expressed per cell among IL-9$^+$ (C) and IL-9$^+$IL-2$^+$ T cells (D) (MFI, mean ± SD). (E-F) Percent inhibition (mean ± SD) induced by hAME over the frequency of IL-9$^+$ and IL-9$^+$IL-2$^+$ T cells (E) and amount of cytokine expressed per cell (F). Results were obtained for eight PBMC samples. Statistically significant differences were considered when $p<0.05$ for the Wilcoxon paired-sample test: * versus PBMC+PMA+Ionomycin; ** for the groups indicated in the figure.

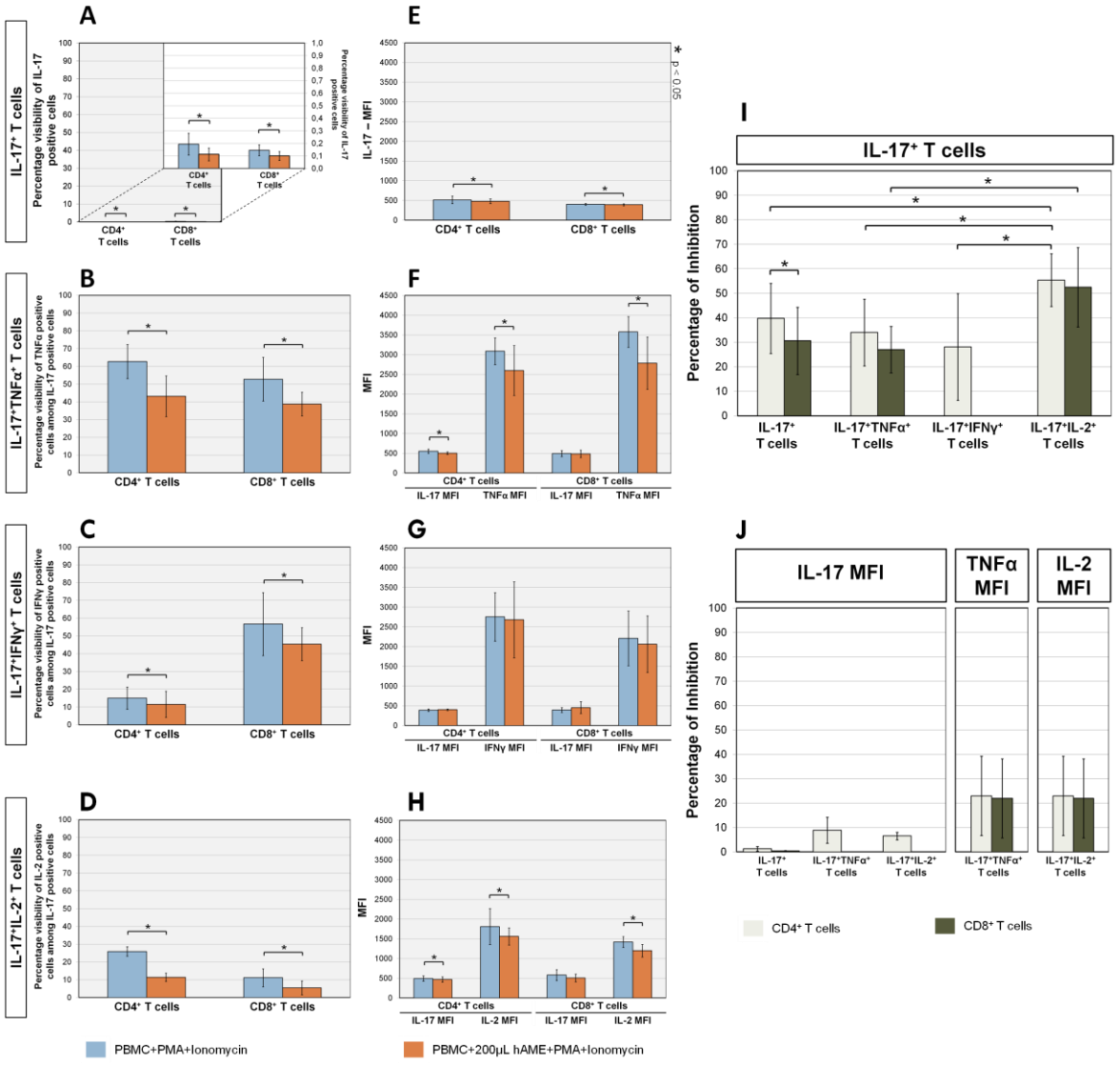

Figure 14. Effect of hAME over IL-17$^+$, IL-17$^+$TNFα$^+$, IL-17$^+$IFNγ$^+$ and IL-17$^+$IL-2$^+$ T cells. (A) Percentage of IL-17 producing cells (mean ± SD), within total CD4$^+$ and CD8$^+$ T cells following PBMC stimulation the absence (PBMC+PMA+Ionomycin) or presence of hAME in the culture medium (PBMC+200µL hAME+PMA+Ionomycin). (B-D) Percentage of IL-17 producing CD4$^+$ and CD8$^+$ T cells that simultaneously express TNFα (B), IFNγ (C) or IL-2 (D). (E-H) Amount of protein expressed per cell among IL-17$^+$ (E), IL-17$^+$TNFα$^+$ (F), IL-17$^+$IFNγ$^+$ (G) and IL-17$^+$IL-2$^+$ T cells (H) (MFI, mean ± SD). (I-J) Percent inhibition (mean ± SD) induced by hAME on the frequency of IL-17$^+$, IL-17$^+$TNFα$^+$, IL-17$^+$IFNγ$^+$ and IL-17$^+$IL-2$^+$ T cells (I) and amount of cytokine expressed per cell (J). Results were obtained for eight PBMC samples. Statistically significant differences were considered when $p<0.05$ for the Wilcoxon paired-sample test: * versus PBMC+PMA+Ionomycin; ** for the groups indicated in the figure.

Moreover the presence of hAME also produced a minor reduction in the amount of cytokine produced per cell (Figures 13 and 14), but this decrease was much less pronounced than the decrease in the frequency of cytokine producing cells; hAME reduced the percentage of IL-9$^+$ and IL-17$^+$ T cells in 44% and 41% (for CD4$^+$IL-9$^+$ and CD8$^+$IL-9$^+$, respectively), and 40% and 31% (for CD4$^+$IL-17$^+$ and CD8$^+$IL-17$^+$, respectively) while the decrease in MFI was substantially smaller; namely, of 5% and 7% (for CD4$^+$IL-9$^+$ and CD8$^+$IL-9$^+$, respectively), and 5% and 3% (for CD4$^+$IL-17$^+$ and CD8$^+$IL-17$^+$, respectively).

The decrease in the frequency of cells that express two cytokines at the same time was also accompanied by a small decrease in the amount of each cytokine produced per cell, with the exception of IL-17$^+$IFNγ$^+$ T cells (CD4$^+$ and CD8$^+$) and for the MFI of IL-17 in IL-17$^+$TNFα$^+$ and IL-17$^+$IL-2$^+$ CD8$^+$ T cells (Figures 13 and 14).

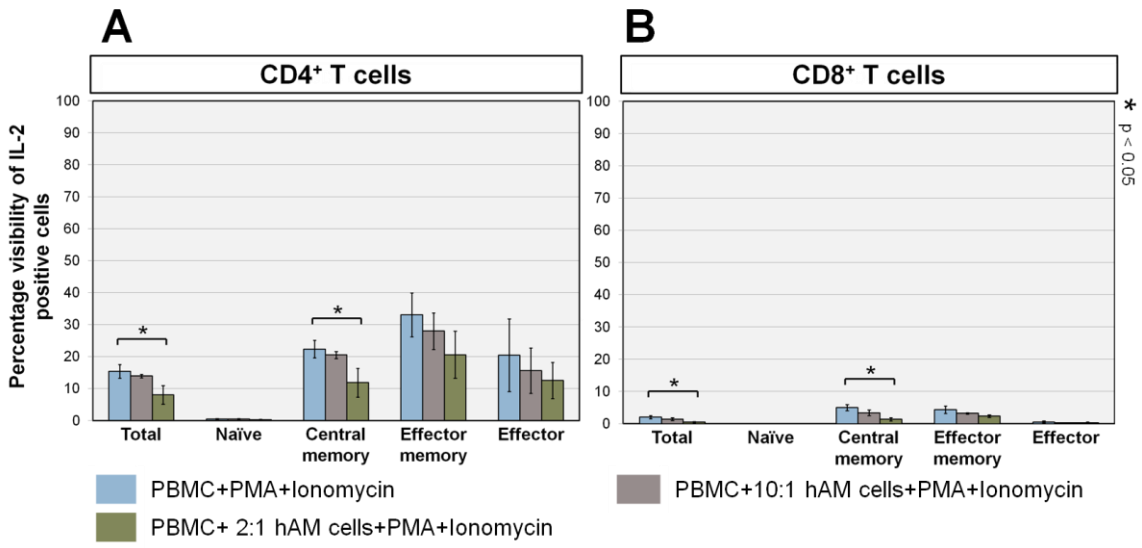

Figure 15. Effect of hAM-derived cells over the frequency of IL-2 expressing T cells. Percentage of IL-2 producing cells among different CD4$^+$ (A) and CD8$^+$ T cell (B) subsets (naïve, central memory, effector memory and effector) (mean ± SD) following PBMC stimulation in the absence (PBMC+PMA+Ionomycin) or presence of hAM-derived cells, in a proportion of PBMC-to-hAM-derived cells of either 10:1 (PBMC+10:1 hAM cells+PMA+Ionomycin) or 2:1 (PBMC+2:1 hAM cells+PMA+Ionomycin), in the culture medium. Results were obtained for two PBMC samples and two replicates. Statistically significant differences were considered when $p<0.05$ for the Wilcoxon paired-sample test: * versus PBMC+PMA+Ionomycin.

Conversely, co-culture of PBMC with hAM-derived cells, at a ratio of PBMC to hAM-derived cells of 10:1 or 2:1, did not produce the same effects; the presence of hAM-derived cells did not significantly reduced the frequency of $TNF\alpha^+$, $IFN\gamma^+$, $IL\text{-}17^+$ or $IL\text{-}9^+$ T cells (data not shown). Interestingly there was a significant reduction in the overall percentage of $CD4^+IL\text{-}2^+$ and $CD8^+IL\text{-}2^+$ T cells (Figure 15), but, among the different T cell subsets, only T_{CM} cells ($CD4^+$ and $CD8^+$) were affected, which is not surprising considering that the previous results, with hAME, pointed to $IL\text{-}2^+$ T_{CM} cells possibly being the more susceptible to inhibition of all the analyzed cytokine producing cell populations.

5.6. mRNA expression in purified T cell populations

Different T cell subsets were isolated from the cultures in the presence or absence of hAME; namely, $CD4^+$ T cells, $CD8^+$ T cells, Treg cells and γδ T cells. The sorted subsets were then analyzed for mRNA expression and the results, like the results from the T cell proliferation assay and quantification of pro-inflammatory cytokine production, demonstrated that hAME exerted a general anti-inflammatory effect over T lymphocytes, by suppressing 'aggressive' T cell phenotypes and promoting and/or inducing suppressor phenotypes among Treg cells and $CD4^+$ T cells.

Flow cytometry results revealed a decrease, among $CD4^+$ T cell subpopulation, in the overall production of pro-inflammatory cytokines typically associated with Th1 cells, when PBMC were cultured in the presence of hAME. Under the same conditions, mRNA expression revealed a decrease in mRNA expression of the Th2-associated transcription factors GATA3 and STAT6, and in the mRNA expression of the Th2-related cytokine IL-4. Simultaneously, there was an increase in Foxp3 and IL-10 mRNA expression (Figure 16). Taken together, these results suggest that hAME skews Th cell polarization 'away' from the Th1 and Th2 phenotypes and towards induced regulatory phenotypes (which express Foxp3 and secrete anti-inflammatory cytokines) such as Tr1 or Th3.

The overall anti-inflammatory effect of hAME is also observed among $CD8^+$ and γδ T lymphocytes. These cell populations demonstrated a decrease in the mRNA expression of the cytolytic protein perforin 1, apoptosis-inducing protein granzime B and activating receptor NKG2D (Figure 16). These results point to a hAME-mediated reduction in T cell activation and cytotoxicity. Additionally, whithin γδ T cells, there was a reduction in the mRNA expression of eomesodermin (EOMES).

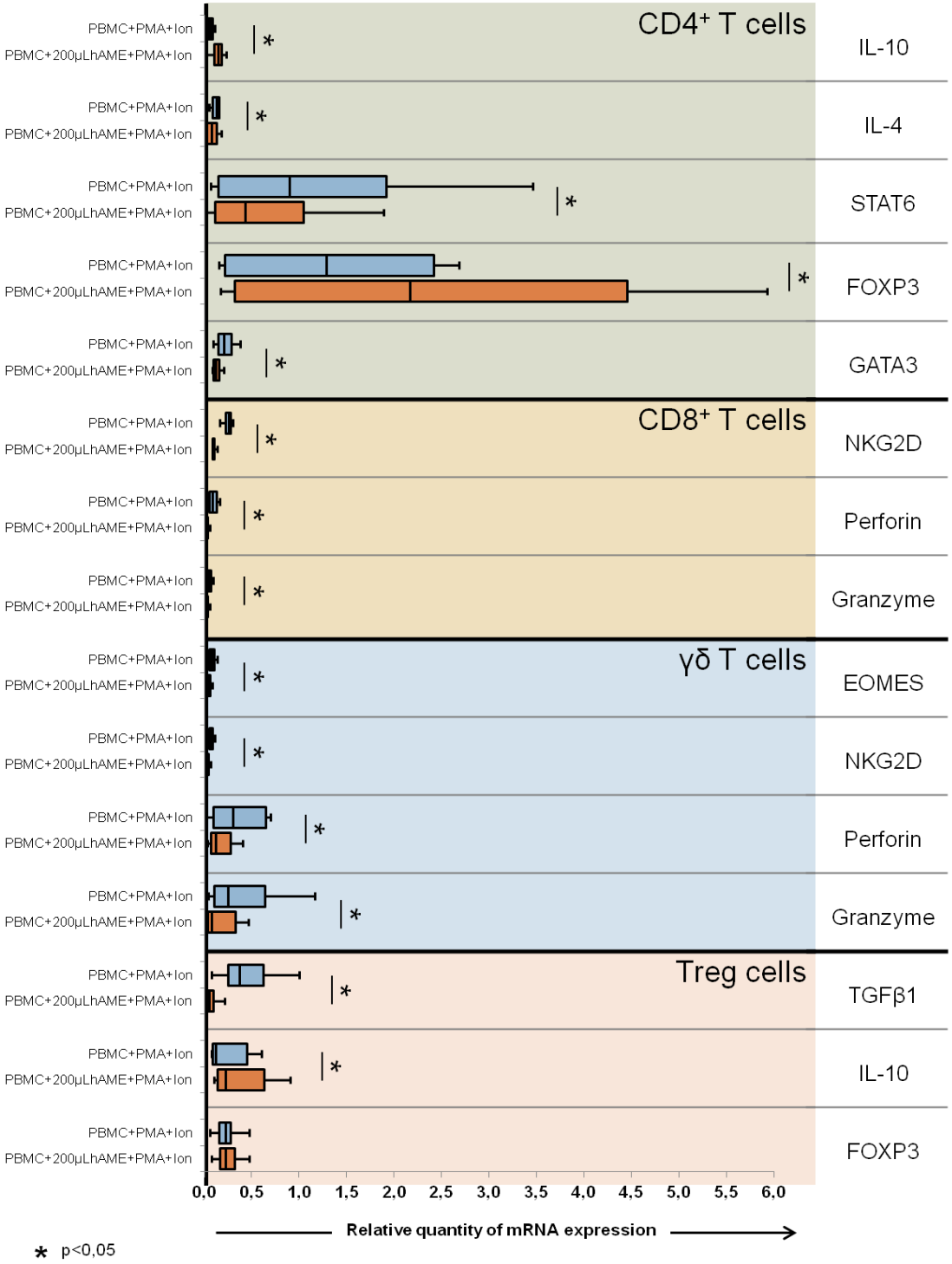

Figure 16. mRNA expression in purified $CD4^+$, $CD8^+$, γδ T and Treg cells. Semi-quantitative analysis of mRNA expression in FACS-purified T cells from two separate culture conditions: PBMC stimulated in the absence (PBMC+PMA+Ion; blue bars) or presence of hAME (PBMC+200µL hAME+PMA+Ion; orange bars). Results were obtained for five PBMC samples. Statistically significant differences were considered when $p<0.05$ for the Wilcoxon paired-sample test: * for the groups indicated in the figure.

Finally, among the purified Treg cell population, the presence of hAME in the culture medium resulted in augmented IL-10 mRNA expression and diminished TGFβ1 mRNA expression, even though both IL-10 and TGFβ are cytokines with by and large anti-inflammatory roles. The Foxp3 mRNA levels, however, remained unchanged despite the presence of hAME (Figure 16).

6. DISCUSSION

The inhibitory effect of hAM and hAM-derived MSCs over T cell proliferation and T cell-mediated immune responses has already been documented for T cells in general and, more recently, for different T helper effector cell populations (mostly Th1, Th2 and Th17), but data regarding the concrete effects over individual T cell subsets is very limited, as is information concerning the 'unconventional' T cell subpopulations, such as γδ T cells, and also Th cell subsets other than the more well known types 1, 2 and 17; for instance, Th9, Th3 and Tr1 cells. This data would be important for understanding, in greater detail, the effect of these cells on immune responses *in vivo*, given that they result from a composite network of different cell populations.

The objective of this study was, therefore, to evaluate the influence of a hAM extract over T cell-mediated responses, focusing on the particular effects over individual T cell subpopulations ($CD4^+$, $CD8^+$, Treg and γδ T cells) and different $CD4^+$ and $CD8^+$ T cell subsets (naïve, central memory, effector memory and effector) from the peripheral blood of healthy blood donors. In general, the hAME displayed an immunosuppressive effect over T cell proliferation, pro-inflammatory cytokine production and mRNA expression among the studied T cell populations.

Morphological and flow cytometry analyses showed that the hAM-derived cells, from which the hAME was obtained, included of two distinct cell populations; one, more abundant, morphologically similar to amniotic epithelial cells (98.2% ± 1.3%) and a minority population of cells, which resembled mesenchymal stromal cells (1.8% ± 1.3%). Both cell populations lacked expression for the endothelial marker CD31, the hematopoietic marker CD45 and for MHC class II molecule HLA-DR. hAM cells have been shown to express HLA-A, -B and –C surface antigens but not HLA-DR (*Hu et al. 2009*), while MSCs can express HLA-DR, under certain conditions, but are usually distinguished as being HLA-DR negative (*Dominici et al. 2006*).

The two distinct cell populations differed from one another in the expression levels for the panel of mesenchymal stromal cell markers; CD73, CD13, CD90 and CD44. The more abundant cell population was negative for all four aforementioned markers, and, thus, likely corresponds to epithelial amniotic cells. The other, scarcer population displayed an expression profile similar to that of MSCs, according to the International Society for

Cellular Therapy (*Dominici et al. 2006*), namely; positive staining for CD13, CD73 and CD90 and for the adhesion molecule CD44, along with the lack of CD45 expression. The relatively high expression of CD90 displayed by this cell population deserves particular attention since, in human MSCs; the $CD90^{high}$ phenotype is correlated with a stronger ability to suppress T cell proliferation, while low CD90 positivity correlates with loss of inhibitory ability (*Campioni et al. 2009*). The presence of this cell population within hAM has led other investigators to speculate whether the immunomodulatory activities of MSCs have an appreciable role in maternal-fetal immune tolerance, but the hypothesis is yet to be proven (*Magatti et al. 2008*).

Either one of the two cell populations (or possibly both), found in the hAM-derived cell samples might be the source of the immunossupressive factors present in the hAME, since both hAM epithelial cells and hAM-derived MSCs possess the ability to suppress T cell proliferation and activation.

By culturing human peripheral blood mononuclear cells in the presence of hAME the first observation was of an anti-proliferative effect over mitogen-activated cells, which seemed to be independent from the dose of hAME, a curious result considering that, in most other studies, the inhibitory effect of hAM-derived MSCs (and conditioned medium) (*Alikarami et al. 2015, Castro-Manrreza & Montesinos 2015, Insauti et al. 2014, Kang et al. 2012, Magatti et al. 2008, Prokop & Oh 2012, Yang et al. 2009,*) or hAM (and conditioned medium) (*Insausti et al. 2014, Liu et al. 2012, Magatti et al. 2008, McDonald et al. 2015, Miki 2011, Rossi et al. 2012*) is dose-dependent.

Inhibition of T lymphocyte proliferation mediated by hAM cells (*Magatti et al. 2008, McDonald et al. 2015, Ueta et al. 2002*), or conditioned medium from hAM cells (*Rossi et al. 2012*), has already been reported for cell proliferation induced by allogeneic target cells or through T-cell receptor engagement, but not for mitogen-induced T cell proliferation, except for concanavalin A-activated mouse splenocytes (*Liu et al. 2012*). This anti-proliferative effect is thought to involve prostaglandins as key effector molecules (*Rossi et al. 2012*). Moreover, amniotic epithelial cells produce soluble HLA-G (particularly HLA-G5) (*Kubo et al. 2001*) that can inhibit T cell proliferation through the suppression of cell cycle progression (*Bahri et al. 2006*).

hAM-derived MSCs, on the other hand, are known to be able to suppress $CD4^+$ and $CD8^+$ T cell proliferation induced by several stimuli, including alloantigens and mitogen

stimulation (*Lindenmair et al. 2012, Pianta et al. 2015*), likely through the release of a variety of mediators, particularly prostaglandin E2 (PGE2) and indoleamine 2,3-dioxygenase (IDO).

The differentiation of T lymphocytes into memory and effector T cells is a crucial event in adaptive immune responses, thus, the effect of hAME over the individual naïve, central memory, effector memory and effector T cell responses was characterized through the identification of the different T cell subsets and evaluation of the frequency of pro-inflammatory cytokine producing cells through flow cytometry. The results showed a relatively strong inhibition (between 30-60%) of the frequency of $CD4^+$ and $CD8^+$ T cells that produce pro-inflamatory cytokines (TNFα, IFNγ, IL-2, IL-17 and IL-9), following activation with PMA plus ionomycin. The extent of that inhibition differed not only between $CD4^+$ and $CD8^+$ T cells, and between different subsets, but also according to the cytokine under study. For instance, the relative susceptibility to inhibition of naïve T cells varied greatly depending on the cytokine in question; among $CD4^+TNF\alpha^+$ T cells, the naïve cells were less significantly affected than $CD4^+$ T_{CM} or T_{EM} cells ($CD4^+$ T_{CM} cells seemingly being more strongly inhibited than the rest), while the opposite was observed for naïve $CD4^+IFN\gamma^+$ T cells, which yielded greater percent inhibition than $CD4^+$ T_{CM} or T_{EM} cells. On the other hand, there was no difference in the effect over the frequency of $IL\text{-}2^+$ T cells between the four different subsets. Still, the results obtained for $TNF\alpha^+$ and $IFN\gamma^+$ point to a differential regulation of each individual T cell subpopulation. As a result, the specific immunomodulatory action of hAME over a given immune response will be dependent on the cellular context.

Overall, the T cell subset most susceptible to hAME-mediated immunosuppression seemed to be the T_{CM} cells, and the most susceptible types of cytokine producing cells were the $IL\text{-}2^+$ T cells, the only group where all subsets were strongly affected and in equal measure. This marked decrease in IL-2 expression among all T cell subsets may aid in the abovemenioned anti-proliferative effect of hAME, given the role of IL-2 in the induction of proliferation in resting T cell (*Liao et al. 2011*).

The other pro-inflammatory cytokines under study were IL-17 and IL-9 and, as before, the presence of hAME decreased the overall frequency of $CD4^+$ and $CD8^+$ T cells that produced IL-17 and IL-9. Additionally, the presence of hAME also reduced the overall frequency multifunctional $CD4^+$ and $CD8^+$ T cells; namely, T cells that simultaneously

produce TNFα together with IL-17, IL-2 with IL-17, IL-2 with IL-9, and also IL-17^+IFN$\gamma^+$$CD4^+$ T cells (the IL-17^+IFN$\gamma^+$$CD8^+$ T cells being unaffected). Once again, the extent of the inhibition varied according the cytokine under study; the highest percent inhibition was observed for the cells that produced IL-2 (IL-17^+IL-2^+ and IL-9^+IL-2^+ T cells) and the lowest for IL-17^+IFNγ^+ T cells.

Within the $CD4^+$ T cell population, the decrease in frequency of Th17-related (IL-17), Th9-related (IL-9), and Th1-related (TNFα, IFNγ and IL-2) cytokine producing cells points to an inhibition of Th17, Th9 and Th1 subsets. Also within this population there was a decrease in the mRNA expression of the Th-2-associated markers GATA-3, STAT6 and IL-4, suggesting a downregulation of the Th2 subset, and an increase in Foxp3 and IL-10 mRNA expression, indicating an induction of regulatory T cells. Although, while hAME seems to directly influence Th2 polarization in a negative manner, there may be an indirect skewing towards type 2 Th cells due to the reduction of IFNγ and increased IL-10 (*Murphy et al. 2013*).

The general effect of hAME over the $CD4^+$ T cell population seems to be the induction of regulatory phenotypes, such as Tr1 (characterized by high IL-10 secretion), at the cost of the inflammatory effector Th subsets.

The hAM has been reported by previous studies to suppress the production type 1 and type 2 cytokines by T cells, in contact-independent manner; *Ueta et al*. (*2002*) observed the inhibition of alloreactive synthesis of IL-2, IFNγ and IL-6 when murine mixed lymphocyte reactions were separated from hAM in by a permeable membrane, however, the group failed to detect the known inhibitory hAM-derived cytokines IL-4, IL-10 and TGFβ in the culture supernatant. The therapeutic benefit of this hAM-mediated reduction in cytokine production was demonstrated by *Bauer* and collegues' study (*2009*), wherein it was reported a decrease in IFNγ and IL-2 production following culture of lymphocytes, from herpes simplex virus (HSV)-1–infected mice, in the presence of hAM. In the same study, treatment of murine corneas with herpetic stromal keratitis with hAM transplantation also resulted in a decrease in IL-2 production (*Bauer et al.2009*).

However, the two aforementioned studies reported a decrease in the production of IL-10, while, in this case, the presence of hAME in the cultures of PBMC upregulated IL-10 mRNA expression among Treg and $CD4^+$ T cells. Similarly, *MacDonald*'s group (*2015*) observed a significant reduction of IFNγ and TNFα, as well as IL-10, by activated T cells

in the presence of hAM epithelial cells, but the group also reported that there was no change in the production of IL-4 or IL-17A *in vitro*, although, *in vivo*, the administration these hAM epithelial cells in an experimental autoimmune encephalomyelitis mouse model decreased IL-17A and increased IL-4 secretion, and resulted in polarization towards a regulatory phenotype, leading to increased numbers of $CD4^+CD25^+Foxp3^+$ Treg cells. This is in agreement with the observed increase in Foxp3 mRNA expression among FACS purified $CD4^+$ T cells. Likewise, it has been shown that treatment of rheumatoid arthritis patients and mice with collagen-induced arthritis with hAM-isolated cells leads to the induction of $CD4^+CD25^+Foxp3^+$ Treg cells, with the ability to suppress T cell responses, along with an increased IL-10 production and reduction of Th1 (TNFα, IFNγ and IL-2) and Th17 (IL-17) cytokines (*Parolini et al. 2014*). These last effects were reported to be due to the production of soluble mediators derived from cyclooxygenase (COX)-1/2 activation (*Parolini et al. 2014*). In support of this hypothesis, prostaglandins have been shown to be crucial for the anti-proliferative effects of conditioned medium from hAM cells (*Rossi et al. 2012*).

He et al. (*2008*) have demonstrated that hAM extract retains the anti-inflammatory properties of hAM and went on to isolate a complex formed between pentraxin 3 and high molecular weight hyaluronic acid (HC-HA/PTX3) that was capable of inhibiting T cell proliferation, inducing $Foxp3^+$ T cells from $CD4^+$ T lymphocytes and suppressing $CD4^+$ T cell polarization into Th1 cells, *in vivo* (*He et al. 2014*). Another study, by *Tan et al.* (*2015*), suggested that hAM epithelial cells-mediated promotion of Treg cell expansion, and differentiation of $Fox3^-$ T lymphocytes into $Foxp3^+$ Treg cells, required TGFβ, and the group successfully prevented the promotion of Foxp3 transcripion in naïve $CD4^+$ T cells by adding anti-TGFβ1 blocking antibodies. The involvement of TGFβ was also reported in a study by *Liu et al.* (*2012*), wherein hAM epithelial cells suppressed splenocyte proliferation, induced by non-specific and antigen-specific stimulation, in a TGFβ and PGE2 dependent manner, but seemingly independent of HGF, NO or IL-10, which were not present in the hAM epithelial cells conditioned medium.

hAM-derived MSCs, and their conditioned medium, have also been shown to, like hAM cells, downregulate the expression of Th1 and Th17-related markers, decrease the production of the pro-inflammatory cytokines TNFα, IFNγ, IL-1β (Th1), IL-5, IL-6 (Th2), IL-9 (Th9), IL-17A and IL-22 (Th17-related), and induce Treg cells among $CD4^+$ T cells

(*Pianta et al. 2015*). In a a study by *Kang et al.* (*2012*), hAM-derived MSCs were able to suppress mitogen-activated T cell proliferation, in a dose dependent manner, and also diminished IL-17 and IFNγ production while increasing IL-10 production by total T lymphocytes. The group demonstrated that these effects were likely linked to IDO, PGE2, TGFβ and HGF production by the hAM-derived MSCs.

An anti-inflammatory effect was also observed among the purified $CD8^+$ and γδ T cells. In these cell populations, there was a significant decrease in the mRNA expression for the cytolytic protein perforin 1, the apoptosis-inducing protein granzyme B, and of the activating receptor NKG2D, which suggests that the hAME can suppress activation and cytotoxic functions of $CD8^+$ and γδ T cells. Among γδ T cells, there was also a reduction in the mRNA expression of eomesodermin (EOMES), a key transcription factor of cytotoxic lymphocyte lineages which contributes to IFNγ production in γδ T cells (*Chen et al. 2007*).

As mentioned, the hAM, especially hAM epithelial cell, expresses soluble HLA-G (*Kubo et al. 2001*), and produce prostaglandins, including PGE2 (*Ueta et al. 2002, Rossi et al. 2012*). Likewise, human MSCs are known to also produce both HLA-G5 (*Ryan et al. 2005, Selmani et al. 2008*) and PGE2 (*Dhingra et al. 2013, Rasmusson et al. 2005, Zhang et al. 2013*), which are thought to contribute for the promotion of Treg cells generation, suppression of inflammatory Th phenotypes and inhibition of cytotoxic cells, including the inhibition $CD8^+$ T lymphocytes activation and suppression of effector $CD8^+$ T cell responses (*Bahri et al. 2006*). Furthermore, *Li et al.* (*2014*) reported MSC-mediated reduction of cytokine production and NKG2D downregulation in $CD8^+$ T cells, resulting in $CD8^+$ T cells, with diminished NKG2D expression, with impaired proliferation after mitogen stimulation. The group managed to partially restore T cell proliferation with a combination of PGE2, IDO and TGFβ inhibitors (*Li et al. 2014*).

Within sorted Treg cells, hAME did not seem to influence the expression of Foxp3 mRNA; however, the augmented expression of IL-10 mRNA suggests that the presence of hAME stimulated immune regulation by Treg cells.

MSC-mediated immunosuppression is known to involve different mechanisms, one of which being the generation functional Treg cells, both through direct cell contact and through the release of soluble factors (*Aggarwal & Pittenger 2005, Luz-Crawford et al. 2013, Pianta et al. 2015, Ren et al. 2008, Tasso et al. 2012*), but, whether these suppressor T cells come from an increased proliferation of natural Treg and/or from the the induction

of Tregs from Th0, Th1 or Th17 cell subsets is still up to debate. In a study by *Luz-Crawford et al.* (*2013*), it was demonstrated that the $CD4^+CD25^+Foxp3^+$ T cells obtained when human PBMC where co-cultured with MSCs had a relatively low expression of neuropilin-1 and protein helios (two markers that allow for the distinction between nTreg and iTreg cells); a phenotype typical for iTreg cells. The group also verified that, along with the Treg generation, IL-10 levels were augmented in the supernatant of the co-culture. The identity of the immunosuppressive factors present in hAME is unknown, but the aforementioned study, *Luz-Crawford* and colleagues (*2013*) identified MSC-derived IL-10, PGE2 and TGFβ1 as important players in the Treg conversion.

hAM epithelial cells share with MSCs this ability to generate functional Treg cells since, as mentioned, hAM-derived cells can increase Treg numbers partially through the promotion of Treg expansion and partially through the Treg induction from $Foxp3^-$ $CD4^+$ T cells (*MacDonald's et al. 2015, Parolini et al. 2014, Tan et al. 2015*).

In line with the abovementioned observations; $CD4^+$ T cells co-cultured with hAME showed an increase in Foxp3 and IL-10 expression at the mRNA level, which may be indicative of the generation of induced $CD4^+Foxp3^+$ T cells. In addition, while the levels of IL-10 mRNA expression where increased by the presence of hAME within the purified natural Treg cells, Foxp3 expression seemed to be unaffected, suggesting that the hAME increases the regulatory T cell phenotypes by inducing iTreg cells, and not nTreg cells, though it was capable of stimulating IL-10 expression by pre-existent nTreg cells.

Interestingly, in parallel with increased IL-10 expression, there was a marked decrease in the mRNA expression of TGFβ1. While TGFβ1 is generally considered an anti-inflammatory, immunosuppressive cytokine, it also plays a major role in fibrotic response by inducing fibroblasts to synthesize extracellular matrix, and thus is a potential target for anti-scarring therapies. In fact, the anti-fibrotic activity of hAM in has been reported to involve suppression of TGFβ expression (*Guo et al. 2011, Lee et al. 2000, Tseng et al. 1999*). Likewise, while both T lymphocytes and MSCs produce TGFβ, co-cultures of the two have been shown to result in a suppression of TGFβ by the lymphocytes (*Zhou et al. 2011*). Furthermore, Xu and colleagues (*2013*) demonstrated that adding TGFβ1 to the culture medium can directly affect MSCs, reversing their anti-inflammatory effects, instead of acting in synergy with MSCs in the suppression of immune responses, as the group had initially expected. On the other hand, *Pianta et al*. (*2015*) showed that co-culture of

alloreactive T lymphocytes with hAM-derived MSC condioned medium (instead of MSC cells) resulted in an induction of Treg cells and increased TGFβ production. However, said increase only ocurred following three or more days of co-culture and the authors observed no change in the TGFβ levels for shorter periods (*Pianta et al. 2015*).

Co-culture of PBMC with the isolated hAM-derived cells did not result in the same inhibitiory effects observed for the extract. In fact, only IL-2 producing cells were significantly affected by the presence of hAM-derived cells, and, even then, statistical significance was only observed among the T_{CM} subset, and only for the lowest ratio of PBMC to hAM-derived cells (2:1). The results obtained for hAME had shown that IL-2^+ T cells were more strongly inhibited than TNFα^+ or IFNγ^+ T cells, and T_{CM} cells were also very susceptible to inhibition, which could explain why only these cells were affected by the presence of hAM-derived cells.

Even though the doses of hAM added to the cell cultures were relatively low in comparison to the number of PBMC (PBMC-to-hAM cell ratios of 10:1 and 2:1) it was still expected inhibition of the frequency of pro-inflammatory cytokines producing cells. *Liu et al.* (*2012*) achieved a dose-dependent inhibition of mitogen-induced lymphocyte proliferation with ratios of 1:5 to 1:10240 of hAM epithelial cells-to-mouse splenocytes, and, in a study by *McDonald* and colleagues (*2015*); hAM epithelial cells suppressed the proliferation of T cells, activated with anti-CD3/CD28, at ratio of 25:1 (human PBMC-to-hAM epithelial cells).

The lack of inhibitory effect over the frequency of pro-inflammatory cytokine producing T lymphocytes observed for hAM-derived cells could be due to the low percentage of the MSCs population within the hAM-derived cell samples (1.8% ± 1.3%) and, consequently, to a PBMC-to-hAM-derived MSCs ratios of, approximately, 500:1 and 100:1. The hAME, on the other hand, consists of an enriched mixture of the factors from hAM-derived MSCs (and hAM epithelial cells), thus possibly 'bypassing' the need for higher MSCs content. To support this hypothesis; in most studies, MSC-mediated immunosuppressive effects only become evident at higher MSC concentrations: in a study by *Yang et al.* (*2009*), human MSCs were only able to inhibit T cell proliferation at a ratio of 20:1, while the 200:1 and 2000:1 ratios were ineffective. *Kang* and colleagues (*2012*) successfully abrogated the proliferation of mitogen-activated T cells by co-culturing PBMC and hAM-derived MSCs in a ratio of 2:1 and 10:1, but failed to do so with a 100:1 ratio. Likewise, *Le Blanc et al.*

(*2003*) observed a decrease in T cell proliferation, in a mixed lymphocyte reaction, when MSCs were added to the culture medium at a 10:1 ratio (human peripheral blood lymphocytes-to-MSCs), but not 100:1. Interestingly, in the same study, allogeneic MSCs could also suppress mitogen-induced proliferation (PHA) at a 10:1 ratio, and not 1000:1, but the study described a slight decrease in proliferation at the lowest MSC concentration when autologous MSCs were used instead, the group concluded that the mechanism of inhibition may differ, at least a bit, between the two (*Le Blanc et al. 2003*).

Furthermore, *Ribeiro et al.* (*2013*) demonstrated that MSCs from umbilical cord matrix, adipose tissue and bone marrow suppressed T cell activation, following mitogenic stimulus, at a ratio of 10:1 (PBMC-to-MSC), however the strenght of this inhibition varied between the three types of MSCs. The group also demonstrated that MSCs from different sources exerted their inhibitory effect at different stages of T cell activation, thus the suppressive mechanism may also vary according to the tissue from which the MSCs are isolated (*Ribeiro et al. 2013*).

There are many discrepancies from study to study regarding the dosage, efficiency and mechanism of MSC-mediated suppression of T cell responses, not only because MSC populations differ slightly depending on the species and tissue of origin, but also because different cell population and immune cells from different species are differentially modulated, and because the behavior of MSCs is environmentally responsive and, thus, is substantially affected by the cell isolation method, specific culture conditions and timing (*Rasmusson et al. 2005*).

7. GENERAL CONCLUSIONS AND FUTURE PRESPECTIVES

T cells are pivotal players in immunity, both by producing specific immune reactions to combat infection and disease, and by directing and regulating immune responses. Because of this, T lymphocytes have a crucial role in inflammatory diseases and autoimmunity. In all these processes, the differentiation of T cells into effector and memory cells and the polarization of T cells onto the different subsets are crucial, given that the overall T cell response depends on the balance between the different T cell subpopulations, each with different functions and different cytokine profiles.

The objective of this study was to characterize the effect of hAME over T-cell responses of naïve, central memory, effector memory and effector $CD4^+$ and $CD8^+$ T cells. The observed effects suggest that the hAME contains bioactive molecules with immunosuppressive effects. The presence of hAME in culture suppressed mitogen-activated cell proliferation and reduced the frequency of pro-inflammatory cytokine producing $CD4^+$ and $CD8^+$ T cells, but to a different extent depending on the subset and cytokine under study. Overall the T_{CM} subset seems to be the most susceptible to inhibition among both $CD4^+$ and $CD8^+$ T cells. Additionally; IL-2^+ T cells appear to be more readily inhibited by hAME than TNFα^+ or IFNγ^+ T cells.

The apparent decrease in the frequency of $CD4^+$ T cells that produce cytokines type 1 (TNFα, IFNγ and IL-2), Th17-related (IL-17) and Th9-related (IL-9), along with the reduction in IL-4 (a type 2 cytokine), GATA3 and STAT6 (Th2-associated transcription factors) mRNA expression and upregulation of Foxp3 and IL-10, suggests that hAME causes a skewing of $CD4^+$ T cell polarization towards regulatory phenotypes, along with the downregulation of the other T helper subsets.

Furthermore, the frequency of pro-inflammatory cytokine producing $CD8^+$ T cells was equally reduced. The hAME also appears hamper the activation and reduce the cytolytic activities of $CD8^+$ and γδ T cells, since it caused a decrease in the mRNA expression of the cytolytic protein perforin 1, the apoptosis-inducing protein granzime B and the activating receptor NKG2D among these cell populations. It may also, possibly hamper IFNγ production by γδ T cells, through the downregulation of eomesodermin.

Finally, the increased Foxp3 expression in purified $CD4^+$ T cells, but not in purified Treg cells, indicates that hAME promotes the generation of iTreg cells. Thus, hAME may

mediate immunosuppression by expanding and sustaining a functional peripheral Treg cell pool through iTreg cell conversion, along with the induction of IL-10 production (in both iTreg and nTreg cells).

Collectively, these results suggest that hAME could be successfully applied in the reduction of inflammatory processes, perhaps providing a convenient cell-free therapy for the treatment of immune disorders. However, hAME differentially regulates different $CD4^+$ and $CD8^+$ T cell subsets, thus, additional controlled studies are needed to determine the impact of this differential regulation over the therapeutic effect of hAME and to confirm effectiveness and safety.

Finally, because the observed anti-inflammatory properties of hAME are not associated with any external stimuli prior to hAME preparation, these properties are intrinsic to the hAM, which, in turn, constitutes an attractive source of immunosuppressive factors without necessity of complex cell preparation procedures. Future works are required to identify the immune modulatory molecules present in hAME.

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