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ISOLATION AND CHARACTERIZATION OF AMNION-DERIVED INHIBITORY FACTORS ON LYMPHOCYTE PROLIFERATION

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ABSTRACT

Background

Cells derived from the amniotic membranes of human term placenta are receiving particular attention because of their characteristics of stemness, multipotency and their immunologycal features, supporting a variety of possible clinical applications in the field of cell transplantation and regenerative medicine. We have previously demonstrated that cells isolated from the mesenchymal region of the human amniotic membrane (human amniotic mesenchymal tissue cells, hAMTC) possess immunoregulatory properties, such as the ability to inhibit lymphocyte proliferation and cytokine production and to suppress generation and maturation of monocyte-derived dendritic cells, as also reported for MSC from other sources. All of the results described above were obtained when hAMTC were cultured either in direct cell contact or in a transwell system, thereby suggesting the involvement of soluble inhibitory factor(s) secreted by these cells. However, the precise factors and mechanisms responsible for the immunoregulatory roles of hAMTC remain unknown.

Aims

Therefore, in this study, we aimed to identify the soluble factors released by hAMTC which are responsible for the anti-proliferative effects of these cells on lymphocytes, and also to gain insight into the mechanisms underlying their actions through in vitro studies.

Materials and Methods

Conditioned medium (CM) was prepared by culture of isolated hAMTC (CM-hAMTC) and fragments of the whole human amniotic membrane (CM-hAM) for 5 days in UltraCulture medium. Chemical and physical properties, such as the thermostability, chemical nature and molecular weight, of the factors associated with the anti-proliferative effects of hAMTC were evaluated. Blocking of specific synthetic pathways involved in the production of immunomodulatory factors, such as nitric oxide, kynurenine and prostaglandins, was achieved by adding specific inhibitors during CM production. Finally, the involvement of certain cytokines in the antiproliferative effects of the CM was evaluated by adding neutralizing antibodies to the CM during lymphocyte proliferation tests.

Results

In this study, we have demonstrated that:

- *the inhibitory factors are temperature-stable, have a small molecular*

weight, and are likely to be non-proteinaceous;

- *only inhibition of the cyclooxygenase pathway partially reversed the anti-proliferative effects of hAMTC, suggesting that prostaglandins may be key effector molecules in this phenomenon;*
- *factors which have been previously documented to play a role in the inhibitory effects of MSCs from other sources (HGF, TGF-β, NO and IDO) were not shown to be involved;*
- *the anti-proliferative effects of the amniotic membrane are intrinsic to this tissue and its derived cells, since these effects are manifested in the absence of stimulating culture conditions, as opposed to MSC derived from the bone marrow, which possess anti-proliferative ability only when cultured in the presence of activating stimuli.*

Conclusions

We have demonstrated that both isolated cells or the amniotic membrane in toto are capable of releasing factors that present with immunomodulatory capacity, thereby providing new insights toward the identification of some of these factors and toward the characterization of other factors which remain to be identified.

SOMMARIO

Introduzione

Le cellule derivate dalle membrane amniotiche della placenta umana a termine stanno ricevendo particolare attenzione per le loro caratteristiche di staminalità, multipotenza e per le loro proprietà immunologiche, suggerendo una varietà di possibili applicazioni cliniche nel campo del trapianto di cellule e della medicina rigenerativa. Nel nostro laboratorio abbiamo precedentemente dimostrato che le cellule isolate dalla regione mesenchimale della membrana amniotica umana (hAMTC per amniotic mesenchymal tissue cells) possiedono, così come riportato anche per MSC isolate da altre fonti, la capacità di inibire la proliferazione dei linfociti e la produzione di citochine da parte degli stessi e di sopprimere la generazione e la maturazione di cellule dendritiche derivate da monociti. Questi risultati sono stati ottenuti sia quando le hAMTC sono state utilizzate in un sistema di co-cultura diretta, sia in un sistema di transwell, suggerendo il coinvolgimento di fattori solubili secreti da queste cellule. Tuttavia, i fattori coinvolti e i meccanismi responsabili di questa azione immunoregolatoria da parte delle hAMTC rimangono tuttora non chiari.

Scopo

In questo studio, attraverso analisi in vitro, abbiamo cercato di identificare i fattori solubili rilasciati da hAMTC e responsabili degli effetti antiproliferativi di queste cellule, oltre che i possibili meccanismi alla base della loro azione.

Materiale e metodi

Il terreno condizionato (CM) è stato recuperato dopo 5 giorni di coltura, in terreno UltraCulture, di hAMTC (CM-hAMTC) e frammenti di membrana amniotica umana (CM-hAM). Abbiamo valutato le proprietà chimico-fisiche, analizzando la termostabilità, la natura chimica e il peso molecolare, dei fattori responsabili degli effetti antiproliferativi. Inoltre, abbiamo valutato il ruolo di molecole note per essere coinvolte nelle azioni immunomodulatorie delle MSC isolate da altre fonti, come l'ossido nitrico, la chinurenina e le prostaglandine, bloccando specificamente le loro vie biosintetiche durante la produzione dei CM. Infine abbiamo valutato il coinvolgimento di selezionate citochine nell'effetto antiproliferativo dei CM, utilizzando anticorpi neutralizzanti specifici durante il test di prliferazione linfocitaria.

Risultati

Abbiamo quindi dimostrato che:

- *i fattori inibitori sono stabili a cambiamenti di temperatura, hanno un basso peso molecolare e sono probabilmente di natura non proteica;*
- *l'inibizione dell'enzima cicloossigenasi induce una parziale riduzione dell'effetto antiproliferativo delle hAMTC, suggerendo che le prostaglandine possano essere importanti molecole effettrici di questo fenomeno;*
- *fattori precedentemente documentati aventi un ruolo negli effetti inibitori di MSC isolate da altre fonti (come HGF, TGF-β, NO e IDO) non sono coinvolti nell'azione delle hAMTC;*
- *gli effetti antiproliferativi della membrana amniotica e delle cellule derivate da essa sono intrinseche in questo tessuto, poiché questi effetti si manifestano in assenza di condizioni di coltura stimolanti, al contrario di MSC derivate dal midollo osseo che possiedono la capacità anti-proliferativa solo quando coltivate in presenza di stimoli attivatori.*

Conclusioni

Abbiamo dimostrato che sia le cellule isolate che la membrana in toto sono in grado di rilasciare fattori solubili con capacità immunomodulatoria. Inoltre abbiamo fornito una parziale, seppur importante, identificazione di alcuni di questi fattori e qualche indicazione sulle caratteristiche delle sostanze ancora sconosciute.

TABLE OF CONTENTS

LIST OF ABBREVIATION

αCD3 or **anti-CD3**, monoclonal Antibody anti-CD3;
α-TCP-PUF; polyuretan foam covered with α Tri-Calcium-Phosphate
BM-MSC, human bone marrow mesenchymal stromal/stem cells;
CM-BM-MSC, conditioned medium generated from the culture of bone marrow-mesenchymal stromal/stem cells;
CM-hAM, conditioned medium from the culture of hAM;
CM-hAMTC, conditioned medium from the culture of hAMTC;
CM-MLR-hAMTC, conditioned medium from mixed lymphocyte reactions performed in the presence of hAMTC;
COX, cyclooxygenase;
FBS, fetal bovine serum;
G-CSF, granulocyte colony-stimulating factor;
GM-CFS, granulocyte-macrophage colony-stimulating factor;
hAEC, human amniotic epithelial cells;
hAM, human amniotic membrane;
hAMSC, human amniotic mesenchymal stromal cells;
hAMTC, human amniotic mesenchymal tissue cells;
hCMTC, huamn chorionic mesenchymal stromal cells;
hCTC, human chorion trophoblastic cells;
HGF, hepatocyte growth factor;
IDO, indoleamine 2,3-dioxygenase;
IL, interleukin;
IP-10, interferon-inducible protein-10;
L-NAME, L-Nitro-Arginine Methyl Ester;
MIP-1α and **β**, monocyte chemoattractant protein-1α and β;
MLR, Mixed Leukocyte Reaction;
MT, DL-Methyl Tryptophan;
NO, nitric oxide;
OSM, oncostatin M;
PBMC, human peripheral blood mononuclear cells;
PG, prostaglandins;
PGE2, prostaglandin E2;
PGD2, prostaglandin D2;
PGF2α, prostaglandin F2α;
PUF, polyuretan foam;
RANTES, regulated upon activation, normal T-cell expressed and secreted;
TGF−β, tumor growth factor-β;
TNF-α, tumor necrosis factor-α.

1. INTRODUCTION

1.1 The placenta

1.1.1 Structure of human term placenta

The placenta is the organ that plays an essential role in development of the growing embryo by facilitating gas and nutrient exchange between the mother and fetus. The placental sac is expelled in the final phase of labour, following its detachment from the uterine wall which is provoked by the quick reduction in uterine volume after the emerging of the neonate.

The human term placenta is round or oval in shape with a diameter of 15-20 cm and a thickness of 2-3 cm. Within the placental tissue, it is possible to distinguish the trophoblast and the fetal membranes *(**Fig. 1.1**)*. The fetal membranes (amnion and chorion) extend from the margins of the trophoblast and enclose the fetus in the amniotic cavity. The umbilical cord insert into the fetal face of the placenta, and from this the fetal blood vessels originate *(**Fig. 1.1**)*.

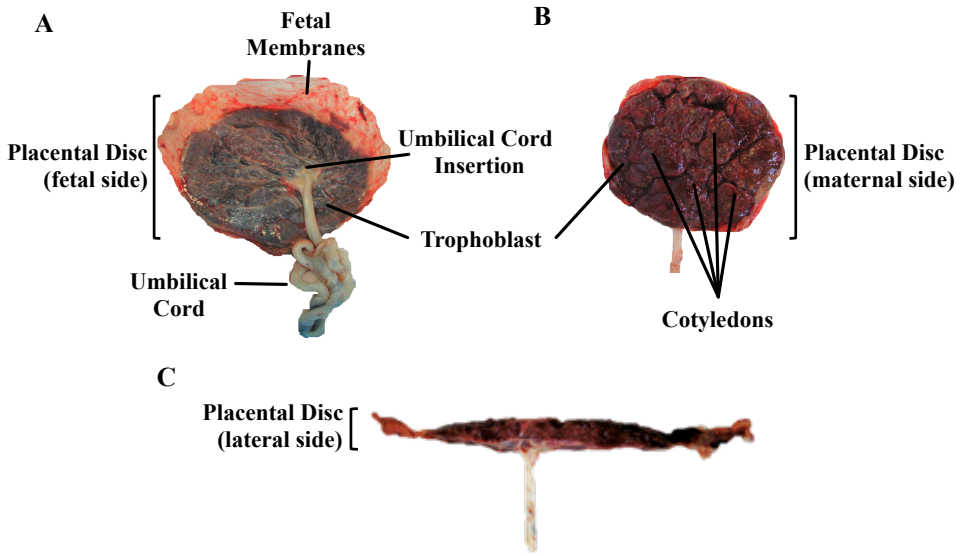


***Figure 1.1. Human term placenta. (A)** Internal surface (in contact with the fetus). The trophoblast becomes thinner toward the edge, continuing to the fetal membranes (amnion and chorion). The umbilical cord inserts into the fetal side of*

the trophoblast, and contains fetal blood vessels. ***(B)*** *External surface (in contact with the maternal uterine wall). The trophoblast becomes subdivided into irregular lobed structures, termed cotyledons.* ***(C)*** *Lateral view of placenta.*

The placenta is a fetomaternal organ, with the maternal portion being composed of the decidua which is derived from the maternal endometrium; while the fetal portion is composed of the placental disc and the amniotic and chorionic membranes.

The placental disc *(**Fig. 1.2**)* is composed of the chorionic plate and the basal plate, which form a cover and bottom, respectively, to surround the intervillous space. The chorionic plate is a multilayered structure that faces the amniotic cavity, and consists of two different structures, namely, the amniotic membrane and the chorion. From the chorionic plate originate the villi that anchor the placenta through the trophoblast of the basal plate to the endometrium. From the maternal side, protrusions of the basal plate within the chorionic villi produce the placental septa, which divide the parenchyma into irregular cotyledons *(**Fig. 1.2**)*.

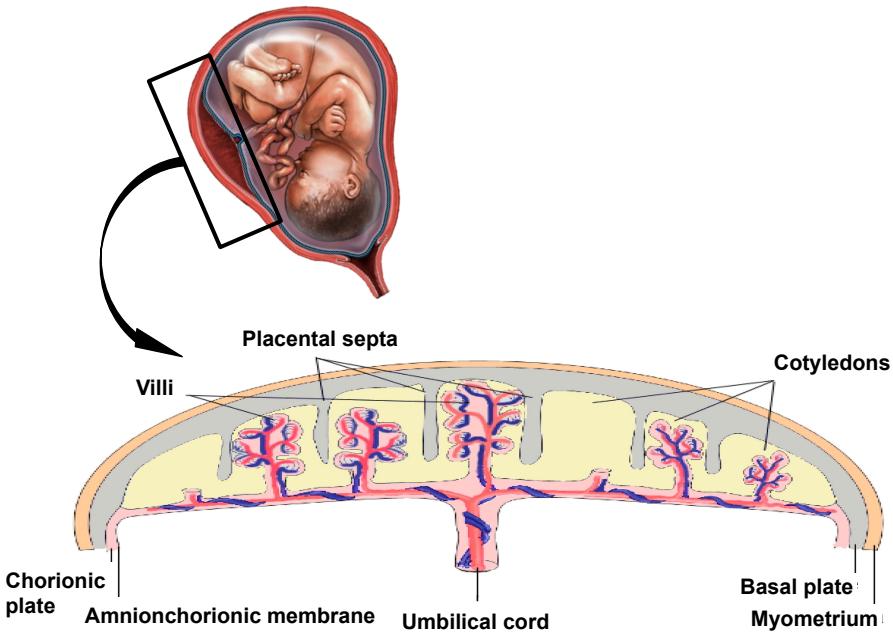


Figure 1.2. Schematic section of human term placenta.

Some villi anchor the placenta to the basal plate, whereas other terminate freely in the intervillous space where maternal blood flows. Inside the villi there are fetal vessels that are connected to the circulatory system via the chorionic plate and the umbilical cord. Fetal blood is carried to the villi via the branches of the umbilical arteries. After circulating through the capillaries of the villi, the fetal blood absorbs oxygen and nutritional materials from, and transfers waste products to the maternal blood through the villous walls. The purified and nourished fetal blood is then carried back to the fetus via the umbilical vein.

At the placental margin, the intervillous space is obliterated so that the chorionic plate and the basal plate fuse with each other forming the chorionic membrane, which together with the amniotic membrane, encloses the fetus in the amniotic cavity.

The amniotic membrane *(**Fig. 1.3**)* or amnion is a thin, avascular membrane composed of an uninterrupted single layer of cuboidal and columnar epithelial cells (termed the amniotic epithelium) which is bathed in amniotic fluid and is contiguous over the umbilical cord with the fetal skin.

The amniotic epithelium is attached to a distinct basement membrane that is, in turn, connected to the amniotic mesoderm, an acellular compact layer composed of collagens I, III and fibronectin. Deeper in the amniotic mesoderm, a network of dispersed fibroblast-like mesenchymal cells and rare macrophages are observed. Next to the amniotic membrane lies the chorionic membrane *(**Fig. 1.3**)*, consisting of the chorionic mesoderm and the chorionic trophoblast. A spongy layer of loosely arranged collagen fibers separates the amniotic and chorionic mesoderm, both of which are similar in composition. The chorionic trophoblast, which lies on the top of the mesodermal layer, is a highly variable layer of extravillous trophoblast cells. Toward the endometrium, the trophoblast interdigitates extensively with the decidua [1,2].

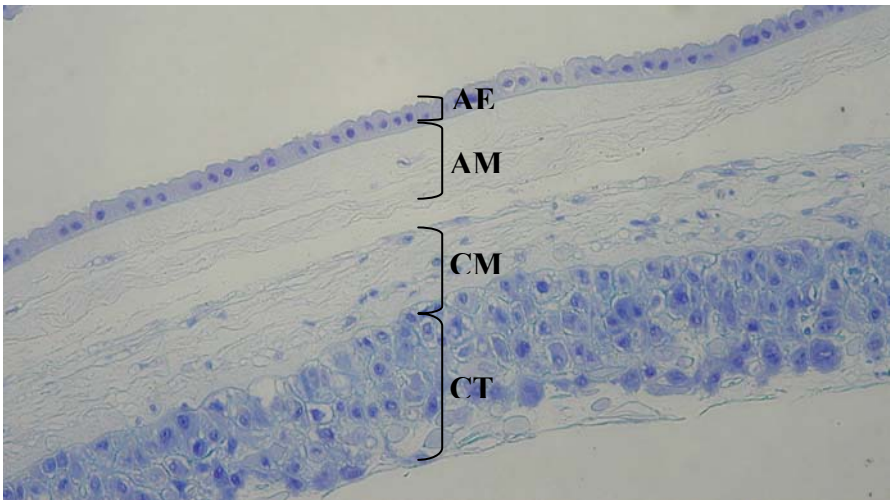


***Figure 1.3. Cross-sectional representation of human fetal membranes (amnion and chorion)**. The amnion is composed of a monolayer of epithelial cells, which rest on a mesodermal layer. The chorionic membrane consists of a mesodermal layer and a layer of extravillous trophoblast cells. Abbreviations: AE, amniotic epithelium; AM, amniotic mesoderm; CM, chorionic mesoderm; CT, chorionic trophoblast. (Giemsa staining)*

1.1.2 Embryological development of the placenta

Development of the placenta begins as soon as the blastocyst implants in the maternal endometrium (6-7 days after fertilization). At this stage, the blastocyst is a flattened vesicle in which most of the cells form an outer wall (trophoblast) which surrounds the blastocystic cavity (blastocoel). A small group of larger cells, known as the inner cell mass, is apposed to the inner surface of the trophoblastic vesicle *(**Fig. 1.4**)*.

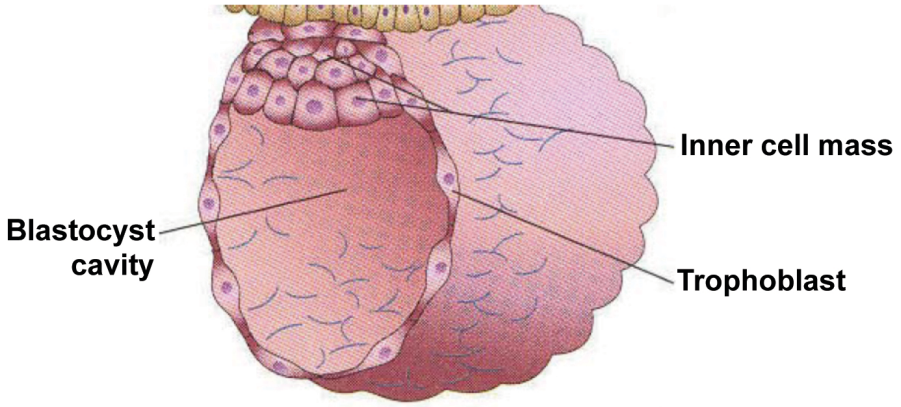


Figure 1.4. Representation of the blastocyst at six days after fertilization. *(Adapted from* [3]*).*

The trophoblast gives rise to the chorion, whereas the fetus, the umbilical cord and the amnion are derived from the inner cell mass.

As the blastocyst adheres to the endometrial epithelium, the invading trophoblast erodes the deciduas, allowing the embedding of the blastocyst. During implantation, the trophoblastic cells of the implanting pole of the blastocyst show increased proliferation, resulting in a bi-layered trophoblast made up of a multinucleated outer syncytiotrophoblast which originates from fusion of neighboring trophoblast cells, and an inner, mononucleated cytotrophoblast layer *(**Fig. 1.5**)*.

The proliferating cytotrophoblast and the syncytiotrophoblast give rise to a system of trabeculae intermingled with hematic lacunae. From these trabeculae are generated the primordial villi.

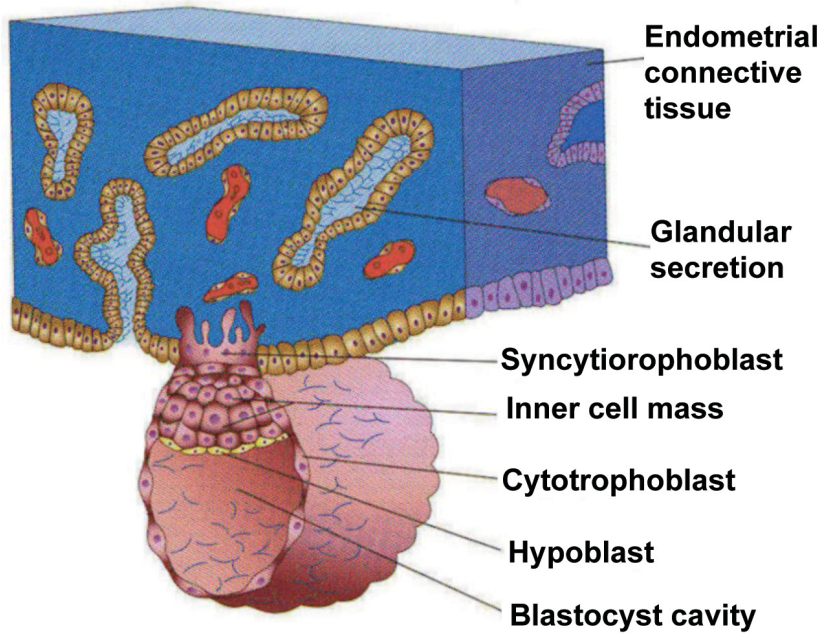


Figure 1.5. Attachment of the blastocyst to the endometrial epithelium. *(Adapted from* [3]*).*

At day 8-9 after fertilization, morphological changes occur in the inner cell mass, which differentiates into two layers termed the epiblast and the hypoblast, that together form the bilaminar embryonic disc ***(Fig. 1.6)***. From the epiblast, some small cells appear between the trophoblast and the embryonic disc and enclose a space that will become the amniotic cavity while the cells themselves will eventually constitute the amniotic epithelium. The three germ layers of the embryo (endoderm, mesoderm, ectoderm) will also originate from the epiblast.

On the opposite side, between the hypoblast and the cytotrophoblast, the exocoelomic membrane and the remainder of the blastocystic cavity modify to form the yolk sac. The extraembryonic mesoderm arranges into a connective tissue that surrounds the yolk sac and the amniotic cavity, giving rise to the mesoderm of both the amnion and the chorion.

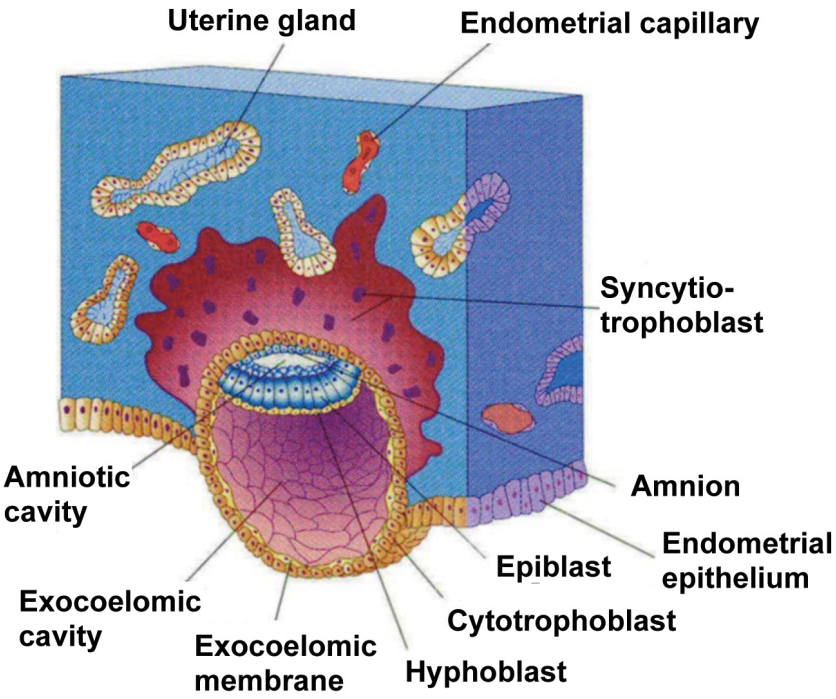


Figure 1.6. Formation of the bilaminar embryonic disc. *(Adapted from* [3]*).*

Both the amnion and chorion originate in the first two weeks after fertilization, before the commencement of gastrulation during the third week, whereby the bilaminar disc differentiates into the three germ layers (ectoderm, mesoderm and endoderm) [2,3] *(**Fig. 1.7**)*.

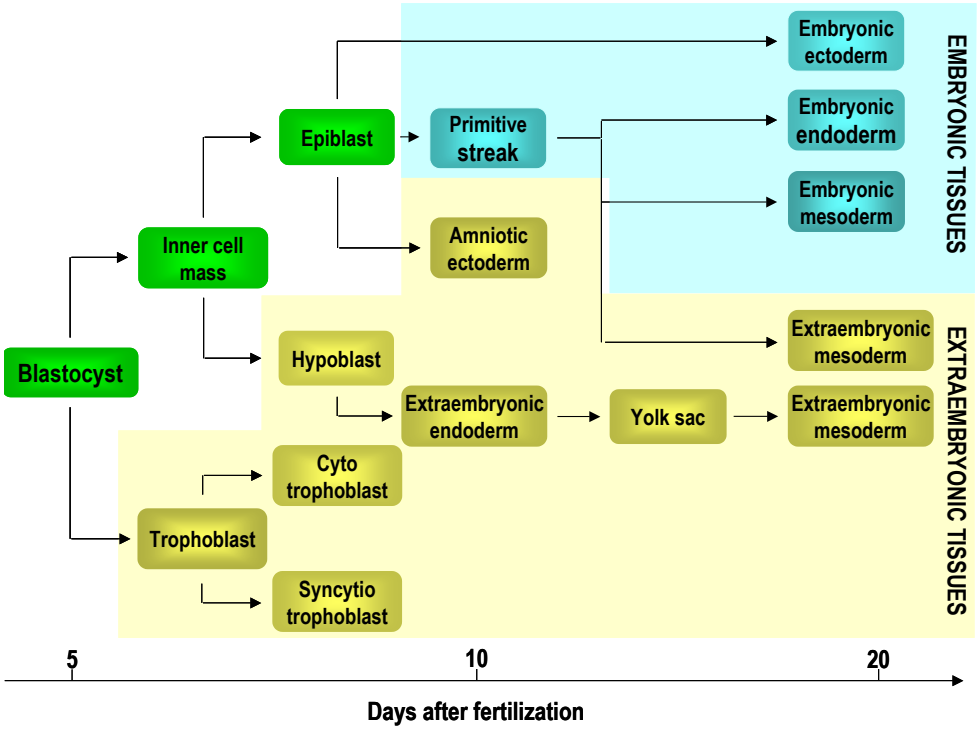


Figure 1.7. *Schematic representation of the development of embryonic and extraembryonic tissues within the first 20 days after formation of the zygote (Adapted from* [3]*).*

1.2 Amniotic membrane-derived cells

As previously described, the amniotic membrane is a thin, avascular membrane composed of an epithelial layer and an outer layer of connective tissue, and is contiguous, over the umbilical cord, with the fetal skin. The amniotic epithelium (AE) is an uninterrupted, single layer of flat, cuboidal and columnar epithelial cells in contact with amniotic fluid. It is attached to a distinct basal lamina that is, in turn, connected to the amniotic mesoderm (AM) *(**Fig. 1.8**)*. In the amniotic mesoderm closest to the epithelium, an acellular compact layer is distinguishable, composed of collagens I and III and fibronectin. Deeper in the AM, a network of dispersed fibroblast-like mesenchymal cells and rare macrophages are observed. Very recently, it has been reported that the mesenchymal layer of amnion indeed contains two subfractions, one having a mesenchymal phenotype and the second containing monocyte-like cells [4].

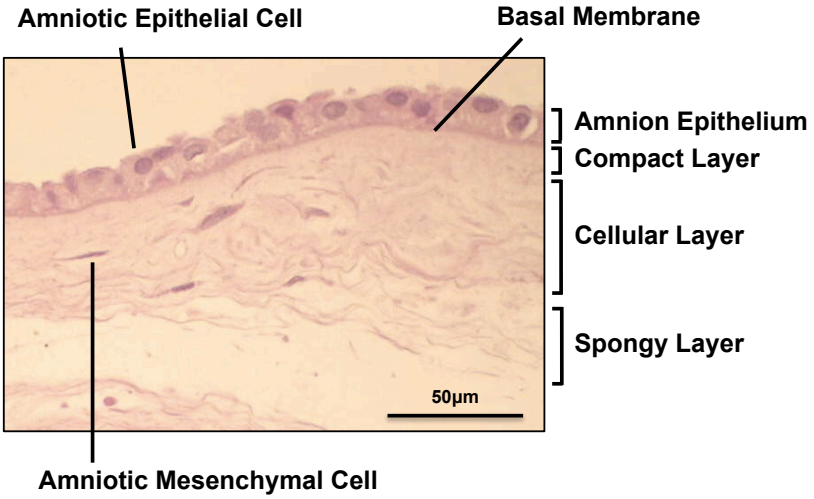


Figure 1.8. Human amniotic membrane composition.

The nomenclature utilized for amniotic membrane-derived cells, i.e. hAEC and hAMSC to indicate human amniotic epithelial cells and human amniotic mesenchymal stromal cells, respectively, was adopted following the *"First International Workshop on Placenta Derived Stem Cells"* in 2007 [5]. From the data presented by the participants of that meeting, the following points, amongst others, were highlighted:

✓ cells isolated from placental tissue should be verified to be of fetal origin;

✓ four regions of fetal placenta can be distinguished, i.e. amniotic epithelial, amniotic mesenchymal, chorionic mesenchymal, and chorionic trophoblastic. From these regions, the following cell populations are isolated: human amniotic epithelial cells (hAEC), human amniotic mesenchymal stromal cells (hAMSC), human chorionic mesenchymal stromal cells (hCMSC), and human chorionic trophoblastic cells (hCTC).

1.2.1 Human amniotic epithelial cells (hAEC)

Human amniotic epithelial cells (hAEC) are cuboidal to columnar cells that arise from the embryonic epiblast and are amongst the first cells to differentiate from the conceptus [5,6].

Recent reports indicate that hAEC express stem cell markers and have the ability to differentiate toward all three germ layers [7]. These properties, the ease of isolation of the amniotic cells and the plentiful availability of placenta as a discard tissue, make the amnion a potentially useful and noncontroversial source of cells for transplantation and regenerative medicine. For isolation of epithelial cells, the amniotic membrane is stripped from the underlying chorion and digested with trypsin [8-11]. A detailed protocol is available providing a step-by-step isolation procedure along with photos of the process [12]. Isolated cells readily attach to plastic or basement membrane-coated culture dishes. Culture is commonly established in a simple medium such as Dulbecco's modified Eagle's medium supplemented with 5-10% serum and epidermal growth factor (EGF), where the cells proliferate robustly and display typical cuboidal

epithelial morphology [10,11,13] *(**Fig. 1.9**)*. Normally, 2–6 passages are possible before proliferation ceases. Cells do not proliferate well at low densities.

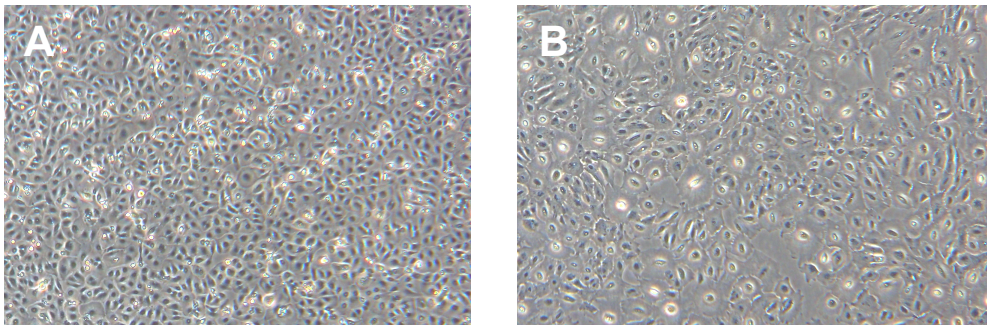


Figure 1.9. *Phase micrographs showing cuboid morphology of cultured hAEC at passage 0* ***(A)*** *and at passage 3* ***(B)****.*

The amniotic membrane contains epithelial cells which express different surface markers, suggesting some heterogeneity of phenotype. Immediately after isolation, hAEC appear to express very low levels of human leukocyte antigen (HLA)-A,B,C [11]; however, by passage 2, significant levels are observed [10,14-16]. Additional cell surface antigens on hAEC include ATP-binding cassette transporter G2 (ABCG2/BCRP), CD9, CD24, E-cadherin, integrins α6 and β1, c-met (hepatocyte growth factor receptor), stage-specific embryonic antigens (SSEAs) 3 and 4, and tumor rejection antigens (TRA) 1-60 and 1-81 [10,17]. Surface markers thought to be absent on hAEC include SSEA-1, CD34, and CD133, whereas other markers, such as CD117 (c-kit) and CCR4, are either negative or may be expressed on some cells at very low levels. Although initial cell isolates express very low levels of CD90 (Thy-1), the expression of this antigen increases rapidly in culture [10,17].

In addition to surface markers, hAEC express molecular markers of pluripotent stem cells, including octamer-binding protein 4 (OCT-4), SRY-related HMG-box gene 2 (SOX-2), and Nanog [10].

The suggestion that hAEC may be pluripotent was supported by the report by Tamagawa et al. [18]. These investigators created a xenogeneic chimera of human amnion cells with mouse embryonic stem cells, *in vitro*, obtaining chimeric aggregates which showed human contribution to all three germ layers. Later studies indicated that differentiation of hAEC can be directed, with Sakuragawa et al. identifying neural and glial markers on cultured hAEC [19]. Later studies reported that cultured hAEC synthesize and release acetylcholine, catecholamines [9,20,21], and dopamine [22,23]. Hepatic differentiation of hAEC has also been reported. In particular, Sakuragawa et al. reported that cultured hAEC produce albumin (Alb) and α-fetoprotein (AFP) and that Alb- and AFP-positive hepatocyte-like cells could be identified integrated into hepatic parenchyma following transplantation of hAEC into the livers of severe combined immunodeficiency (SCID) mice [24]. The hepatic potential of hAEC was later confirmed and extended [10,25,26], whereby in addition to Alb and AFP production, other hepatic functions, such as glycogen storage and expression of liver-enriched transcription factors, such as hepatocyte nuclear factor (HNF) 3γ and HNF4α, CCAAT/enhancer-binding protein (CEBP α and β), and several of the drug metabolizing genes (cytochrome P450) could be demonstrated. The wide range of hepatic genes and functions identified in hAEC suggests that these cells may be useful for liver-directed cell therapy.

Differentiation of hAEC to another endodermal tissue, pancreas, has also been reported. Wei et al. [27] reported that after culture of hAEC for 2–4 weeks in the presence of nicotinamide to induce pancreatic differentiation, transplantation of the insulin-expressing hAEC corrected the hyperglycemia in streptozotocin-induced diabetic mice.

1.2.2 Human amniotic mesenchymal stomal cells (hAMSC)

The mesodermal region of the amniotic membrane contains mesenchymal cells that are dispersed in an extra-cellular matrix which is composed largely of collagen and laminin. The mesenchymal cells themselves are derived from the extraembryonic mesoderm [6], and originate during the pre-gastrulation stages of embryogenesis before the delineation of the three primary germ layers [5,6].

In accordance with the guidelines set out during the *"First International Workshop on Placenta Derived Stem Cells"* in 2007 [5], as well as the criteria proposed by Dominici et al. for bone marrow-derived mesenchymal stromal cells [28], mesenchymal cells isolated from amniotic membranes are termed amniotic mesenchymal stromal cells (hAMSC).

Indeed, the minimal criteria for defining hAMSC are as follows:

✓ adherence to plastic;

✓ formation of fibroblast colony-forming units;

✓ a specific pattern of surface antigen expression *(**Table 1.1**)*;

✓ differentiation potential toward one or more lineages (including osteogenic, adipogenic, chondrogenic, and vascular/ endothelial);

✓ fetal origin.

Positive (>95%)	Negative (<2%)
CD90	CD45
CD73	CD34
CD105	CD14
	HLA-DR

***Table 1.1. Specific surface antigen expression** at passages 2-4 for amniotic mesenchymal stromal cells (Adapted from* [5]*).*

hAMSC can be isolated from first-, second-, and third-trimester mesoderm of amnion [14,15,29-32]. Here, when using the terms hAMSC we refer to cells from term placenta unless otherwise specified.

Isolation of hAMSC is usually performed with term amnion dissected from the deflected part of the fetal membranes to minimize the presence of maternal cells. hAMSC populations can be obtained by different procedures, which are based essentially on digestion of the amniotic membrane with collagenase [33] or collagenase and DNase [34]. hAMSC show a fibroblastoid shape, and are able to adhere to and proliferate on tissue culture plastic and can be kept until passages 5–10 *(**Fig. 1.10**)*.

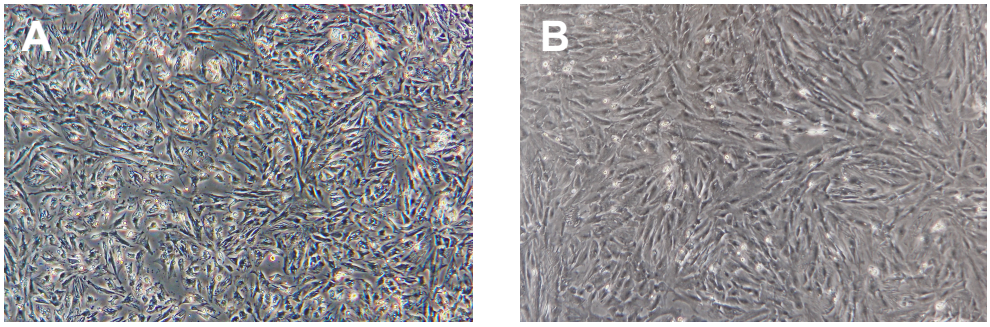


***Figure 1.10.** Phase micrographs showing fibroblastoid morphology of cultured hAMSC at passage 0 **(A)** and at passage 3 **(B)**.*

Expression of CD49d (α4 integrin) on hAMSC distinguishes them from hAEC [15], while, in culture, neither vimentin nor cytokeratin 18 expression is specific for hAMSC or hAEC, respectively. Transmission electron microscopy of hAMSC shows mesenchymal and epithelial characteristics, and this hybrid phenotype is interpreted as a sign of multipotentiality. Transmission electron microscopy of hAMSC show a simpler cytoplasmic organization of these cells, the most notable features of which include the

presence of stacks of rough endoplasmic reticulum cisternae, dispersed mitochondria and glycogen lakes. Meanwhile, features of higher specialization, such as presence of assembled contractile filaments, prominence of endocytotic traffic, and junctional communications, are lacking [35]. The surface marker profile of cultured hAMSC and mesenchymal stromal cells (MSC) from adult bone marrow are similar, in that all express typical mesenchymal markers *(**Table 1.1**)* but are negative for hematopoietic (CD34 and CD45) and monocytic markers (CD14) [14,15,30,32]. While surface expression of SSEA-3 and SSEA-4 and RNA for OCT-4 has been reported [15,27,36-38] in hAMSC, immunofluorescence staining of mesenchymal tissue itself has failed to detect SSEA-3 or SSEA-4 [39].

hAMSC differentiate toward “classic” mesodermal lineages (osteogenic, chondrogenic, and adipogenic) [14,15,30,32,36], and differentiation of hAMSC to all three germ layers - ectoderm (neural) [14,31], mesoderm (skeletal muscle [16,36], cardiomyocytic [37,40] and endothelial [36]) and endoderm (pancreatic) [27] - has been reported. However, questions remain as to the proportion of hAMSC that exhibit plasticity and whether the progeny are indeed able to undergo full maturation [39].

Human amniotic cells have been reported to successfully and persistently engraft in multiple organs and tissues *in vivo*. Indeed, human chimerism has been detected in the brain, lung, bone marrow, thymus, spleen, kidney, and liver after either intraperitoneal or intravenous transplantation of human amnion and chorion cells into neonatal swine and rats, indicating active migration of these cells, which was consistent with their observed expression of adhesion and migration molecules (L-selectin, VLA-5, CD29, and P-selectin ligand 1), as well as cellular matrix proteinases (MMP-2 and MMP-9) [41].

1.2.3 Human amniotic mesenchymal tissue cells (hAMTC)

As described above cells derived from the mesenchymal region of the amnion showed the characteristics of hAMSC when cultured; however, a defined population of HLA-DR-expressing cells with macrophage-monocyte phenotypic characteristics has also been described in the mesenchymal layer of the amnion [42-44], thus suggesting the presence of populations capable of active immune function within these tissues.

Indeed, Magatti et al. [4] have confirmed the presence of two distinct subpopulations in the mesenchymal region of amnion which differ in their expression of CD45, CD14, and HLA-DR. Considering that mesenchymal stromal cells from different origins are agreed to be negative for these markers [5,28], the unfractionated population derived from the mesenchymal region of amnion, which includes both HLA-DR-positive and -negative cells, has therefore been designed as human amniotic mesenchymal tissue cells (hAMTC). Furthermore, since freshly isolated cells at passage 0 are described in this study, we will adopt the nomenclature hAMTC.

1.3 Immunology and placenta

1.3.1 Fetomaternal tolerance

Immunologically, pregnancy is an exceptional phenomenon whereby the fully competent immune system of the mother is rendered tolerant to the immunologically distinct fetal allograft, thereby creating a situation in which rejection, which would normally ensue in an allogeneic transplantation setting in the absence of immunosuppression, is avoided. The paradox of maternal immunological tolerance to the fetus was first raised more than 50 years ago by Peter Medawar, who proposed three hypotheses to explain the phenomenon: *i)* that there is a physical separation between the mother and the fetus, *ii)* that the fetus is antigenically immature, and *iii)* that the mother possesses an immunological inertness [45]. Since the time of Medawar, it has become evident that these mechanisms cannot fully explain why the fetus is not rejected by the mother, and other site-specific immune suppression mechanisms must therefore be considered. Indeed, several studies have indicated that the fetal placental barrier may be less inert or impervious than previously envisioned, with evidence presented for cellular trafficking in both directions across the fetal/maternal interface [46-48]. With regard to the second mechanism of Medawar's paradox, it has been shown that fetal cells do in fact express MHC I and MHC II which are antigenically mature and detectable in maternal circulation [49]. Finally, it is also now clear that the maternal immune system is not inert during pregnancy, and it can in fact respond to and eliminate fetal cells that enter the maternal circulation [47,50].

Many local mechanisms that contribute to protection of the fetus from the maternal immune system have been identified at the fetomaternal interface, although it is not yet clear how these mechanisms interact with each other.

For example, It has been shown that trophoblastic cells express the non classical HLA molecules HLA-E, and HLA-G [51]. In particular, HLA-G has been shown to inhibit natural killer cells that could induce fetal rejection

[49]. Meanwhile, it has been hypothesised that the effect of HLA-G on NK cell activity is not induced directly, but rather, that it also requires the expression of HLA-E on trophoblastic cells [52,53]. In addition, soluble HLA-G molecules produced by placenta induce apoptosis of activated CD8+ T cells and inhibit CD4+ T-cell proliferation [54]. Moreover, the interaction of HLA-G with dendritic cells through KIR-related leukocyte Ig-like receptors may have an indirect effect on the immune response by tolerizing dendritic cells and facilitating the generation of regulatory T cells [55,56].

Other explanations have also been proposed regarding the regulation of maternal T-cell proliferation at the maternal interface. One such suggestion is that Indoleamine 2,3-dioxygenase (IDO) plays a role in promoting fetomaternal tolerance [57]. This tryptophan catabolising enzyme is produced by some cells of the syncytiotrophoblast, and it is thought to prevent immune responses to the fetus by inhibiting maternal T cell activation either by depriving T cells of tryptophan [58] or by producing catabolites of tryptophan (kynurenine) which prevent activation and proliferation of T cells, B cells and NK cells [59]. However, in IDO knockout mice, fetus rejection was not observed [60] and so, although IDO activity has been suggested as a key mechanism for protecting the allogenic fetus, it is not the sole determinant in this process, and other mechanisms can compensate for the loss of IDO activity during gestation.

Other studies have shown that trophoblast cells express Fas ligand, which is able to induce apoptosis of activated maternal Fas-expressing lymphocytes [61]. Therefore, this Fas/FasL system could also contribute to the maintenance of fetomaternal tolerance.

1.3.2 Immunologic properties of fetal membranes

Whether the fetus participates actively in suppressing maternal allogeneic immune responses and, if so, which fetal placental tissues exert these immunomodulatory effects, are both questions which remain to be

addressed. Evidence which provides support to the hypothesis that fetal membranes are non-immunogenic comes from clinical studies in which amniotic membrane has been used for treatment of skin wounds, burn injuries, chronic leg ulcers, and prevention of tissue adhesion in surgical procedures [62-68]. Amniotic membrane is also used in ocular surface reconstruction to promote the development of normal corneal or conjuntival epithelium [69,70] without acute rejection in absence of immunosuppressive treatment. Moreover, several clinical trials in humans have proven that allogeneic transplantation of amniotic membrane [71-73] or hAEC [8,74] does not induce acute immune rejection in the absence of immunosuppressive treatment.

Intriguingly, *in vitro* experiments using supernatants from short term cultures of human placenta have demonstrated inhibition of PHA-(phytoheamaglutinin-) driven lymphocyte activation, mixed lymphocyte reaction and natural killer activity [75], while additional experiments have shown that fragments of amniotic membrane exert immunosuppressive properties in mixed lymphocyte reactions [76].

Furthermore, both human amniotic membrane and human amniotic epithelial cells have been shown to survive for prolonged periods of time after xenogeneic transplantation into immuno-competent animals, including rabbits [77], rats [51], guinea pigs [78] and bonnet monkeys [79]. Additionally, long-term engraftment has been observed after intravenous injection of human amniotic and chorionic cells into newborn swine and rats, with human microchimerism detected in several organs [41], suggesting active migration and tolerogenic potential of the xenogeneic cells. Finally, long-term survival of rat amnion-derived cells, with no evidence of immunological rejection or tumor formation, has been observed after allogeneic in utero transplantation of these cells into the developing rodent brain [80].

1.3.3 The paracrine immunomodulatory action of amnion membrane and its derivatives

Several lines of evidence support the possibility that placental tissue or cells may be capable of exerting immunomodulatory actions. For example, considering the fundamental role played by the placenta in the phenomenon of fetomaternal tolerance, it is conceivable that cells isolated from placental tissues might have immunomodulatory properties. Meanwhile, given that mesenchymal cells present in the bone marrow stroma have been shown to exert immunomodulatory effects, it is plausible that this property could also be displayed by mesenchymal cells that are isolated from placental tissues.

Indeed adult mesenchymal stromal/stem cells (MSC), which were first identified in bone marrow (BM) [81], but have since been successfully isolated from various other sources, including umbilical cord blood [82], adipose tissue [83], peripheral blood [84], amniotic fluid [85], Wharton's Jelly [86,87], and more recently from different tissue of human placenta [5], have been extensively studied both for their ability to differentiate towards multiple lineages, such as cells of the bone, cartilage, muscle, adipose, tendons and central nervous system [88-90], as well as for their interesting immunoregulatory properties [91-93]. Numerous *in vitro* studies have demonstrated that MSC can target and modulate the function of different cells of the immune system, such as T cells [94], B cells [95], natural killer cells [96] and dendritic cells [97,98].

These *in vitro* immunomodulatory properties have generated enormous interest in the potential application of MSC *in vivo* as an immunosuppressive cellular therapy. Successful results have been obtained with the use of MSC for both the prevention of graft-versus-host disease (GVHD) in solid organ transplantation [99,100] and for the treatment of steroid-resistant acute GVHD, which arises after allogeneic hematopoietic cell transplantation [101,102]. Moreover, systemic infusion of MSC has been shown to ameliorate the clinical and histopathological severity of experimental

autoimmune encephalomyelitis [103], multiple sclerosis [104], and colitis [105].

In recent years, our laboratory (*Centro di Ricerca E. Menni, Fondazione Poliambulanza-Istituto Ospedaliero, Brescia*) has demonstrated that cells with characteristics of MSC can be successfully isolated from the human amniotic and chorionic membranes [32,41].

In particular, we have shown that a mix of cells isolated from the mesenchymal region of both human amnion and chorion fail to induce allogeneic T-cell responses and actively suppress T-cell proliferation induced by alloantigens [41]. Moreover, after intravenous injection of heterogeneous human amniotic and chorionic cells into newborn swine or rats, human microchimerism was detected in bone marrow, brain, lung, and thymus, suggesting active migration and integration into specific organs, and therefore indicating active tolerance [41].

Interestingly, Magatti et al. have shown, through *in vitro* studies, that human amniotic mesenchymal tissue cells (hAMTC) not only fail to induce allogeneic lymphocyte activation *in vitro*, but also actively suppress lymphocyte proliferation in an allogeneic mixed lymphocyte reaction (MLR) or induced by T-cell receptor engagement. This effect was observed when the hAMTC were present both in direct cell contact or when cultured in a transwell system (which impedes cell contact) [4].

In addition, this same group has demonstrated that the presence of hAMTC strongly inhibited differentiation of monocytes into iDC, preventing the expression of the DC marker CD1a and reducing the expression of HLA-DR, CD80, and CD83 [97]. Moreover, exposure to LPS of monocytes cultured with GM-CSF and IL-4 and in the presence of hAMTC resulted in lack of CD83 and CD80 upregulation, reduced CD86 and HLA-DR expression, and a final cell phenotype very similar to the original monocytes. It was also observed that the presence of hAMTC in differentiating DC cultures results in the arrest of the cells to the G0 phase

and abolishes the production of inflammatory cytokines such as TNF-α, CXCL10, CXCL9 and CCL5.

Through co-culture experiments, Magatti and co-workers also revealed that hAMTC significantly reduced the proliferative activity of different tumor cell lines, and that this effect was cell-dose-dependent [106]. This anti-proliferative effect was observed both when hAMTC and tumor cells were co-cultured in contact or physically separated in a transwell system. Notably, the anti-proliferative effect of hAMTC were shown not to be exerted through induction of tumor cell apoptosis, but rather, through cell cycle arrest in the G0/G1 phase [106].

All of the results described above were obtained when hAMTC were cultured either in direct cell contact or in a transwell system, thereby suggesting the involvement of soluble inhibitory factor(s) secreted by these cells, although the identities of such factors remain to be elucidated.

In support of a key role of soluble factors in the suppressive action of amniotic membrane-derived cells, Cargnoni and colleagues reported use of a mouse model of lung fibrosis to show that a reduction of severity and progression of the disease can be obtained not only after the transplantation of amniotic membrane-derived cells, but also after the injection of their conditioned medium generated from culture of these cells [107,108]. Indeed, the extent, severity and progression of lung fibrosis in mice after bleomycin instillation were reduced in mice which had also been treated with conditioned medium from hAMTC culture (CM-hAMTC) when compared to animals of the control group [108].

Likewise, Cargnoni and colleagues demonstrated that application of an amniotic membrane fragment (hAM) onto ischemic rat hearts could significantly reduce postischemic cardiac dysfunction [109], with amniotic membrane-treated rats showing higher preservation of cardiac dimensions and improved cardiac contractile function in terms of higher left ventricle ejection fraction, fractional shortening, and wall thickening. However, no engraftment of amniotic cells was detected into host cardiac tissues, all

together suggesting that the amniotic membrane may constitute a convenient vehicle for supplying cells that produce cardioprotective soluble factors, and also reinforcing the notion that this tissue constitutes a cell source with clinical potential that has yet to be completely revealed.

Moreover, Sant'anna and co-workers have demonstrated that hAM patching onto livers of rats subjected to bile duct legation (BDL) reduced the severity and progression of BDL-induced fibrosis [110]. Indeed, while fibrosis progressed rapidly in untreated control rats, leading to cirrhosis within 6 weeks, fibrosis in hAM-treated rats was confined at the portal/ periportal area with no signs of cirrhosis, while collagen deposition was reduced to about 50% of levels observed in control rats. In addition, the hAM was able to significantly slow the gradual progression of the ductular reaction and reduce the area occupied by activated myofibroblasts at all time points analyzed in the study [110]. Interestingly, like Cargnoni and colleagues, these authors also failed to detect donor cells in host liver.

In summary, it is clear that the beneficial effects observed after the application of the amniotic membrane onto the ischemic rat heart [109] and the fibrotic rat liver [110] are very likely induced by paracrine actions exerted on host tissues by bioactive molecules secreted by the amniotic cells, further suggesting the involvement of soluble factors as the key actors in the anti-inflammatory and anti-fibrotic effects of the amniotic cells.

2. AIM OF THE STUDY

Both *in vitro* and *in vivo* studies have provided evidence to support the immunoregulatory role of MSC [94-105]. However, the exact mechanisms underlying this immunomodulatory function still remain to be fully elucidated. Factors such as indoleamine 2,3-dioxygenase (IDO) enzyme, nitric oxide (NO), prostaglandin E2 (PGE2), tumor growth factor (TGF)-β, interleukin (IL)-10, hepatocyte growth factor (HGF) and galectins have all been reported to be associated with the immunosuppressive effects of human MSC isolated from bone marrow [92]. Nevertheless, conflicting results have been reported to date regarding the specific contribution of these factors in MSC-mediated immuno-regulatory processes [92,93]. In addition, even though is widely accepted that the mechanism of action of MSC derived from the amniotic membrane is also associated with the release of soluble factors, no studies so far have reported the characterization of these factors.

Therefore, the main aim of this study was to identify the various immunomodulatory soluble factors released by hAMTC and to shed light on the mechanisms underlying the effects of these factors on lymphocyte proliferation.

To this end, we firstly prepared conditioned medium (CM) from the culture of hAMTC and fragments of the whole human amniotic membrane (hAM), and then tested whether the immunomodulatory properties of these conditioned media were affected by either addition of specific antibodies against certain cytokines, blockage of specific biochemical pathways, or different chemical treatments.

We first analyzed the thermostability, chemical nature, and molecular weight of the factors associated with the anti-proliferative effects of hAMTC. We then assessed the effects of blocking the activity of specific molecules, and also investigated the role of factors known to be involved in the immunomodulatory actions of MSC from other sources.

Finally, through the blockage of distinct biochemical pathways, such as those responsible for IDO metabolism, or nitric oxide production or prostaglandin production, we gained insight into the identity of the factors involved in the immunomodulatory proprties of amniotic membrane-derived cells.

3. MATERIAL AND METHODS

3.1 Ethics statement

Human term placentas, bone marrow (BM) aspirates and peripheral blood were obtained from donors after informed consent according to the guidelines of Ethical Committee of the Catholic Hospital (CEIOC). For all samples used throughout our study, informed written consent was sought and obtained from each single donor. The research project was authorized by Centro di Ricerca E. Menni-Fondazione Poliambulanza Commitee, which specifically approved this study.

3.2 Human sample collection, cell isolation and culture

3.2.1 Isolation and culture of human amniotic mesenchymal tissue cells (hAMTC)

Human term placentas were processed immediately after birth using a previously described protocol [32], with some modifications. The amnion was manually separated from the chorion and washed extensively in phosphate-buffered saline (PBS; Sigma, St Louis, MO, USA) containing 100 U/ml penicillin and 100 µg/ml streptomycin (herein referred to as P/S), (both from Euroclone, Whetherby, UK) and 2.5 µg/ml amphotericin B (Lonza, Basel, CH). Afterwards, the amnion was cut into small pieces (3 × 3 cm^2). Amnion fragments were sterilized by a brief incubation in PBS + 2.5% Eso Jod (Esoform, Italy) and 3 min in PBS containing 500 U/ml penicillin, 500 µg/ml streptomycin, 12.5 µg/ml amphotericin B and 1.87 mg/ml Cefamezin (Pfizer, Italy). Sterilized amnion fragments were then incubated for 9 min at 37°C in HBSS (Lonza, Basel, CH) containing 2.5 U/ml dispase (Roche, Mannheim, Germany). After a 3 min resting period at room temperature in RPMI 1640 medium (Cambrex, Verviers, Belgium) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sigma), 2 mM L-glutamine (Cambrex) and P/S, the fragments were digested with 0.94 mg/ml collagenase (Roche) and 20 µg/ml DNase (Roche) for 2.5-3 hrs

at 37°C. Amnion fragments were then removed, mobilized cells were passed through a 100 µm cell strainer (BD Falcon, Bedford, MA), and collected by centrifugation at 280 x g for 10 min. These cells, as reported above, are referred to as hAMTC, for human amniotic mesenchymal tissue cells.

To obtain hAMTC at different passages, freshly isolated cells were plated at a density of 50 x $10^3/cm^2$. Upon reaching confluency, adherent cells were trypsinized and then sub-cultured at a density of 20 x $10^3/cm^2$ untill passages 3.

3.2.2 BM-derived MSC isolation and culture

Bone marrow (BM) aspirates were extracted from the femoral heads of patients undergoing orthopedic surgery and processed as follows. Briefly, BM samples were diluted (1:4) in PBS + P/S, transferred to 50 ml conical tubes (Falcon) and centrifuged at 1500 x g for 15 min. After discarding of the fat layer and supernatant, the cells were layered on a density gradient (Lymphoprep, Axis Shield) and centrifuged at 670 × g for 30 min. Recovered mononuclear cells were then collected, washed twice with PBS and centrifuged at 280 × g for 10 min before being plated at a density of 8 x $10^5/cm^2$ in DMEM (Lonza), supplemented with 10% heat-inactivated FBS, L-glutamine and P/S and maintained in culture at 37°C with 5% CO_2. After 3 days, non-adherent cells were removed. Upon reaching confluency, adherent cells (BM-MSC) were trypsinized and sub-cultured at a density of 8 x $10^3/cm^2$, and were used for experiments at passage 3.

3.2.3 Human peripheral blood mononuclear cells (PBMC)

PBMC were obtained from heparinized whole blood samples or buffy coats from healthy subjects using density gradient centrifugation (Lymphoprep, Axis-Shield).

3.3 Flow cytometry analysis

For evaluation of cell phenotype, cell suspensions were incubated for 20 minutes at 4°C with fluorescein isothiocyanate or phycoerythrin-conjugated antibodies specific for human CD90 (clone 5E10), CD73 (clone AD2), CD13 (clone L138), CD44 (clone L178), CD105 (clone SN6), CD166 (clone 3A6), CD45 (clone H130), HLA-DR (clone TU36), and CD34 (clone AC136), or isotype controls IgG1 (clone X40), IgG2a (clone X39), and IgG2b (clone MG2b-57). All monoclonal antibodies were obtained from BD Biosciences (San Jose, CA,) except for isotype control IgG2b, which was obtained from Biolegend. Samples were analyzed with a FACSCalibur instrument and the CellQuest software (BD Biosciences).

3.4 Polyuretan foam scaffold production

Prof. Tanzi and colleagues (Biomaterials Laboratory, Politecnico of Milan) provided PUF matrix (EF) that was synthesized by reacting a polyether-polyol mixture with MDI prepolymer, using Fe-acetyl-acetonate as a catalyst, and water as an expanding agent, essentially as previously described [112,113], with the exception that the polyether-polyol mixture used in this case had higher hydrophilicity than that previously described. Density, porosity and average pore size, by micro CT (Skyscan 1172, Aartselaar, Belgium), were then evaluated *(**Fig.3.1**)*.

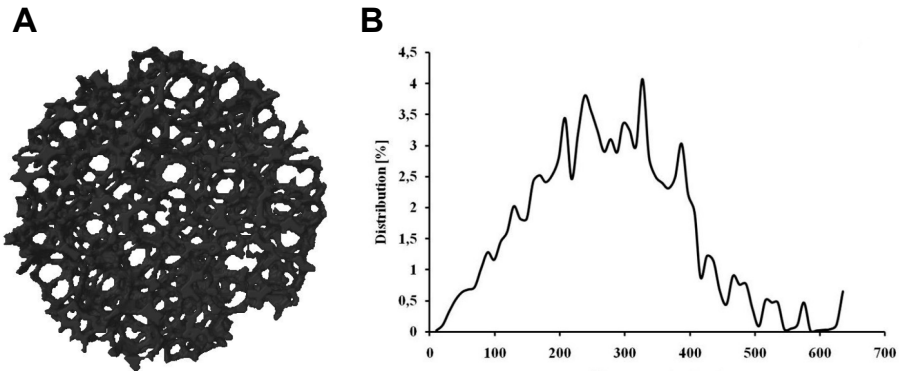


Figure 3.1. Micro CT scan of EF PUF foam. (A) *3D model of the EF PUF foam generated through the micro CT algorithm adaptive rendering;* ***(B)*** *trend of the foam average pore size distribution.*

For experimental approach used in this study, 2 different type of scaffolds were employed, namely, uncovered EF PU foam or EF PU foam covered with Tri-Calcium-Phosphate (α-TCP), made by fixing EF discs (Ø=6 mm) in circular slots in a polymeric mesh, followed by coating through immersion in α-TCP suspension under magnetic stirring.

3.5 In vitro hAMTC osteogenic differentiation

hAMTC, isolated from human placenta as previously described, were suspended in culture medium at a density of 5 x 10^6/ml. Cell suspension (100 µl/well) were seeded into glass two-well chamber slides or onto each EF PUF specimen, placed in an incubator (5% CO_2, 37°C) and maintained under static culture conditions for the entirety of the study (3 weeks).
Osteogenic differentiation was assessed by incubating plated cells (in glass chambers slides or on EF PUF scaffolds) in NH OsteoDiff Medium (Miltenyi Biotec) or in Eagle's Minimum Essential Medium (EMEM), as control, for 3 weeks at 37°C with 5%CO_2. Media was changed every 3 days.

3.6 Osteogenic differentiation evaluation

3.6.1 Histological and cytological stainings

After 3 weeks EF PUF foam samples which had been cultured with hAMTC, were fixed in formalin for 24h, dehydratated in a graded series of ethanol and embedded in paraffin. Sections of 3μm thickness were then stained with hematoxylin-eosin for scaffold colonization and cell distribution analysis, or with Alizarin Red (Sigma) or von Kossa (Bio-Optica) for calcium deposit evaluation.

At the same time point, the cells which had been differentiated in glass chamber slides were fixed with Bio-Fix (Bio-Optica) and air-dry for the cytological analysis. Osteoblasts were identified by cytochemical staining for alkaline phosphatase (ALP), using a commercial kit (86-R; Sigma) as previously described [114]. Calcium deposits, characteristic of osteogenic differentiation in cultures, were visualized by alizarin red (Sigma) and von Kossa staining (Bio-Optica) according to the manufacturer's instructions.

3.6.2 RNA extraction and RT-PCR analysis

Both on the day of isolation, and also at the end of osteogenic differentiation culture in glass chamber slides, hAMTC were resuspended in RLT buffer (Qiagen) for the molecular analysis. Total RNA was isolated from the undifferentiated and the osteogenic differentiated hAMTCs using the EZ1 RNA Cell Mini Kit (Qiagen) according to the manufacturer's instructions, and the yield was determined spectrophotometrically at 260/280 nm. Equal amount of RNA (100 ng) from each sample were reverse transcribed into cDNA (Improm II Reverse Transcription System, Promega) and PCR was performed using a GoTaq polymerase kit (Promega). Oligonucleotide primers and an amplification profile specific to each gene marker analyzed in this study are presented in ***Table 3.1***. RT-PCR products were run on a 1.5% agarose gel and visualized by staining with ethidium bromide.

Target gene	Primers sequence	Product size (bp)	Annealing temperature (°C)	Number of cycles
GAPDH	5'-GAAGGTGAAGGTCGGAGT-3' 5'-GAAGATGGTGATGGGATTTC-3'	275	58	25
Alkaline phosphatase	5'-ACGTCGCTAAGAATGTCATC-3' 5'-CTGGTAGGCGATGTCCTTA-3'	475	55	35
Collagen I	5'-TGACGAGACCAAGAATCTG-3' 5'-CCATCCAAACCACTGAAACC-3'	599	55	35
Collagen II	5'-TCTGCAACATGCAGACTGGC-3' 5'-GAAGCAGACAGGCCCTATGT-3'	517	58	40
Sox9	5'-AACATGACCTATCCAAGCGC-3' 5'-ACGATTCTCCATCATCCTCC-3'	143	55	35
PPAR-2γ	5'-ATGGGTGAAACTCTGGGAG-3' 5'-CCTTGCATCCTTCACAAGC-3'	481	56	40
Mushashi-I	5'-GCCCAAGATGGTGACTCG-3' 5'-ATGGCGTCGTCCACCTTC-3'	114	58	35

Abbreviation: GAPDH=Glyceraldehyde 3-phosphate dehydrogenase; Sox9=SRY-box 9; PPAR-2γ=Peroxisome proliferator-activated receptor γ2

Table 3.1. Primers sequences and amplification profile.

3.7 Production of conditioned medium (CM)

The different conditioned media (CMs) used in this study were obtained as follows.

3.7.1 CM generated from freshly isolated hAMTC

hAMTC (obtained from amniotic membranes of at least 80 different donors) at passage 0 were re-suspended in an opportune volume of complete UltraCulture medium, composed of UltraCulture medium (Lonza) supplemented with P/S, and plated in 24-well plates (Corning Inc., Corning, NY) at different concentration: usually at 1 x 10^6 cells/well in a final volume of 1 ml (referred to as CM-hAMTC) or, when otherwise specified, 0.5 x 10^5 cells/ well in 1ml (CM-hAMTC 0.5*10^6/ml), 0.25 x 10^5 cells/well in 1ml (CM-hAMTC 0.25*10^6/ml) or 0.125 x 10^5 cells/well in 1ml (CM-hAMTC 0.125*10^6/ml).

3.7.2 CM generated from hAMTC at passage 3 (CM-hAMTC p3)

hAMTC at p3 (obtained from 3 different placentas) were plated in 24-well plates at a density of 2.5 x 10^5 cells/well in 0.5 ml of complete UltraCulture medium.

3.7.3 CM generated from the culture of fragments of the amniotic membrane (CM-hAM)

Sterilized fragments (3 cm x 3 cm) from freshly separated amniotic membranes (obtained from at least 50 different donors) were plated (1 fragment in each well) in 6-wells plates (Corning Inc., Corning, NY) covered with a glass slide (Bio-Optica) and cultured in presence of 1 ml of complete UltraCulture medium. One fragment of amniotic membrane contains a number of hAMTC comparable to that used for the production of CM-hAMTC ($8 \pm 4 \times 10^5$ hAMTC/ml).

3.7.4 CM generated from BM-MSC (CM-BM-MSC)

BM-MSC (obtained from 3 different BM donors) cultured untill passage 3, were plated in 24-well plates at a density of 2.5×10^5 cells/well in 0.5 ml of complete UltraCulture medium.

3.7.5 CM from cells that have been treated to block specific synthetic pathways

hAMTC (at passage 0) and hAM fragments (both obtained from at least 8 different donors), were cultured in presence of the inhibitor of IDO activity (0.5 mM DL-methyl-tryptophan), the inhibitor of NO synthase (1mM L-NAME) or the inhibitors of cyclooxygenase (indomethacin, ketoprofen, niflumic acid), all purchased from Sigma-Aldrich. To determine the optimal dose of COX inhibitors able to decrease the quantity of all prostanoids in both CM-hAMTC and CM-hAM, we previously performed experiments with different concentrations of the different COX inhibitors (*i.e.* 1, 5, 10, 50 and 100 µM), (data not shown). We found that the highest reversion of the inhibitory effect was obtained when using a concentration of 10 µM, which was the dose selected for further analysis.

All the cultures described above were performed at 37°C and 5% CO_2 atmosphere, for 5 days. At the end of this culture period, the different CMs

were collected, centrifuged at 300 x g, filtered through a 0.8 µm sterile filter (Sartorius), opportunely aliquoted under sterile conditions and frozen at -80°C until use.

Each CM preparation was tested for its ability of inhibiting lymphocyte proliferation, as explained below in the paragraph describing lymphocyte proliferation test. For temperature stability analysis, proteinase K treatment and CM fractionation (described below), in order to obtain results that were less influenced by single donor variability and more representative of soluble factors released by hAMTC and hAM, we pooled 8 to 10 different CM-hAMTC or CM-hAM and used these pools for each specific analysis.

3.8 Temperature stability analysis

CM-hAMTC and CM-hAM were subjected to different temperature changes. In particular, they were: *i)* incubated for 30 min at 60°C or 80°C; *ii)* subjected to three frost-defrost cycles, which were carried out by putting these CMs in liquid nitrogen and subsequently in a thermostatic bath at 37°C.

3.9 Proteinase K treatment

Complete UltraCulture medium, CM-hAMTC and CM-hAM (1 ml of each sample) were treated with 1 U of proteinase K linked to agarose beads (Sigma-Aldrich), for 2 and 6 hours at 37°C. The digestion was then blocked by removing proteinase K through brief centrifugation. Proteinase-treated CMs were loaded on SDS-PAGE gels that were then stained by Coomassie blue. The efficacy of protein digestion was evaluated monitoring the reduction of the density of the band corresponding to BSA protein, which is the most abundant in CMs and complete UltraCulture medium. The band intensity was quantified using the Quantity One software (Bio-Rad).

3.10 CM fractionation

To obtain fractions potentially enriched in soluble factor(s) with inhibitory effects, different CM-hAMTC (n=11 pools) were fractionated using Centrifugal Filter Devices with different NMWL (Nominal Molecular Weight Limit) Ultracel membranes (Millipore). First, 3 ml of CM-hAMTC were loaded onto a Centrifugal Filter Device with Ultracel membranes with a 100 kDa cut-off and centrifuged at 2700 x g for 35 min. Then, the solute was re-suspended in the same volume of complete UltraCulture medium as the loaded sample, while the eluate was loaded onto a column with a 30 kDa membrane cut-off. After centrifugation at 2700 x g for 30 min, the solute was resuspended in complete UltraCulture (in the same volume as the loaded sample), while the eluate was reloaded onto a column with a 3 kDa membrane cut-off and centrifuged at 2700 x g for 35 min. Finally, the eluate was harvested and the solute was resuspended in complete UltraCulture medium. To analyze the potential interactions between factors, for several experiments we mixed different fractions. All the fractions were immediately tested for the ability to inhibit lymphocyte proliferation, as described below.

3.11 Lymphocyte proliferation test

Lymphocyte proliferation was induced either by stimulating PBMC (1 x 10^5/well in 96-well-plate) by the addition of anti-CD3 (Orthoclone OKT3, Janssen-Cilag), (final concentration of 12.5 ng/ml) or by the co-culture with irradiated allogeneic “stimulator” PBMC [mixed leukocyte reaction (MLR)] at a 1:1 ratio. All cultures were carried out in triplicate in complete UltraCulture medium, and lymphocyte proliferation was assessed by adding [^{3}H]-thymidine for 16-18 hours after 3 (for PBMC + anti-CD3 stimulation) or 5 (for MLR) days of culture.

Proliferation inhibitors tested in these experiments were: (*a*) irradiated (3000 cGy) hAMTC or BM-MSC (1 x 10^5/well) put in contact or transwell with PBMC, in the absence or presence of 0.5 mM DL-methyl-tryptophan; (*b*) different amounts (50, 75, 100 µl/well) of CM-hAMTC, CM-hAM or CM-

BM-MSC, with or without the addition of FBS (5%) and essential/non-essential amino acids (Sigma); (*c*) different amounts of CM-hAMTC and CM-hAM obtained after temperature- or proteinase K- treatment or after column fractionation; (*d*) different amounts of CMs generated after culture of hAMTC or hAM in presence of specific inhibitors; (*e)* 100 µl of different CMs after the addition of indomethacin (10 µM), DL-methyl-tryptophan (0.5 mM), L-NAME (1 mM), a mixture of PGs (mix 1: PGE2 500 ng/ml + PGD2 100 ng/ml + PGF2α 25ng/ml or mix 2: PGE2 25 ng/ml + PGD2 6 ng/ml + PGF2α 10 ng/ml, all purchased from Cayman Chemical) or neutralizing antibodies against IL-10 (1 µg/ml, BD), IL-6 (1 µg/ml, BD), HGF (1 µg/ml, R&D Systems) and TGF-β (1 µg/ml, R&D Systems); (*f*) different amounts (from 10 pg/ml to 5 µg/ml) of PGE2, PGD2, PGI2, PGF2α or a mixture of these PGs (mix 1: PGE2 500 ng/ml + PGD2 100 ng/ml + PGF2α 25ng/ml; mix 2: PGE2 25 ng/ml + PGD2 6 ng/ml + PGF2α 10 ng/ml).

3.12 NO quantification

NO production in CM-hAMTC and CM-hAM was measured as nitrite (NO_2-) accumulation, using the Griess reagent (G4410-Sigma Aldrich) and following manufacturer's instructions. Serial $NaNO_2$ dilutions in water were used to produce a standard curve. Absorbance was measured at 540 nm with a microplate reader (DV990/BV6-GDV).

3.13 Prostanoids quantification

Total prostanoids, prostaglandin (PG) E2, PGD2 and PGF2α quantification in CM-hAMTC and CM-hAM were obtained using the *Cox Inhibitor Screening Assay Kit, Prostaglandin E2* EIA kit, *Prostaglandin D2* EIA kit and *Prostaglandin F2α* EIA kit respectively (all purchased from Cayman Chemical) according to the manufacturer's instructions. Absorbance was measured at 405 nm with a microplate reader. Since the manufacturer specifies that PGD2 monoclonal antibodies possess 92% cross reactivity with PGF2α, the amount of PGD2 was calculated by subtracting 92% of the value obtained for PGF2α from the value obtained with the PGD2 antibody.

3.14 Cytokine assay

The levels of different cytokines in CM-hAMTC and CM-hAM were determined using a multiple cytometric beads array system (CBA Flex set, BD Biosciences), according to the manufacturer's instructions. The following cytokines were measured: IL-2, IL-6, IL-8, tumor necrosis factor (TNF)-α, Fas-l, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CFS), interferon-inducible protein (IP)-10, monocyte chemoattractant protein (MIP)-1α and β, oncostatin M (OSM) and RANTES (regulated upon activation, normal T-cell expressed and secreted). Samples were acquired with a FACSAria and analyzed with FCAP Array software (BD Biosciences).
The levels of TGF-β and IL-10 were measured by sandwich enzyme-linked-immunosorbent assay (ELISA) using a commercially available coupled antibody (respectively 555052-555053, and 554499-554497 from BD Biosciences) according to the manufacturer's instructions. Concentrations were calculated from standards curves, and absorbance was measured at 405 nm with a microplate reader.

3.15 Kynurenine/tryptophan quantification

For these experiments the following were tested: CM-hAMTC, CM-hAM and CM derived from control MLR (CM-MLR) or from MLR performed in the presence of hAMTC (CM-MLR-hAMTC), with or without the addition of 0.5 mM DL-methyl-tryptophan (CM-MLR-hAMTC+MT), which were harvested after 5 days of culture.
One ml of phosphate buffer at pH 6.5 and 2 ml trichloroacetic-acid were added to 100 µl of CM samples. The different CMs were incubated for 30 min at 50°C and then centrifuged for 15 min at 4000 rpm. Two ml of the supernatant were then used for solid phase extraction (SPE) using OASIS® HLB cartridges 1cc. Elution was ensued with 90% acetonitrile. The eluate was then dried by nitrogen and re-dissolved in 200 µl mobile phase for

HPLC analysis. IDO activity was measured as the ratio between kynurenine and tryptophan concentration determined by HPLC.

3.16 Statistical analysis

Data are expressed as mean ± SD. Student's *t*-test were use to assess differences between groups. Raw P-values were adjusted by Holm-Bonferroni's procedure for multiple comparison and differences were considered statistically significant for p values < 0.05.

4. RESULTS

4.1 hAMTC phenotype and differentiation potential

4.1.1 hAMTC phenotypic characterization

Freshly isolated human amniotic mesenchymal tissue cells (hAMTC) were phenotypically characterised by FACS analysis. As shown in ***Table 4.1***, at passage 0 hAMTC showed high positivity for CD90, CD73, CD13 and CD44, low positivity for CD105, CD166, CD45, HLA-DR, CD14 and negativity for CD34.

Markers	hATMC p0
CD90	82±3%
CD73	66±6%
CD13	89±2%
CD44	47±18%
CD105	6±4%
CD166	14±4%
CD45	6±3%
HLA-DR	6±3%
CD34	1±1%

Table 4.1. Phenotypical FACS analysis of freshly isolated hAMTC*. Data are express as mean and standard deviation of positive cells percentage to considered markers.*

4.1.2 hAMTC osteogenic differentiation

In order to evaluate the differentiation potential of hAMTC, we induced these cells to undergo osteogenic differentiation *in vitro*.

After 3 weeks of culture on glass slides in the presence of osteogenic medium, hAMTC showed calcium deposition, which was visualized by positive staining for alizarin red and von Kossa, as shown in ***Figure 4.1A***. On the contrary, calcium deposits were not present in cells cultured in control medium (***Fig. 4.1A***). Furthermore, *in vitro* osteoblastic differentiation, evaluated by the presence of alkaline phosphatase (ALP) activity in cultured cells, was observed for hAMTC which had been cultured under osteogenic conditions, while hAMTC which had been cultured in control medium displayed no enzyme activity *(**Fig. 4.1A**)*.

In order to evaluate gene expression during *in vitro* osteogenic differentiation, we analyzed the mRNA levels of different markers which are specific to the three mesenchymal lineages (osteogenic, chondrogenic and adipogenic) in hAMTC cultured in osteogenic or control conditions.
Interestingly, freshly isolated hAMTC present with basal level expression of ALP, Col-I, Col-II, Sox9, PPAR-2γ and Musashi-1 *(**Fig. 4.1B**)*. Through mRNA expression analysis, we observed that, after the 3 weeks of culture, the expression of osteogenic-related genes (ALP and Col I) was upregulated in hAMTC which had been cultured in differentiation medium as compared to cells that had been cultured in control medium *(**Fig. 4.1B**)*. On the contrary, we observed a down regulation in the expression of chondrogenic-related genes (Sox9 and Col-II) in the osteogenic differentiated cells (***Fig. 4.1B***). Moreover, we found that the mRNA of Musashi-1, a putative stem cell marker, is present in freshly isolated cells, and its expression was maintained after 3 weeks of culture in control medium, but decreased dramatically after culture in osteogenic differentiation medium *(**Fig. 4.1B**)*. Unexpectedly, the PPAR-2γ (Peroxisome Proliferator Activated Receptor-2γ) gene, which has been reported to be specific to the adipogenic lineage, presented with a high level of expression in hAMTC which had been cultured in osteogenic medium *(**Fig. 4.1B**)*.

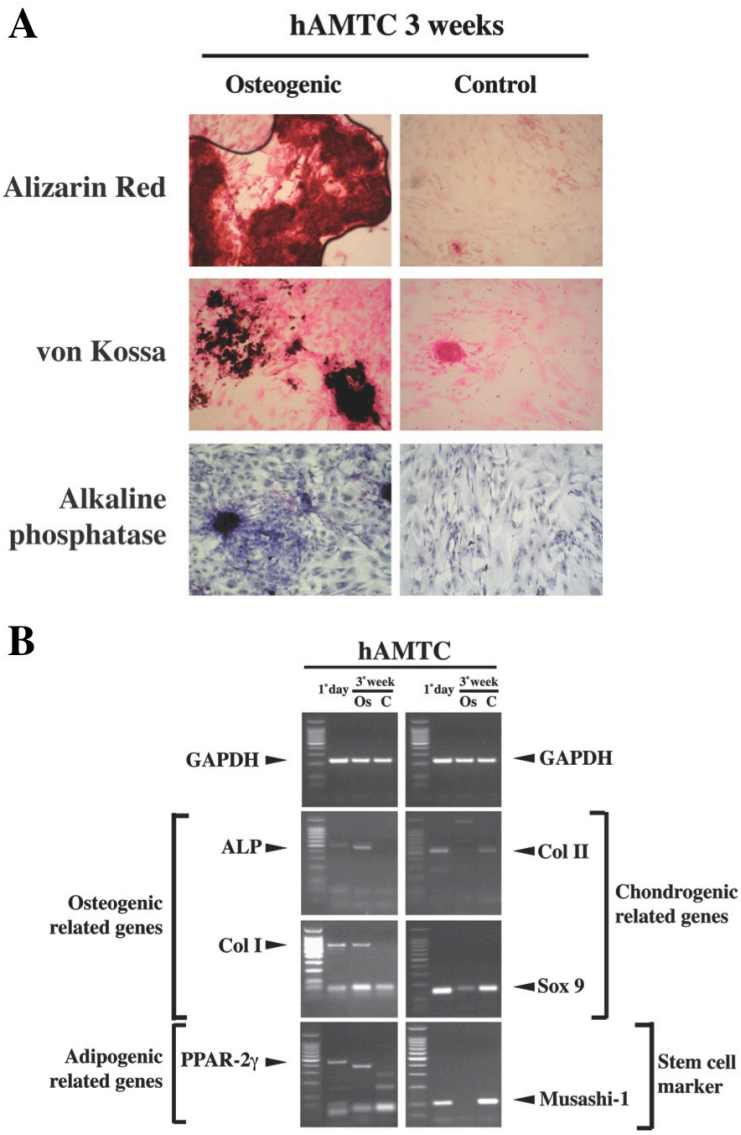


Figure 4.1. In vitro osteogenic differentiation of hAMTC. (A) *Representative cytological images of Alizarin red, von Kossa and Alkaline phosphatase staining of hAMTC cultured in presence of osteogenic or control medium for 3 weeks. (100x magnification).* ***(B)*** *Gene expression analysis on mRNA extracted from hAMTC on the day of cell isolation and after 3 weeks of culture in osteogenic or control medium. (ALP: alkaline phosphatase, Col I: collagen I, Col II: collagen II; Marker: 100bp DNA Ladder).*

In collaboration with Prof. Tanzi's group, we have evaluated the osteogenic differentiation potential of hAMTC which had been plated on 2 different polyuretan scaffolds, namely, EF PU foam or EF PU foam covered with Tri-Calcium-Phosphate (α-TCP).

At 3 weeks post-seeding, a clear adhesion of hAMTC to the pore surface of PU foam was observed, in particular on EF PU foam which had been covered with α-TCP *(**Fig. 4.2A**)*. Indeed, the pore surface was well colonized by the cells, which showed a well spread morphology *(**Fig. 4.2B**)*. Interestingly, alizarin red and von Kossa staining revealed calcium deposition in hAMTC plated on EF PU α-TCP even when the cells were cultured in the absence of osteoinductive medium. On the contrary, the cells which had been seeded on EF PU foam seems unable to produce a matrix containing high level of calcium, even when cultured under osteogenic induction conditions *(**Fig. 4.2A**)*. As shown in ***Figure 4.2A**,* the best osteogenic differentiation was observed in cells which had been seeded on EF PU foam covered with α-TCP and cultured in osteogenic medium, suggesting the important role of the scaffold during osteogenic differentiation.

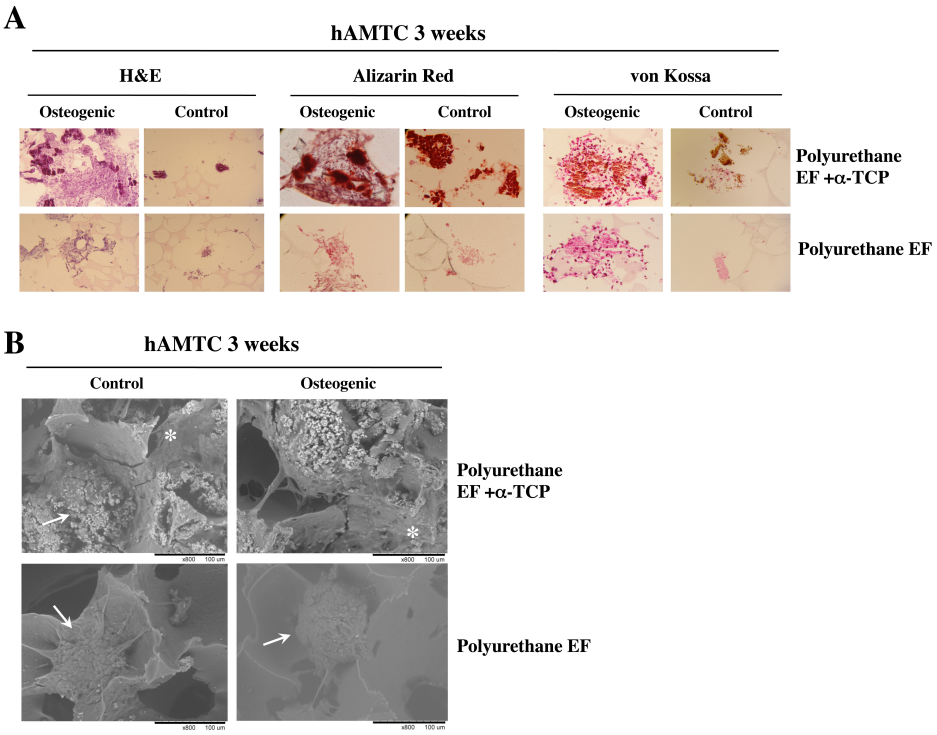


Figure 4.2. In vitro osteogenic differentiation of hAMTC plated on PUF and α-TCP-PUF. (A) *Representative histological images of hematoxylin-eosin, alizarin red and von Kossa staining of hAMTC cultured on α-TCP-PUF or PUF in presence of osteogenic or control medium for 3 weeks. (100x magnification).* ***(B)*** *SEM images of PUF or α-TCP-PUF 3 weeks after hAMTC seeding in osteogenic or control medium. Cells are indicated by arrows while the extracellular matrix (ECM) is matked with *.*

4.2 Inhibitory effect of human amniotic membrane derivatives

4.2.1 Inhibitory effect of hAMTC, CM-hAMTC and CM-hAM on lymphocyte proliferation

It has previously been demonstrated that freshly isolated hAMTC inhibit lymphocyte proliferation *in vitro* [4]. Here we confirm that, under our experimental conditions, these cells inhibit lymphocyte proliferation, both when added in contact and in transwell settings ***(Fig. 4.3)***, thereby suggesting that the anti-proliferative effects of hAMTC are associated with the production of yet unknown soluble factor(s). Herein we demonstrate that conditioned medium (CM) generated from the culture of freshly isolated non-stimulated hAMTC (CM-hAMTC) and from the culture of fragments of the amniotic membrane (CM-hAM), possess the ability to inhibit anti-CD3 stimulated-T lymphocyte proliferation in a dose dependent manner ***(Fig. 4.4A-B)***. We have also observed that when hAMTC are plated at lower concentration, the resulting CM present with lower inhibitory capacity when compared to CM derived from higher cell concentrations ***(Fig. 4.4A)***.

Furthermore, the addition of essential and non-essential amino acids and FBS (5%) to the different CMs generated from hAMTC did not change the CMs' inhibitory ability ***(Fig. 4.4C)***.

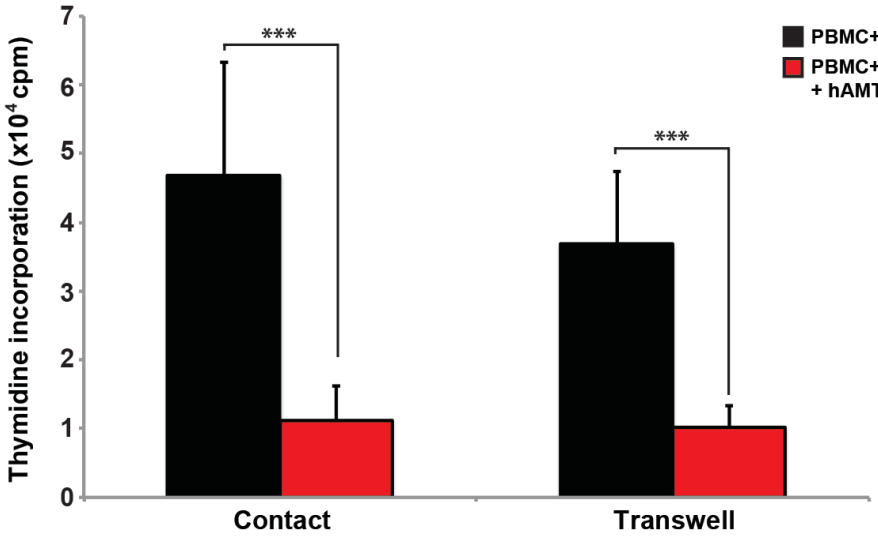
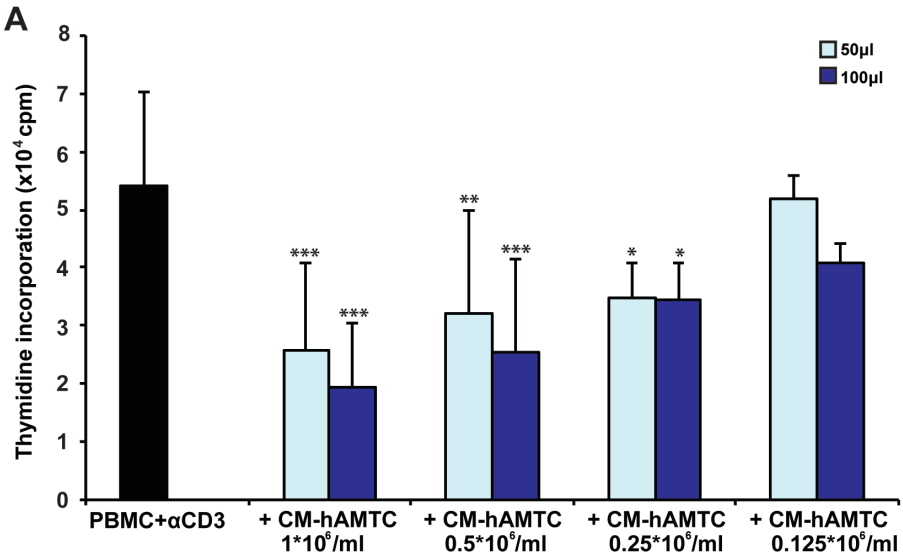


Figure 4.3. Analysis of the antiproliferative effects of hAMTC. *Effects of hAMTC on the proliferation of anti-CD3-stimulated lymphocytes (PBMC + αCD3) when added in a direct contact or transwell system. Data represent the mean and standard deviation (SD) of at least four independent experiments. ***=p<0.001.*



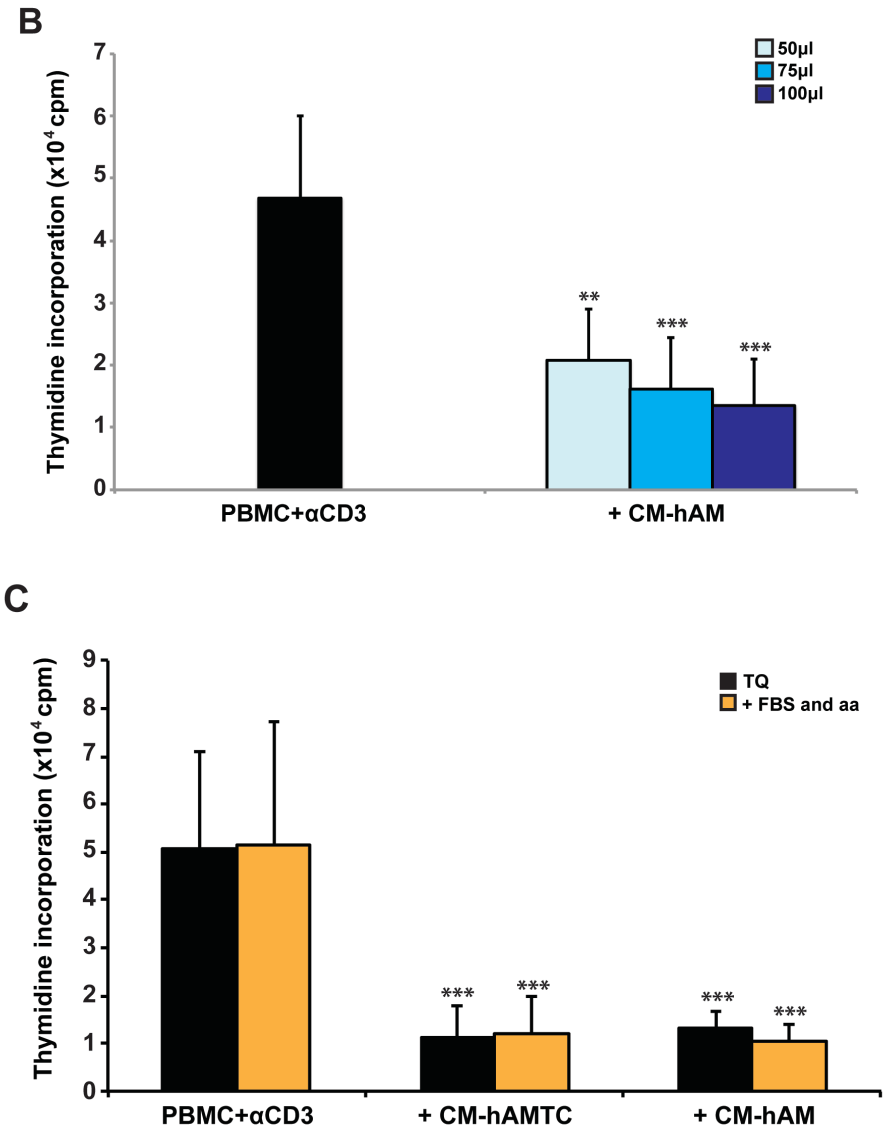


Figure 4.4. Analysis of the inhibitory effects of CM-hAMTC and CM-hAM. *Effects of the following on the proliferation of anti-CD3-stimulated lymphocytes (PBMC + αCD3):* ***(A)*** *different amounts (50-100 µl) of CM derived from hAMTC plated at different concentration;* ***(B)*** *different amounts (50-75-100 µl) of CM-hAM;* ***(C)*** *100 µl of control medium or CM with (orange bars) or without (black bars) the addition of FBS (5%), essential and non-essential aminoacids. Data represent the mean and standard deviation (SD) of at least six independent experiments. *=p<0.05, **=p<0.01, ***=p<0.001 versus PBMC + αCD3.*

Moreover we observed that both hAMTC at passage 3 and the CM derived from these cells (CM-hAMTC p3) maintain their anti-proliferative effects *(**Fig. 4.5**)*. In contrast, even though BM-MSC were also able to inhibit T cell proliferation at passage 3, CM derived from the culture of these cells was not able to reduce T cell proliferation *(**Fig. 4.5**)*, in accordance to previously reported findings [115,116].

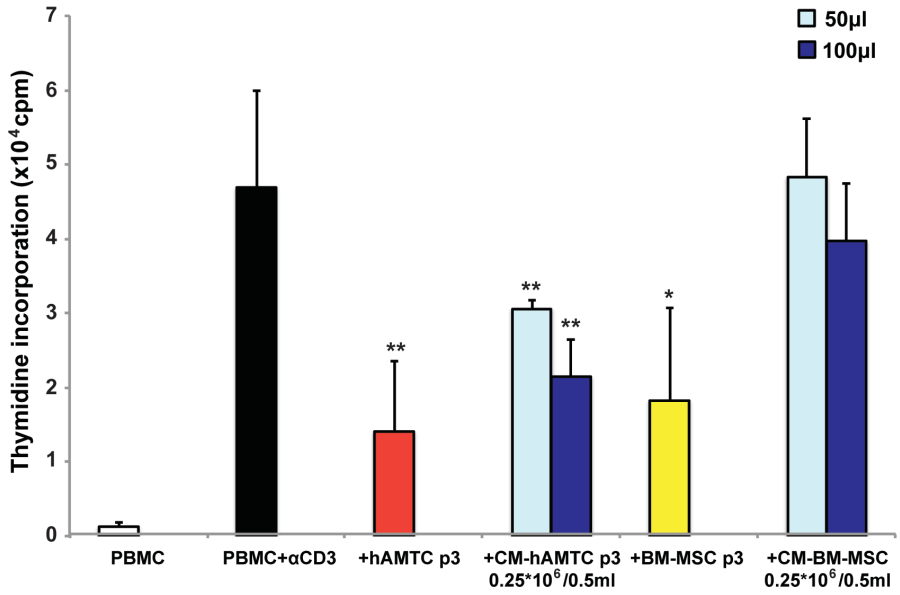


Figure 4.5. Analysis of the inhibitory effect of hAMTC p3, BM-MSC p3 and of the respective CMs. *Effects of the following on the proliferation of anti-CD3-stimulated lymphocytes (PBMC + αCD3): hAMTC at passage 3 (red bar), BM-MSC at passage 3 (yellow bar) and different amounts (50-100 µl) of CM derived from the culture of these cells. Data represent the mean and standard deviation (SD) of at least four independent experiments. *=p<0.05, **=p<0.01, ***=p<0.001 versus PBMC + αCD3.*

4.2.2 Inhibitory soluble factor(s) characterization

4.2.2.1 Cytokine quantification and neutralization assay

We firstly quantified the levels of a panel of inflammation-related molecules (see ***Table 4.2***). Both CM-hAMTC and CM-hAM contain TNF-α, OSM, RANTES, GM-CFS, G-CFS, IL-6, IL-8, MIP-1α, MIP-1β, and TGF-β, but not Fas-ligand, IL-2 and IP-10. In contrast to CM-hAMTC, CM-hAM does not contain IL-10.

Cytokine	UltraCulture		CM-hAMTC		CM-hAM	
	Median	MAD	Median	MAD	Median	MAD
TNF-a	5	4	44	38	49	43
Fas-l	0	0	8	8	6	6
G-CFS	0	0	2271	287	2789	190
GM-CFS	0	0	916	602	322	14
IL-2	12	2	17	4	25	7
IL-6	0	0	>10000	ND	>10000	ND
IL-8	0	0	2591	162	11527	673
IP-10	0	0	10	9	0	0
MIP-1α	0	0	12583	10006	7368	1095
MIP-1β	3	1	3838	1519	5493	898
OSM	0	0	190	174	70	37
RANTES	0	0	265	152	53	3
TGF-β*	0	0	464	204	209	209
IL-10*	4	4	285	155	9	9

MAD: Median Absolute Deviation
Values are expressed in pg/ml * quantified by ELISA

Table 4.2. Cytokine quantification *in UltraCulture medium, CM-hAMTC and CM-hAM.*

To evaluate the involvement of several cytokines known to elicit anti-proliferative effects when secreted by MSC from other sources (such as IL-10, IL-6, HGF, TGF-β) [92], we performed lymphocyte proliferation tests with CM-hAMTC and CM-hAM in the presence of neutralizing antibodies against IL-10, IL-6, HGF and TGF-β.

The neutralization assay revealed that HGF and TGF-β are not involved in the CM inhibitory mechanism ***(Fig. 4.6B-C)***. Meanwhile, the addition of neutralizing antibodies against IL-10 and IL-6 showed a minimal reversion

effect without reaching a statistical difference *(**Fig. 4.6, panel A and D**)*. Similar minimal reversion was seen also when we used a cocktail of neutralizing antibodies against IL-10, IL-6, HGF and TGF-β *(**Fig. 4.6E**)*.

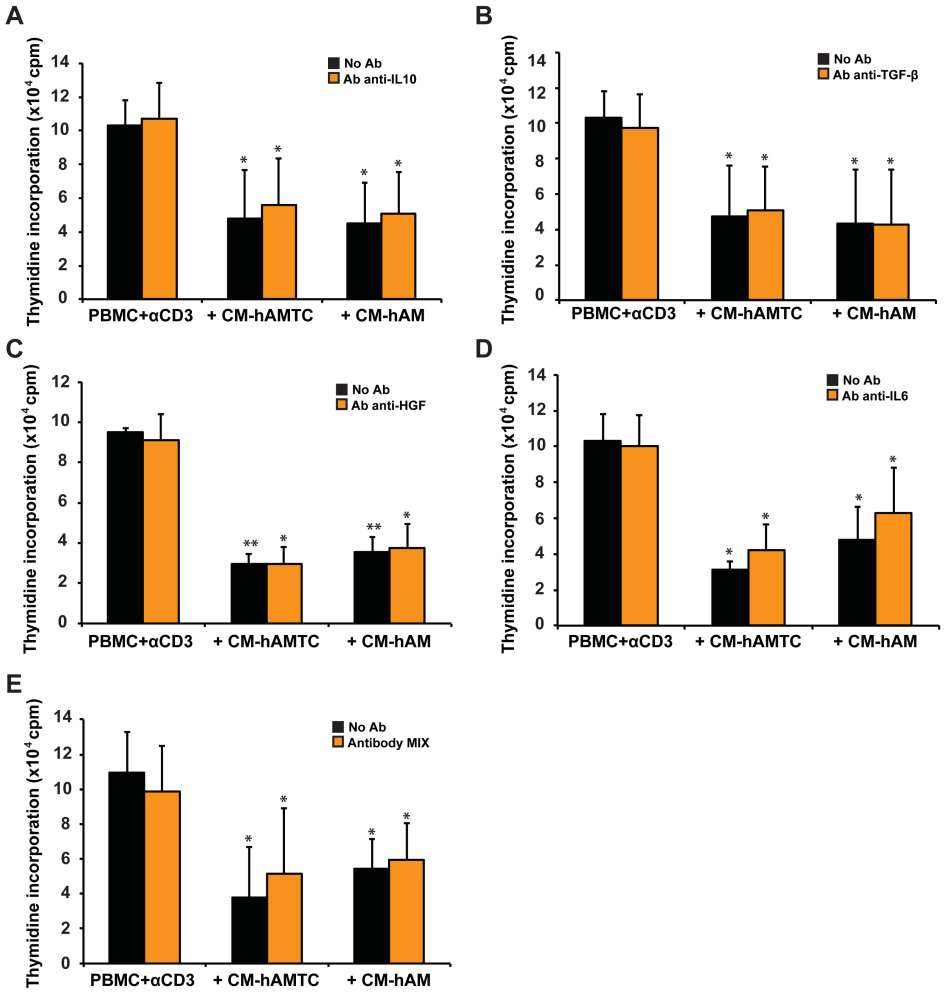


Figure 4.6. Inhibitory effect of CM-hAMTC and CM-hAM in presence of neutralizing antibodies. *Lymphocyte proliferation, stimulated with anti-CD3 (PBMC + αCD3), in presence of 100 µl of control medium or CM with (orange bars) or without (black bars) the addition of neutralizing antibodies against IL-10 **(A)**, TGF-β **(B)**, HGF **(C)**, IL-6 **(D)**, mix of antibodies anti-IL-10, -TGF-β, -HGF and -IL-6 **(E)**. Data represent the mean and SD of at least four independent experiments. *=p<0.05, **=p<0.01 versus PBMC + αCD3.*

4.2.2.2 Temperature stability analysis and proteinase K treatment

To investigate whether the inhibitory potential of CM-hAMTC and CM-hAM is temperature-sensitive, we studied the anti-proliferative action of these CMs after exposuring them to temperature changes. After three frost-defrost cycles, CM-hAMTC and CM-hAM showed the same inhibitory effect as the corresponding untreated CMs *(**Fig. 4.7A**)*. CMs treated for 30 min at 60°C or 80°C also still retained their anti-proliferative effect *(**Fig. 4.7B**)*. The inhibitory potential was also preserved when these CMs were stored at -80°C for more than 1 year (data not shown).

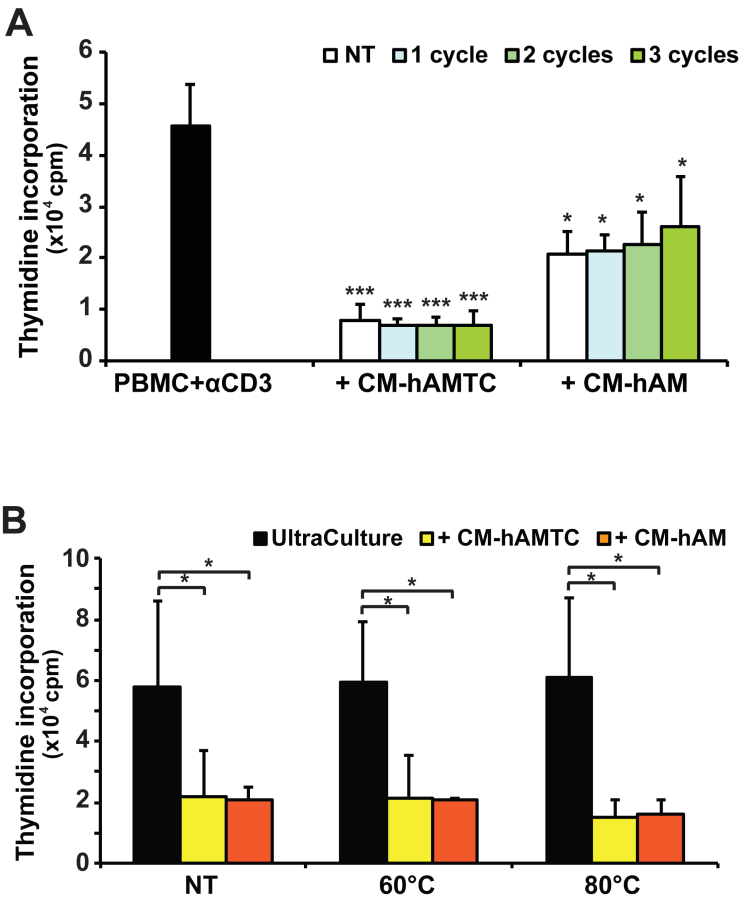
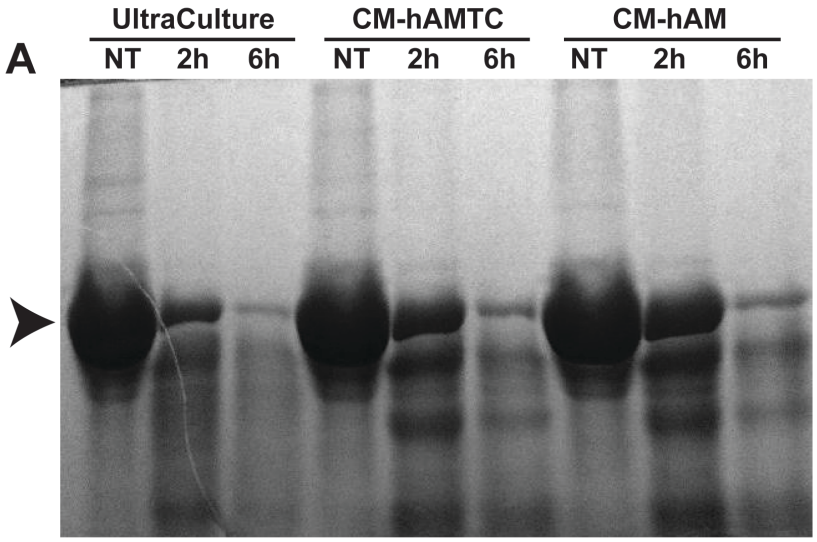


Figure 4.7. Effects of temperature changes on the anti-proliferative potential of CM-hAMTC and CM-hAM. *Effects of the following on the proliferation of anti-CD3-stimulated lymphocytes (PBMC + αCD3):* ***(A)*** *100 µl of untreated CM-hAMTC*

and CM-hAM (white bars) and of the corresponding CMs subjected to different frost-defrost cycles (coloured bars); ***(B)*** *100 µl of untreated (NT) control medium (complete UltraCulture), CM-hAMTC, CM-hAM, and after incubation for 30 min at 60°C or 80°C. Data represent the mean and SD of at least three independent experiments. *=p<0.05, ***=p<0.001 versus PBMC + αCD3.*

Moreover, to determine whether the inhibitory component(s) in CM-hAMTC and CM-hAM has a proteinaceous feature, we developed a protocol to remove protein molecules from CMs based on proteinase K treatment. In our experimental condition six hours of proteinase K treatment caused the digestion of more than 90% of proteins present in the two CMs *(**Fig. 4.8A**)*. Despite protein depletion, CM-hAMTC and CM-hAM maintained their anti-proliferative activity *(**Fig. 4.8B**)*.



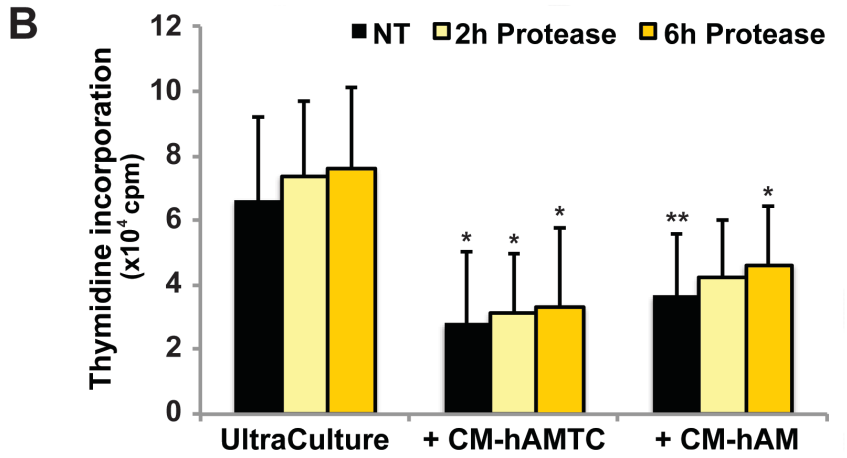


Figure 4.8. Effect of proteinase K treatment on the inhibitory activity of CM-hAMTC and CM-hAM. (A) *Representative SDS-PAGE of untreated and proteinase K-treated (for 2 and 6 hrs) UltraCulture medium, CM-hAMTC and CM-hAM. Digestion level was calculated as the reduction of the density of the BSA band (arrow).* ***(B)*** *Inhibitory effect on lymphocyte proliferation, induced by anti-CD3 stimulation, of 100 µl of untreated (NT) and proteinase K-treated UltraCulture medium, CM-hAMTC and CM-hAM. Similar results were also obtained using 50 µl of CMs (data not shown). Data represent the mean and SD of at least five independent experiments. *=p<0.05, **=p<0.01 versus UltraCulture.*

4.2.2.3 Molecular weight of the inhibitory factor(s)

To establish the potential molecular weight of soluble inhibitory factor(s) present in the CM-hAMTC we subjected the CM to column fractionation. All the obtained fractions were then tested for their effects on lymphocyte proliferation.

The fraction containing molecules with a molecular weight < 3 kDa *(**Fig. 4.9A, grey bars**)* was the only fraction maintaining an inhibitory effect. Meanwhile, fractions obtained by unconditioned medium and used as controls did not show any inhibitory effect *(**Fig. 4.9B**)*. The results represented are obtained using 100 µl of CM, however the same trend was observed using 50 or 75 µl of CM (data not shown).

The level of inhibition achieved with the fraction containing molecules < 3 kDa was significantly reduced with respect to the level of the unfractionated CM *(**Fig. 4.9A, white bar**)*.

In order to exclude the loss of components during fractionation protocol, all the fractions were re-pooled together and tested *(**Fig. 4.9A, CM-hAMTC fraction mix, white-grey-striped bar**)*. CM-hAMTC fraction mix showed the same anti-proliferative effect of the unfractionated CM *(**Fig. 4.9A, white bar**)*.

Then, to find out whether soluble factor(s) present in different fractions may cooperate to exert an inhibitory ability, we mixed the single fractions two by two and tested the effects of these new preparations on T cell proliferation. All the mixes containing the fraction < 3 kDa had an inhibitory effect *(**Fig. 4.9A, black-grey-striped bars**)*, whilst only the mix containing the fraction of molecules > 100 kDa and < 3 kDa exerted an inhibitory effect statistically higher than that of the fraction < 3 kDa alone. Furthermore, this mix had an inhibitory ability comparable to that of the unfractionated CM. The preparation obtained by mixing the fraction 100-30 kDa and the fraction < 3 kDa also had an inhibitory effect higher than the fraction < 3 kDa alone, but this difference did not reach a statistical significance.

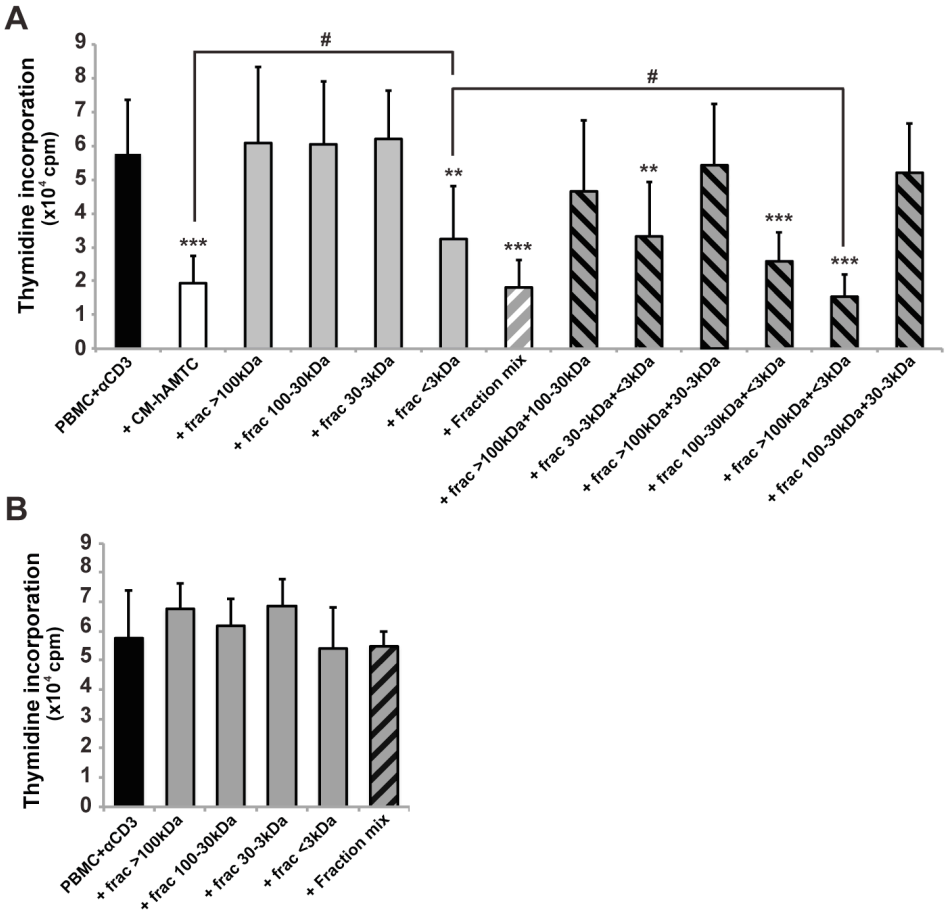


Figure 4.9. Study of the potential molecular weight of the inhibitory factor(s). ***(A)*** *Lymphocyte proliferation, stimulated by anti-CD3, in presence of 100 µl of CM-hAMTC (white bar), CM-hAMTC-fraction-mix (grey bars) or mix of fractions with different molecular weights (white-grey- and black-grey-striped bars).* ***(B)*** *Lymphocyte proliferation, stimulated by anti-CD3, in presence of 100 µl of complete UltraCulture medium (black bar) or its fractions (grey and black-grey-striped bars). Data represent the mean and SD from at least six independent experiments. **=p<0.01, ***=p<0.001 versus PBMC + αCD3; #=p<0.05.*

4.2.2.4 Evaluation of Prostaglandins, Nitric Oxide and IDO involvement in CM-hAMTC and CM-hAM inhibitory effect

Based on the results obtained from the studies of the molecular weight and the indication of the non-proteinaceous nature of the unknown inhibitory molecule(s), our next goal was to unravel the identity of these molecule(s). In order to do so, we evaluated the involvement of known actors in immunomodulation that have low molecular-weight and are not proteins, such as NO, kynurenine (a tryptophan metabolite) and PGs, in the anti-proliferative ability of CM-hAMTC and CM-hAM.

During CM production we therefore specifically inhibited: *i)* the NO synthesis, using L-NAME (L-Nitro-Arginine Methyl Ester), which is an inhibitor of NO synthase; *ii)* the activity of IDO, which is one of the key enzymes of kynurenine biosynthesis [117], using DL-methyl-tryptophan and *iii)* the PGs production, using cyclooxygenase (COX) inhibitors (such as indomethacin, ketoprofen and niflumic acid). CMs obtained after inhibition of these factors were then tested for their effect on activated T-lymphocyte proliferation.

First, we observed that both CMs contain a low amounts of NO, basically the same concentration as that found in the control medium *(**Fig. 4.10A**)*. In addition, CM derived from hAMTC or hAM cultured in presence of 1mM L-NAME had similar inhibitory effect of CMs derived from untreated samples *(**Fig. 4.10B**)*. Taken together, these data indicate that NO is not responsible for the inhibitory effect of CM-hAMTC and CM-hAM, neither directly nor as mediator for the production of other inhibitory factors.

The measurement of IDO activity in CM-hAMTC (determined as the ratio kynurenine/tryptophan) revealed that hAMTC possess IDO activity when cultured alone, which increases when the cells are co-cultured with a MLR (as measured in the CM-hAMTC-MLR) *(**Fig. 4.10C**)*. Despite the fact that the addition of DL-methyl-tryptophan during co-culture experiments dramatically decreases the IDO activity as measured in the CM (CM-MLR-hAMTC+MT) *(**Fig. 4.10C**)*, the inhibitory effect of hAMTC on lymphocyte

proliferation did not decrease in presence of DL-methyl-tryptophan *(**Fig. 4.10D**)*.

To further support the finding that the inhibitory effect of CM-hAMTC and CM-hAM seems independent of IDO activity, we added DL-methyl-tryptophan during preparation of CMs (i.e. during hAMTC and hAM cultures). These CMs showed comparable inhibitory ability with respect to the CMs derived from untreated samples *(**Fig. 4.10E**)*.

Quantification of prostanoids in CM-hAMTC and CM-hAM revealed that amniotic cells produce a high amount of COX products that could be partially responsible for the anti-proliferative effect of CM *(**Table 4.3**)*. Indeed, CM-hAMTC and CM-hAM contain PGE2 (390±146 ng/ml and 24±2 ng/ml, respectively), PGD2 (67±1 ng/ml and 6±1 ng/ml, respectively), and PGF2α (27±13 ng/ml and 10±1 ng/ml, respectively). The involvement of PGs in the CM's inhibitory effect was confirmed by the addition of COX-inhibitor during hAMTC or hAM culture. The indomethacin treatment decreased the concentration of all prostanoids in both CM-hAMTC and CM-hAM, as shown in ***Table 4.3***, and interestingly, the anti-proliferative ability of these CMs were partially reverted *(**Fig. 4.10F**)*. Similar results were also obtained using other COX inhibitors, such as ketoprofen and niflumic acid *(**Fig. 4.10G-H**)*. Even treatment with three COX inhibitors together (indomethacin, ketoprofen and niflumic acid) during CM-hAMTC and CM-hAM production did not lead to a complete reversal of inhibitory effect *(**Fig. 4.10I**)*.

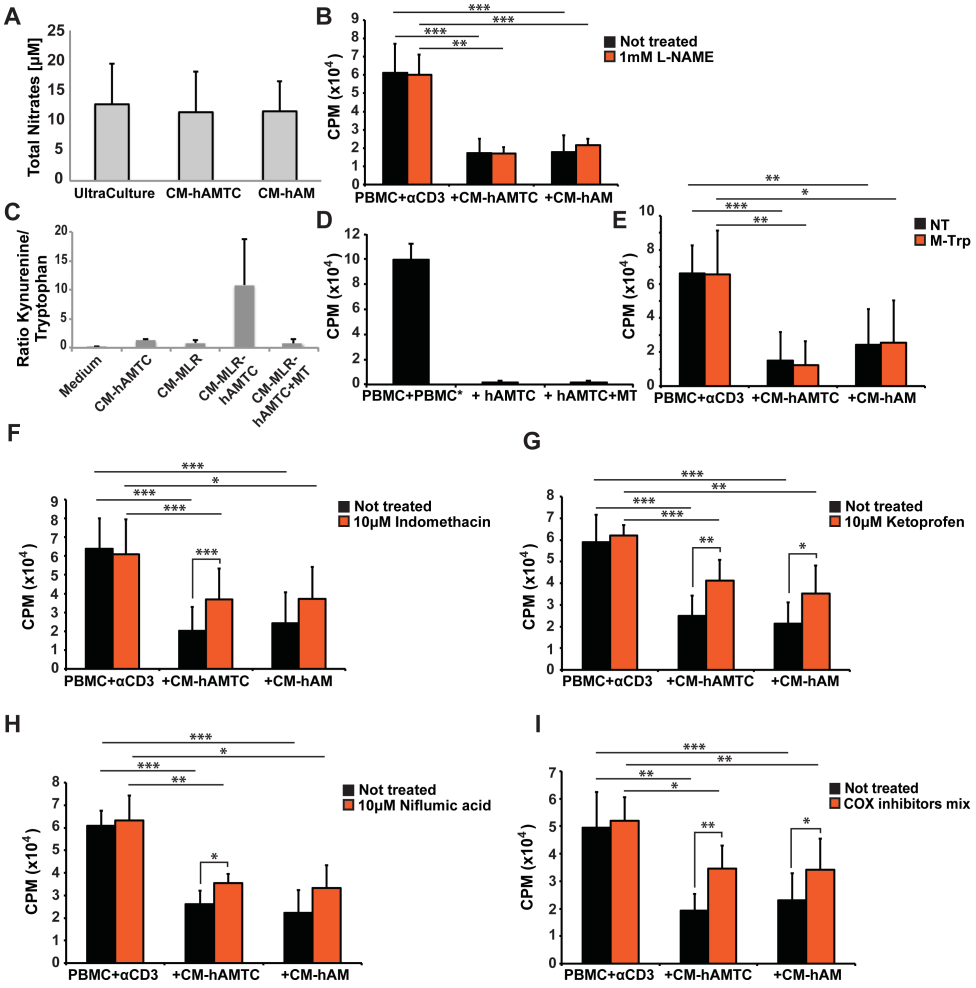


Figure 4.10. NO, kynurenine, and prostaglandins (PGs) in CM-hAMTC and CM-hAM. (A) *NO quantification (total nitrate quantification) in UltraCulture, CM-hAMTC, and CM-hAM performed by means of the Griess test.* ***(B)*** *Lymphocyte proliferation (expressed in cpm) after stimulation with anti-CD3 and in presence of 100 µl of complete UltraCulture medium, CM derived from untreated (black bar), or L-NAME-treated (white bar) hAMTC and hAM cultures.* ***(C)*** *IDO activity, shown as the ratio between the concentration of kynurenine and tryptophan, in the control medium, CM-hAMTC, CM-MLR or CM-MLR-hAMTC, and in the presence of 0.5 mM DL-methyl-tryptophan (CM-MLR-hAMTC+MT).* ***(D)*** *Lymphocyte proliferation (expressed in cpm), induced by MLR, in the presence of hAMTC cultured with or without the addition of DL-methyl-tryptophan.* ***(E)*** *Lymphocyte proliferation (expressed in cpm), stimulated with anti-CD3, in presence of 100 µl of control*

medium or CM derived from untreated (black bar) or 0.5mM DL-methyl-tryptophan-treated (white bar) hAMTC and hAM cultures. ***(F-I)*** *Lymphocyte proliferation (expressed in cpm) after stimulation with anti-CD3, in presence of 100 µl of control medium or CM derived from untreated (black bar) or indomethacin-* ***(F)****, ketoprofen-* ***(G)****, niflumic acid-* ***(H)*** *or inhibitors-mix-* ***(I)*** *treated (white bars) hAMTC and hAM cultures. Data represent the mean and the SD of at least three independent experiments. *=p<0.05, **=p<0.01, ***=p<0.001.*

Prostanoids	UltraCulture	CM-hMATC		CM-hAM	
	NT	NT	Indomethacin treated	NT	Indomethacin treated
Total prostanoids	<1	542±280	5.8±4	69±6	16±5
PGE2	<1	390±146	<1	24±2	<1
PGD2	<1	67±1	<1	6±1	<1
PGF2α	<1	27±13	<1	10±1	<1

Values are expressed in ng/ml

Table 4.3. Prostanoids quantification *in UltraCulture medium and CM derived from untreated or indomethacin treated hAMTC and hAM.*

In order to determine if PGs are partially or entirely responsible for the CMs' inhibitory effect, we added exogenous PGs to anti-CD3 stimulated T cells. While the addition of PGI2 did not inhibit T cell proliferation, the addition of PGF2α, PGD2 and PGE2 had a slight inhibitory effect which was detectable when used at concentrations of 10000 ng/ml, 1000 ng/ml and 5 ng/ml, respectively. Thus, PGF2α and PGD2 display an inhibitory effect when used at a higher concentration than that we observed in CMs *(**Fig. 4.11A** and **Table 4.3**)*, suggesting the lack of involvement of these in CM inhibitory effect. Meanwhile, PGE2 induces a slight reduction of T cell proliferation also when used at concentration comparable to that found in CM-hAMTC (390±146 ng/ml) and CM-hAM (24±2 ng/ml) *(**Fig. 4.11A** and **Table 4.3**)*. Interestingly, the addition of PGE2 induced a consistent and

unchanging inhibitory effect on cell proliferation in the range between 1000 ng/ml and 25 ng/ml *(**Fig. 4.11A**)*.

With the aim to mimic the composition of PGs present in CM-hAMTC or in CM-hAM, and to evaluate if there could be some cooperation between the different PGs, we sought out to determine the effects that a mix of PGs has on T cell proliferation. As shown in ***Figure 4.11B-C*** the addition of a mix of PGs (PGE2 500 ng/ml + PGD2 100 ng/ml + PGF2α 25 ng/ml or PGE2 25 ng/ml + PGD2 6 ng/ml + PGF2α 10 ng/ml) on stimulated PBMC inhibited the cell proliferation, but clearly at lower than that observed with CM-hAMTC and CM-hAM.

To further address the role of PGs in the CMs' inhibitory effect, we also added PGs to anti-CD3 stimulated T-cells in the presence of CMs prepared following COX inhibition. We observed that the addition of a mix of PGs restored the inhibitory effect of the COX-inhibited CMs to approximately the level observed with CM from untreated sample *(**Fig. 4.11B-C**)*. This suggests that some other factor(s) present in the CMs could partecipate with PGs to the CMs' inhibitory effects.

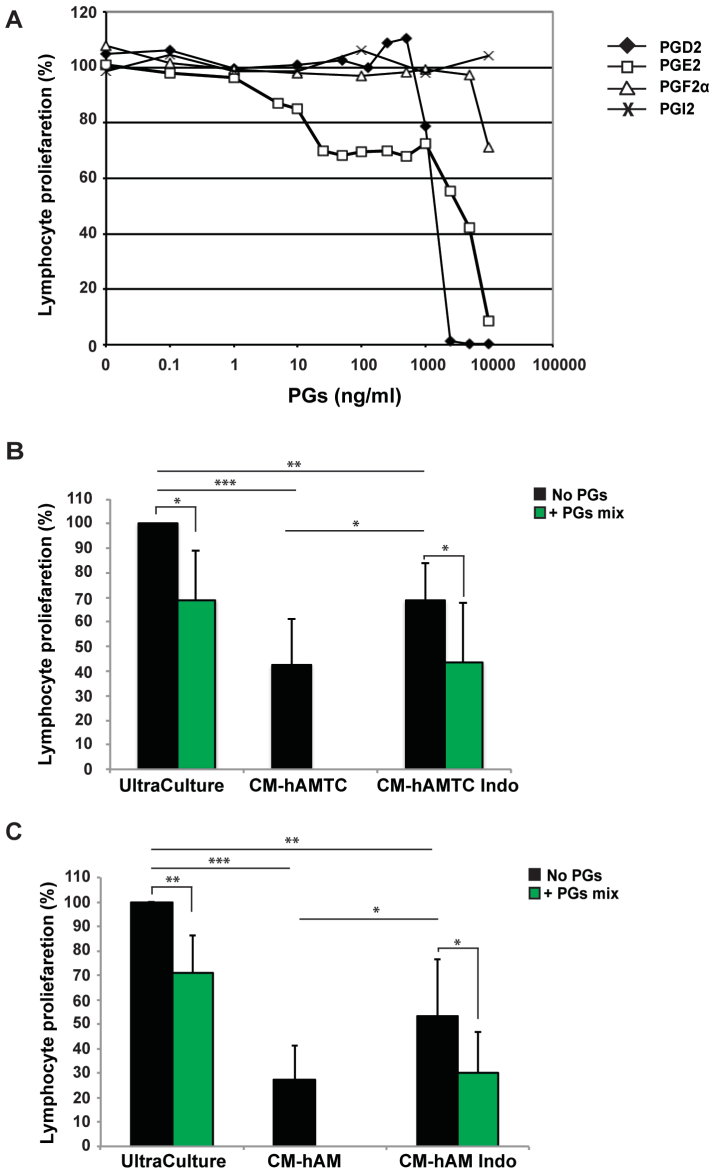
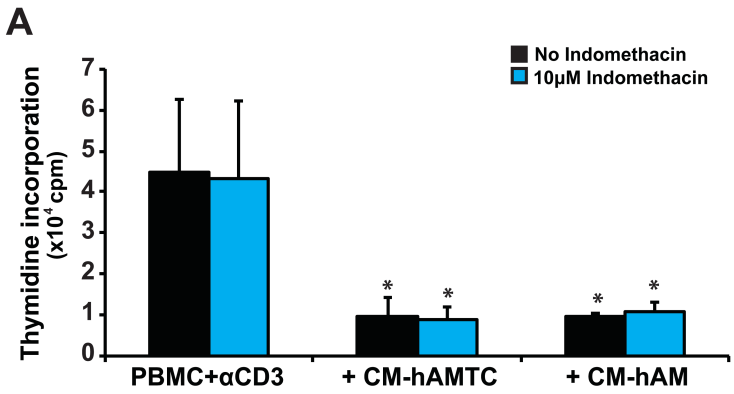


Figure 4.11. Effect of PGs on the proliferation of stimulated PBMC in presence or absence of CMs obtained from hAMTC or hAM treated or not with indometahcin. (A) *Lymphocyte proliferation, stimulated with anti-CD3, in presence of different amount of prostaglandin (PGs): PGD2 (◆), PGE2 (□), PGF2α (Δ) or PGI2 (X).* ***(B)*** *Lymphocyte proliferation, stimulated with anti-CD3, in presence of 100 µl of control medium (UltraCulture) or CM derived from untreated*

(CM-hAMTC) or indomethacin treated (CM-hAMTC Indo) hAMTC with (green bars) or without (black bars) the addition of a mix of PGs (PGE2 500 ng/ml + PGD2 100 ng/ml + PGF2α 25 ng/ml). ***(C)*** *Lymphocyte proliferation, stimulated with anti-CD3, in presence of 100 µl of control medium (UltraCulture) or CM derived from untreated (CM-hAM) or indomethacin treated (CM-hAM Indo) hAM with (white bars) or without (black bars) the addition of a mix of PGs (PGE2 25 ng/ml + PGD2 6 ng/ml + PGF2α 10 ng/ml). *=p<0.05, **=p<0.01, ***=p<0.001.*

4.2.2.5 Evaluation of CM-hAMTC and CM-hAM effect on PGs, NO and kynurenine production in leukocytes

After having studied which molecules are directly released by hAMTC or hAM and associated with inhibitory effect, we questioned whether the CM inhibitory effect could be due to the secretion of these immunomodulatory molecules from PBMC. We therefore added CM-hAMTC or CM-hAM on PBMC stimulated with anti-CD3 in the presence of the inhibitors indomethacin, L-NAME or DL-methyl-tryptophan. The addition of indomethacin did not shown any difference in the proliferation level *(**Fig. 4.12A**)*, suggesting that PBMC are not induced to produce PGs in presence of CMs. Similar results were obtained after the addition of L-NAME and DL-methyl-tryptophan *(**Fig. 4.12B-C**)*, suggesting the lack of involvement of IDO and NO in immunomodulation of the CMs.



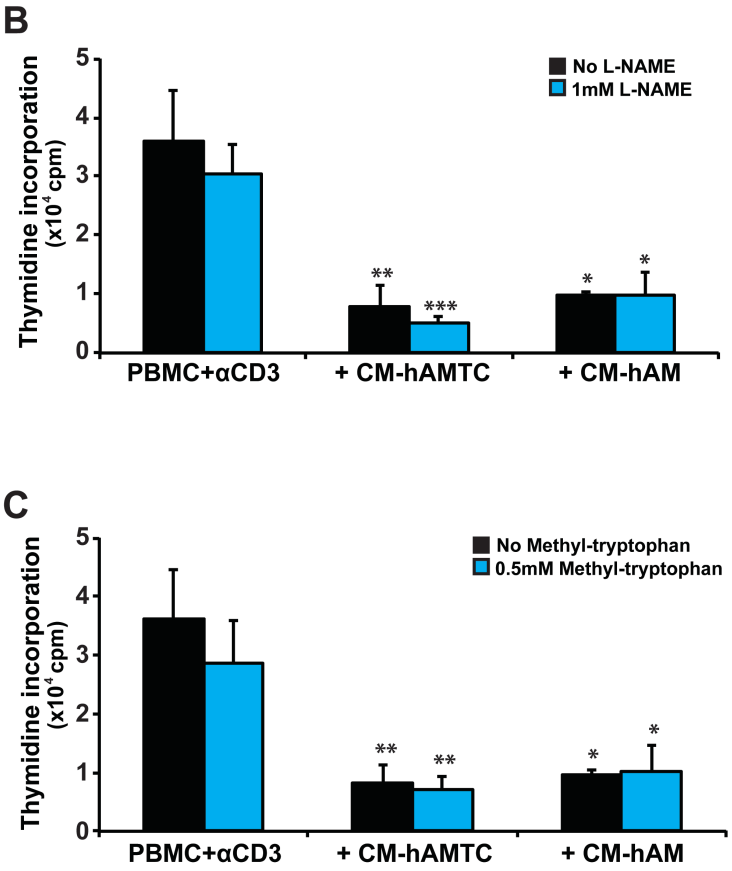


Figure 4.12. Inhibition of production of NO, kynurenine, and prostaglandins (PGs) in PBMC culture. *Lymphocyte proliferation, stimulated with anti-CD3, in presence of 100 µl of control medium or CM-hAMTC and CM-hAM with (blue bars) or without (black bars) the addition of indomethacin* ***(A)****, L-NAME* ***(B)*** *or DL-methyl-tryptophan* ***(C)****. Data represent the mean and SD of at least three independent experiments. *= p<0.05, **= p<0.01, ***= p<0.001 versus PBMC + αCD3.*

5. DISCUSSION

In this study we have demonstrated that soluble factors released in CM from the cultures of hAMTC and hAM possess the ability to inhibit T lymphocyte proliferation. Importantly, hAMTC and hAM were not stimulated prior to CM collection. This is in contrast to MSC derived from BM [115,116], which possess an anti-proliferative ability only when cultured in the presence of activating stimuli, such as IL-1β, TNF-α or IFN-γ [93]. This underlines the functional differences when using MSC from different sources, most likely due to the heterogeneity of the MSC populations.

In our study, aimed at defining the inhibitory factor(s) produced by hAMTC, we began with the analysis of IL-6, IL-10, TGF-β and HGF, which are already documented to be released by and involved in the immunoregulatory activity of MSCs from other sources [94,118-121]. We isolated hAMTC from over 80 donors, produced CM from each cell population/donor and used them in our analyses. Specific inhibition of selected molecules with neutralizing antibodies revealed that HGF and TGF-β do not participate in the anti-proliferative effect exerted on T lymphocytes by CM-hAMTC and CM-hAM, while neutralization of IL-6 and IL-10 showed a slight reversion which did not reach significative difference.

In order to characterize the nature of the factor(s) in question, we analyzed the thermostability, proteinaceous composition, and the molecular weight of molecules contained in the CM-hAMTC. We have demonstrated that the inhibitory factor(s) are temperature-stable, possess a small molecular weight (<3 kDa) and are most likely of a non-proteinaceous nature. We favour the non-proteinaceous nature of some of these compounds because CM treatment with protease which induced 90% protein degradation still did not result in a significant reduction in the capability of the CM to decrease T-cell proliferation. These observations brought us to focus on the potential involvement of small molecules. Some authors have reported that NO and IDO play critical roles in the suppression of T-cell proliferation when using

BM-MSC [122,123]. Our results clearly demonstrate that these factors are not involved, neither directly nor as mediators in the production of other inhibitory factors, as proven by blocking their synthesis pathways using selective inhibitors for IDO and NO synthase (*e.g.* methyl-tryptophan and L-NAME, respectively). No effects were seen either when the inhibitors were used on hAMTC during CM production, or during the incubation of CM-hAMTC with CD3-stimulated PBMC, thus implying that PBMC do not produce NO or IDO in reaction to (after coming in contact with) stimuli found in the CM of hAMTC.

Interestingly, we have shown that hAMTC and hAM release PGs in culture and that these are responsible for a significant part of the inhibitory effect. Moreover, the chemical-physical nature of PGs is in agreement with the high stability and low molecular weight of the factors we characterized in CM-hAMTC. Nevertheless, treatment with COX inhibitors was unable to completely abolish the inhibitory effect of CM-hAMTC and CM-hAM, suggesting the involvement of other factors. Consistent with this hypothesis is the fact that the addition of a mix of PGs (that mimics those present in CM) on stimulated PBMC induces an inhibition of proliferation but at a lower level than that observed with CM-hAMTC and CM-hAM. Furthermore, we have shown that the addition of a mix of PGs to anti-CD3 stimulated T cells in the presence of CM prepared following COX inhibition rescues the inhibitory effect to the level observed with CM from untreated hAMTC. Taken together, these results indicate that PGs are just one of the key effector molecules involved in the immunomodulatory activity of the amniotic membrane. The influence of other factors is also suggested by our investigations on the molecular weight of soluble factors in CM-hAMTC. Indeed, taken individually, the only fraction able to significantly decrease lymphocyte T cell proliferation was the one < 3 kDa, even though not to the extent of the unfractionated CM-hAMTC, thus suggesting that other components are also responsible for the inhibitory effect. The inhibitory effect obtained with unfractionated or the pool of fractions was also

observed by mixing the two fractions < 3 kDa and >100 kDa, suggesting the contribution of molecules within these molecular weight ranges to the immunomodulatory effect of the CM from the amniotic-derived cells.

Some reports suggest that factors such as NO, PGs, and IDO activity are usually active in a multi-cellular environment, which includes not only MSC but also PBMC or other cell types [124,125]. In order to distinguish which cells could produce PGs (MSC or PBMC), we used COX inhibitors both during the generation of CM from hAMTC and from hAM, and also during the culture of the generated CM (CM-hAMTC and CM-hAM) with stimulated PBMC. We observed that PGs are produced and detected in CM-hAMTC and CM-hAM, while it is not produced by PBMC stimulated by the CM.

It is noteworthy that at present we have performed our analyses only with CM derived from unstimulated cells. This is because our first aim was to understand which inhibitory factor(s) are produced by hAMTC in the absence of particular stimuli or particular culture conditions. Eventually, in follow-up studies we aimed to determine for the conditions that can change/ enhance the spectrum of factors released by these cells. Actually, it has been reported that the exposure of human amniotic mesenchymal cells to inflammatory stimuli (such as to the action of IFN-γ or IL-1β), induces changes in the expression of some cellular surface markers [126,127] and the upregulation of secretion of factors involved in inflammation (such as TGF-β [128], IDO [127,128], IL-8 [126], IL-6 [126], serpin E1 [126], IP10 [126], sCD54 [126], MCP-1 [126] and PGs [128,129]). Moreover, this exposure also increases the anti-proliferative ability of human amniotic mesenchymal cells when co-cultured in contact with PBMC [126,127]. These fragmented results suggest that the state of activation of amnion-derived mesenchymal cells may also affect their capability to modulate cell proliferation. Likewise, we have previously demonstrated that hAMTC inhibit the proliferation of T lymphocytes [4] and also of cancer cell lines from hematopoietic and non-hematopoietic origin [106], in both cell-cell contact and transwell co-cultures, and with a higher level of inhibition in a

cell-cell contact settings. It could be possible that the contact between cells leads to the release of some other factors involved in the inhibition of the lymphocytes proliferation. Therefore, it remains to be elucidated if there is any qualitative and/or quantitative difference in the pattern of components of the supernatants, including PGs, when obtained from stimulated cells and from cells that are cultured in cell-cell manner.

This study did not reveal any appreciable differences concerning the inhibition of lymphocyte proliferation between the CM derived from the whole amniotic membrane and the CM derived from cells isolated from the membrane. This suggests that the amniotic membrane could be an interesting source of soluble factors, without referring to extensive cell preparation. Even though the presence of the cells derived from both the mesenchymal and the epithelium layers of amniotic membrane might contribute to produce a supernatant with different composition.

In conclusion, in this study we have shown for the first time that the anti-proliferative effect of hAMTC is not related to the presence of external stimuli. hAMTC and hAM are indeed able to release soluble factors with inhibitory effects when cultured in non-stimulated conditions, proving that the effect is an intrinsic characteristic. Moreover, we have also provided evidence that this effect seems to be mediated by low molecular weight, non-protein, thermostable compound(s) present in CM-hAMTC and CM-hAM. Finally we provide evidence that prostaglandins are one of the key effector molecules in the immunomodulatory activity of the human amniotic membrane, while this activity minimally involves IL-6 and IL-10, but not IDO, NO, TGF-β or HGF instead proposed to be involved in anti-proliferative action of MSC of other sources.

6. FUTURE PROSPECTS AND BIOMEDICAL APPLICATIONS

Further studies are clearly necessary for complete characterization of the soluble factors released by mesenchymal cells derived from the amniotic membrane. Indeed, full identification of the factors involved in the antiproliferative effects of hAMTC will undoubtedly be essential for a complete understanding of the mechanisms responsible for this phenomenon.

Characterization of these mechanisms will be of particular importance for the development of future clinical approaches using soluble factors derived from mesenchymal cells of the amniotic membrane, in particular for the treatment of diseases characterized by an altered inflammatory response.

Moreover, of particular significance is the possibility that a cell-free treatment based on the use of CM-hAMTC could potentially replace cell transplantation, particularly when tissue repair via paracrine bioactive molecules is required. This strategy also offers a series of added advantages:

- CM-hAMTC can be produced easily and in large quantities;
- it can be stored efficiently and easily;
- as a cell-free treatment, it can drastically reduce the risk of adverse immunological reactions, infectious risks and other potential long-term negative effects associated with the presence of exogenous cells;
- CM-hAMTC could be administered easily via intravenous injection, avoiding clot formation and capillary entrapment.

In this context, further study of the CM-hAM as a tool for regenerative medicine is also warranted due to its low production cost and minimal requirement for manipulation in order to obtain soluble factors.

In addition, the study of how different stimulatory environments may modulate the production of immunomodulatory factors by amnion-derived cells also represents another avenue of interest, as it is conceivable that the anti-proliferative efficacy of supernatants could be optimized through

manipulation of hAMTC culture conditions (eg. concentration, days of culture, cytokine stimulation, etc.).

Finally, studies both *in vitro* and in selected animal models will no doubt provide valuable information regarding the immunomodulatory effects of the CM-hAMTC and CM-hAM compared to those observed when using cells isolated from the amniotic membrane.

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And now I prefer to switch to Italian for the other thanks.

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9. ANNEXES

Scientific production relative to present work

- **Rossi D.**, Pianta S., Parolini O. *Characterization of the conditioned medium from amniotic membrane cells: prostaglandins as key effectors of its immunomodulatory activity.* PLoS ONE 2012; **7** (10).
- Cargnoni A., Ressel L., **Rossi D.**, Poli A., Arienti D., Lombardi G., Parolini O. *Conditioned medium from amniotic mesenchymal tissue cells reduces progression of bleomycin-induced lung fibrosis.* Cytotherapy. 2012 Sep; **14** (2):153-161.
- Bertoldi S., Farè S., Denegri M., **Rossi D.**, Haugen H.J., Parolini O., Tanzi MC. *Ability of polyurethane foams to support placenta-derived cell adhesion and osteogenic differentiation: preliminary results.* J Mater Sci Mater Med. 2010 Mar; **21** (3):1005-1011.

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- Magatti M., Cargnoni A., **Rossi D.,** Vertua E., Bonassi Signoroni P., De Munari S., Ricci E., Cotti Piccinelli E., Pianta S., Parolini O. *Human term placenta: a new source of stem cells for regenerative medicine?* J Cancer Res Ther. 2012 Jan; **8** (S1):S7.
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- **Rossi D.***, Bertoldi S.*, Farè S., Denegri M., Parolini O., Tanzi M.C. *Ability of polyurethane foams to support human placenta-derived mesenchymal cell adhesion and osteogenic differentiation.* First meeting of the International Placenta Stem Cell Society (IPLASS): "From fetomaternal tolerance to immunomodulatory properties of placenta-derived cells in cell therapy", Brescia, October 3rd - 6th, 2010.