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IMMUNOLOGICAL PROPERTIES OF CD117^{+} AMNIOTIC FLUID STEM CELLS OBTAINED DURING DIFFERENT TRIMESTERS OF GESTATION

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SUMMARY

ABSTRACT

Amniotic Fluid Stem (AFS) cells are multipotent stem cells achievable through the positive selection and ex-vivo expansion of CD117 (c-Kit)-expressing cells derived from amniotic fluid. Given the broad differentiation potential toward adipogenic, osteogenic, myogenic, endothelial, neuronal and hepatic lineages, AFS cells have raised great interest as new therapeutic tool. However, their immunogenicity and immunomodulatory properties need to be assessed before clinical use. To this aim, we analyzed the immunological effects resulting from the interaction between AFS cells of different gestational age and a number of immune effector cells (IECs), i.e. T, B and NK cells.

Resting 1st trimester-AFS cells showed lower expression of HLA class-I molecules and NK-activating ligands than 2nd and 3rd trimester-AFS cells. This feature was associated to lower sensitivity of 1st trimester-AFS cells to NK cell-mediated lysis. Nevertheless, inflammatory priming of AFS cells by IFN-γ and TNF-α enhanced the resistance of all AFS cell types to NK cell cytotoxicity.

AFS cells modulated lymphocyte proliferation in a different manner according to gestational age: 1st trimester-AFS cells significantly inhibited T and NK cell proliferation, while 2nd and 3rd trimester-AFS cells were less efficient. In addition, only inflammatory-primed 2nd trimester-AFS cells could suppress B cell proliferation, which was on the contrary unaffected by the other AFS cells.

Indolamine 2,3 dioxygenase (IDO) pathway was not significantly involved in 1st trimester-AFS cells-mediated T cell suppression, while it was the main inhibitory mechanism in 2nd and 3rd trimester-AFS.

Overall, this study revealed a number of significant qualitative and quantitative differences among AFS cells of different gestational age in terms of phenotype, immunological functions and immunogenicity, which all have to be taken into consideration in view of AFS cell clinical application.

INTRODUCTION

Stem cell-based regenerative medicine, aimed at generating biological substitutes and improving tissue functions (tissue engineering), has been recently suggested as a potential alternative to current therapeutic modalities, such as medical therapy, surgery, organ transplantation, and mechanical devices. Three essential factors are necessary for tissue engineering: stem cells retaining the capacity to self-renew and restore damaged tissues due to their high ability to proliferate and differentiate; the use of scaffolds supporting stem cell survival, proliferation and differentiation; the use of growth and differentiation factors to favor stem cell functions.

Stem cells isolated from adults or developing embryos (embryonic stem cells) are currently considered a source of cells for regenerative medicine. However, despite their therapeutic potential, both adult and embryonic stem cells present a number of limitations to their clinical application[1]. For instance, not always an adequate number of autologous adult stem cells can be easily achieved from the patient. On the other hand, human embryonic stem cells can quickly proliferate in culture and differentiate into a large number of adult tissues, but their growth is still poorly prone to be controlled and leads inevitably to tumor formation; in addition, the expression of embryonic and fetal antigens may elicit immune alloreaction and graft rejection; furthermore, the ethical issues related to the use of embryos are still unsolved, although some promising improvements will probably come from the use of induced pluripotent stem cell (iPSC) technology.

Fetal stem cells are a relatively new source in the field of stem cell research, exhibiting unique and fascinating features belonging to both embryonic and adult stem cells, and therefore representing a new putative strategy for regenerative medicine. These cells can be obtained from the fetus or extra embryonic tissues, such as amniotic fluid[2] **[Figure1]**. The latter represent an appealing choice due to easiness of cell harvesting during pregnancy without affecting fetal tissue integrity.

AMNIOTIC FLUID

Amniotic fluid allows the fetus to freely grow and move inside the uterus and acts as a vehicle for the exchange of body chemicals with the mother. In humans, the amniotic fluid starts to appear at the beginning of week 2 of gestation as a small film of liquid between the cells of the epiblast. Between days 8 and 10 after fertilization, this fluid gradually expands and separates the epiblast from the amnioblast, thus forming the amniotic cavity. Thereafter, it progressively increases in volume, completely surrounding the embryo after week 4 of pregnancy. Over the course of gestation, amniotic fluid volume changes markedly from 20 ml in week 7 to 600 ml in week 25, 1.000 ml in week 34 and 800 ml at birth. During the first half of gestation, the amniotic fluid results from the active sodium and chloride transport across the amniotic membrane and the non-keratinized fetal skin, with concomitant passive movement of water. In the second half of gestation, the amniotic fluid is represented by fetal urine, gastrointestinal excretions, respiratory secretions and substances exchanged through the sac membranes.[3]

The amniotic fluid is primarily composed of water and electrolytes (98-99%) but also contains chemical substances (e.g. glucose, lipids, proteins, hormones and enzymes), suspended materials (e.g. vernix caseosa, lanugo hair and meconium) and cells.

Cells begin to appear in the amniotic fluid at about 14 weeks of gestation and the total number increases steadily during pregnancy, although the proportion of viable cells decreases. Amniotic fluid cells derive both from extra-embryonic structures (i.e. placenta and fetal membranes) and from embryonic and fetal tissues. Although amniotic fluid cells are known to express markers of all three germ layers, their origin is still debated; they mainly consist of cells shed in the amniotic cavity from the developing skin, respiratory apparatus, urinary and gastrointestinal tracts. **[Figure 2A]** Amniotic fluid cells display a broad range of shapes and functions, varying with gestational age and fetal development. In normal conditions the number of amniotic fluid cells increases with gestation; if a fetal disease is present, amniotic fluid cell counts can be either dramatically reduced (e.g. intrauterine death and urogenital atresia) or abnormally elevated (e.g. anencephaly, spina bifida and exomphalos). On the basis of their morphological and growth characteristics, viable adherent cells from the amniotic fluid are classified into three main groups: epithelioid (33.7%), amniotic fluid specific cells (60.8%) and fibroblastic type cells (5.5%). In the event of fetal abnormalities, other cell types can be found in the amniotic fluid, e.g. neural cells, in presence of neural tube defects, and peritoneal cells, in case of abdominal wall malformations. The majority of cells in the amniotic fluid is terminally differentiated and has limited proliferative capacity. In the 1990s, however, two groups demonstrated the presence of a small subset of cells in the amniotic fluid harboring proliferation and differentiation potential. First, Torricelli et al.[4] reported the presence of hematopoietic progenitors in the amniotic fluid collected before week 12 of gestation. Then, Streubel et al.[5] achieved differentiation of amniotic fluid cells into myocytes, thus suggesting the presence in the amniotic fluid of non-hematopoietic precursors. These results triggered new interest in the amniotic fluid as an alternative source of cells for therapeutic applications.

AMNIOTIC FLUID STEM CELLS

Originally, the German biologist Haeckel in 1868 used the term ‘stem cell’ -*stammzelle*- to define the unicellular ancestor cell of all multicellular organism develop.[6]

Presently, only the zygote up to 1-3 days after fertilization is considered totipotent, i.e. capable of giving rise from each cell to identical complete individuals consisting of complete embryonic and extra-embryonic tissues. After that point, blastocystis develops and differentiation capability declines towards pluripotency in the inner cell mass (i.e. capability of differentiating into tissues of 2 or 3 germ layers) and then multipotency after birth in adult stem cells (i.e. capability of differentiating into different tissues belonging to only 1 germ layer). Fetal stem cells may be considered in-between as far as their differentiation potential is concerned. Although amniotic membrane and amniotic fluid are physically separate, cells from the amniotic membrane can be found in the amniotic fluid and will be here referred to as amniotic fluid stem cells. Amniotic membrane may be collected during villocentesis at the first trimester or after delivery, while amniotic fluid is achievable through amniocentesis for prenatal diagnosis between 15th and 20th week and after cesarean section [7].

The first evidence that the amniotic fluid could contain pluripotent stem cells was provided in 2003 by Prusa et al.[8], who described the presence of a distinct sub-population of proliferating amniotic fluid cells (0.1-0.5%) expressing the pluripotency marker Oct4 (octamer, binding transcription factor 4) at both transcriptional and protein levels, as well as MSCs markers, such as CD90, CD105, CD73, CD166, while lacking hematopoietic markers, such as CD45, CD34, CD14. Oct4 is a nuclear transcription factor that plays a critical role in maintaining embryonic stem cell differentiation potential and self-renewal capacity.[9] Besides embryonic stem cells, Oct4 is specifically expressed by germ cells, where its inactivation results in apoptosis, and by embryonic carcinoma cells and tumors of germ cells origin, where it acts as an oncogenic fate determinant. While its role in stem cells of fetal origin has not been completely addressed, it has been recently demonstrated that Oct4 is neither expressed nor required by somatic stem cells and progenitors[10].

Nevertheless, different groups confirmed the expression of Oct4 and its transcriptional targets (e.g. Rex-1) in the amniotic fluid[11].

Remarkably, Karlmark et al.[12] transfected human amniotic fluid cells with the green fluorescent protein gene under either Oct4 or Rex-1 promoter and found that some amniotic fluid cells were thus capable of activating these promoters. Several authors subsequently harvested amniotic fluid cells displaying features of pluripotent stem cells[13]. Thereafter, the presence of a cell population capable of generating clonal cell lines differentiating into lineages representative of all three embryonic germ layers was definitively demonstrated[14] **[Figure 2B]**; these cells were not tumorigenic, retained long telomeres, and maintained a normal karyotype for over 250 population doublings.[14] Amniotic fluid stem cells are characterized by the expression of the surface antigen c-kit (CD117), the type III tyrosine kinase receptor of the stem cells factor.[15].

Maguire et al., in search of markers defining the origin of amniocytes, performed in a large number of samples immunostaining, flow cytometry, clonal analysis, qPCR and RNA-seq whole genomic profiling [16]. After clonal analysis, most of the amniotic fluid samples were positive for the transcription factor OCT4 (at cytoplasmic and nuclear level), SOX2, NANOG, KLF and WDR5, a key factor that interacts with OCT4. The expression pattern of SSEA1, SSEA3, SSEA4, TRA1-60 and TRA1-81 was heterogeneous, as a large cell population was positive for SSEA4, while a smaller cell group was double positive for SSEA1/SSEA4, Tra1-60/SSEA4 and TRA1-81/SSEA4. The peculiar, broad differentiation potential of amniotic fluid stem cells is shown by the co-staining of more than 75% of these cells with at least two multilineage surface proteins, such as SOX17 endodermal marker, $SM_{22}\alpha$ mesodermal (smooth muscle) marker and Tubb3 ectodermal (neuronal) marker. A recent study reported that c-Kit$^+$ Lin- cells derived from human amniotic fluid may display a multilineage differentiation potential in vitro also towards the hematopoietic lineages[17].

DIFFERENTIATION

Amniotic fluid stem cells display multi-differentiation capabilities towards tissues or organs deriving from all the three embryonic germ layers[14,18].

ENDODERM

To induce liver-specific differentiation, amniotic fluid stem cells were cultured in medium containing hepatocyte growth factor, insulin, oncostatin M, dexamethasone and fibroblast growth factor-4. Consequently, they displayed the expression of albumin, transcription factor HNF4$_{\alpha}$, c-met receptor MDR membrane transporter and α-fetoprotein[18]. The potential for lung-specific differentiation was also revealed by ex vivo culture of human amniotic fluid stem cells in mouse embryonic lungs, where injected cells could integrate into the epithelium and express the thyroid transcriptional factor 1, an early human differentiation marker [19]

ECTODERM

Amniotic fluid stem cells can be induced to differentiate into neuron-like cells through culture in medium containing dimethyl sulfoxide, butylated hydroxyanisole and neuronal growth factor. During the differentiation culture, amniotic fluid stem cells change their shape into large, flat, small and bipolar cell types, which is followed by the appearance of cone-like terminal expansions and expression of neural-specific proteins, including neuroepithelial and neuronal markers as well as some glial markers[14].

MESODERM

Myogenic differentiation can be induced by treating amniotic fluid stem cells with 5-azacytidine and then culturing them on Matrigel® -coated culture dishes, in medium supplemented with horse serum and chick embryo extract. Differentiated cells form myotubules and express sarcomeric tropomyosin and desmin, whereas these markers are not expressed in the original progenitor cell population[14]. Adipogenic differentiation is induced in media containing 3-isobutyl-1-methyl-xanthine, insulin and indomethacin, as confirmed by accumulation of intracellular lipid-rich vacuoles[14].

Endothelial differentiation can be induced by culturing amniotic fluid stem cells on dishes coated with gelatin in endothelial basal medium, which contains epithelial growth factor, vascular endothelial growth factor, fibroblast growth factor 2, insulin-like growth factor 1, hydrocortisone, heparin and ascorbic acid. This treatment induced the expression of human specific endothelial cell surface markers, such as factor VIII and kinase inert domain-containing receptors, as well as morphological changes, such as cobblestone and capillary-like structures on 2 and 3 dimensional culture substrates, respectively[14].

Osteogenic differentiation can be induced by media containing dexamethasone, β-glycerophosphate and ascorbic acid-2-phosphate, as confirmed by calcium precipitation and alkaline phosphatase activity [14].

Chondrogenic differentiation can be induced by culturing cell pellets on alginate hydrogel with media containing dexamethasone, ascorbic acid-2-phosphate, sodium pyruvate, proline and transforming growth factor-β1, thus showing sulfated glycosaminoglycan and type II collagen expression [20].

IMMUNOPHENOTYPE

Amniotic fluid stem cells exhibit a typical MSC marker expression, such as CE90, CD73, CD105, CD29, CD166, CD49e, CD58 and CD44, as determined by flow cytometry [13,21,22]. Additionally, cells express class I HLA antigens, but not hematopoietic markers (CD34 and CD45), endothelial markers (CD31), and class II HLA molecules. [13,21,23]. More importantly, most cultured amniotic fluid mesenchymal stem cells (MSC) express pluripotency markers, such as the Oct4, the homebox transcription factor Nanog, and SSEA-4 [13,14,21].

Actually, amniocytes contain also a small population of CD117-positive cells that can be clonally expanded in culture[14]. The differentiation properties of CD117$^+$ amniotic fluid stem cells were tested for the first time in vivo, thus proving their stem cell nature[14]. Amniotic fluid stem cells seem to derive from spindle-shaped fibroblastoid cells [24].

In a recent study Moorefield et al. demonstrated that direct contact with amniotic fluid stem cells inhibits lymphocyte activation; similar results were obtained with cell-free supernatants derived from amniotic fluid stem cells primed with total blood monocytes or IL-1β (a cytokine released by monocytes and essential in mediation of the inflammatory response). Further investigation with amniotic fluid stem cell-free supernatants by protein array revealed the secretion of multiple factors shared by MSCs that are involved in immune regulation, including growth-related oncogene and monocyte chemotactic protein family members, as well as IL-6. Amniotic fluid stem cells activated by peripheral blood mononuclear cells release several additional cytokines, as compared to bone marrow MSCs, including macrophage inflammatory protein-3α, MIP-1α and Activin. Amniotic fluid stem cells also released higher level of MCP-1 and lower level of MCP-2 compared to bone marrow MSCs, in response to IL-1β activation. This suggest that there may be some amniotic fluid stem cells specific mechanism of inhibition of lymphocyte activation[25].

In an attempt to analyze amniotic fluid stem cells subpopulations, Roubelakis et al. identified recently two morphologically distinct population of amniotic fluid stem cells of mesenchymal origin, with different proliferation and differentiation properties, termed as spindle-shaped an round-shaped [26]. Both subsets express MSC markers at similar levels. However, spindle-shaped colonies expressed higher levels of CD90 and CD44 antigens, as compared to round-shaped colonies.

Recently, our group applied highly standardized methods to compare the immunological properties of human bone marrow MSCs, olfactory ectomesenchymal stem cells, leptomeningeal stem cells, and three different c-Kit-positive stem cells, i.e. cardiac stem cells, lung stem cells and amniotic fluid stem cells. We found that all these stem cell types share a common pattern of immunological features, including expression of activation markers (ICAM-1, VCAM-1, HLA-ABC, and HLA-DR), modulatory activity towards purified immune effectors (T, B, and NK cells), lower immunogenicity following priming with inflammatory cytokines as compared to the resting conditions, and molecular inhibitory pathways (mainly related to indoleamine-2, 3-dioxygenase-activation). Moreover, all the stem cell types analyzed exert an anti-apoptotic effect towards resting immune effector cells, thus showing that the inhibitory effect is not a constitutive property of stem cells, but it is acquired as a consequence of immune effector cell activation.[27]

TRANSCRIPTOMICS

A functional analysis of the gene expression signature of amniotic fluid MSCs, as compared to bone marrow, cord blood and amniotic membrane MSCs, was initially performed by Tsai et al.[28]. Genes expressed in MSCs from all the three sources could be categorized in groups related to (i) extracellular matrix remodeling and tissue inhibitor metalloproteinase 1, (ii) cytoskeletal regulation, (iii) chemokine regulation and adhesion, (iv) plasmin activation, (v) transforming growth factor β receptor signaling and (vi) genes encoding E3 ubiquitin ligases[28]. The upregulated genes in amniotic fluid MSCs, as compared to bone marrow, cord blood and amniotic membrane MSCs, included molecules involved in uterine maturation and contraction, such as oxytocin receptor and regulation of prostaglandin synthesis, such as phospholipase A2. Other upregulated genes in this group were involved in signal transduction related to (i) thrombin triggered response, (ii) hedgehog signaling, and (iii) G-protein related pathways, regulator of G protein signaling 5 and 7, and phospholipase C beta 4[28].

Recently, Kim et al. described for the first time by illumine microarray analysis the gene expression changes in total amniotic fluid stem cells population during different passages; 1970 differentially expressed genes were detected and categorized according to their expression profiles into 9 distinct clusters[29]. Genes with gradually increasing expression levels included chemokine ligand 12, cadherin 6, and folate receptor 3. Down-regulated genes included cyclin D2, keratin 8, IGF2, natriuretic peptide precursor B and cellular retinoic acid binding protein 2.[29]. To obtain further information, chip data analysis on aging genes was performed and revealed the up-regulation of gene transcripts, such as nerve growth factor beta, insulin receptor substrate 2, insulin-like growth factor binding protein 3 and apolipoprotein 3.

Wolfrum et al. performed a global gene expression analysis of amniotic fluid stem cells compared to iPSCs derived from amniotic fluid and embryonic stem cells.[30]. Among these, genes related to self-renewal and pluripotency were detected in amniotic fluid stem cells[30]. Furthermore, the authors examined the expression of senescence and telomere-associated genes in amniotic fluid stem cells of early and late expansion passages to study the effect of reprogramming on cell senescence. 64 genes were identified as differentially expressed in amniotic fluid stem cells, as compared to amniotic fluid iPSCs lines. Of these, telomere-associated and cell cycle-regulating genes were down-regulated in amniotic fluid stem cells, as compared to amniotic fluid-derived iPSCs and embryonic stem cells.[30].

Recently, Moschidou et al. used microarray-based transcriptome analysis to investigate whether 1st and 2nd trimester c-Kit$^+$ amniotic fluid stem cells show similar gene expression profiles. These cell populations were related but differed as far as cell size and molecular signature were concerned, with a cell-specific gene expression signature including 366 genes for 1st trimester and 340 genes for 2nd trimester amniotic fluid stem cells[31].

PROTEOMICS

Proteomic studies on the total amniotic fluid stem cell populations, including epithelioid, amniotic fluid-specific and fibroblastic cells, revealed 2400 spots that resulted in the identification of 432 different gene products. The majority of the proteins was localized in the cytoplasm, mitochondria and nucleus and represented mainly enzymes and structural proteins. A relatively high percentage of membrane associated proteins were also present[32].

Recent studies provided evidence that the metabolic enzyme pattern in the amnion cells is involved in metabolic and genetic syndromes, thus their detection might be important for prenatal diagnosis.

A proteomic analysis was also performed on different cultures passages of CD117$^+$ amniotic fluid stem cells, exhibiting variations in protein expression that mainly occurred in early passages. 23 proteins were differentially expressed between early and late passages[33].

In 2007, the proteomic map of human amniotic fluid MSCs was drawn and directly compared to the one derived from bone marrow MSCs[21]. 261 different proteins were identified in amniotic fluid MSCs with the majority of proteins localized in the cytoplasm. Amniotic fluid MSCs expressed a number of proteins related to proliferation and cell maintenance, such as ubiquilin-1 (controlling cell cycle progression and cell growth), the proliferation-associated protein 2G4 (a nucleolar growth regulating protein), the acidic and rich in cysteine secreted protein (regulated during embryogenesis and involved in the control of cell cycle and cell adhesion).[21]. Other proteins expressed at high levels in amniotic fluid MSCs were related to development and cytoskeletal organization and movement.

SECRETOME

Recently, the analysis of the secreted proteins from amniotic fluid stem cells has significantly improved. Amniotic fluid stem cell secretome was responsible for enhancing vasculogenesis and capable of evoking a strong angiogenic response in murine recipient[34]. According to this study, a detailed analysis with Luminex's MAP Technology of the amniotic fluid stem cell-conditioned media revealed the presence of proangiogenic and antiangiogenic factors. Vascular endothelial growth factor, stromal cell-derived factor 1, interleukin 8, monocyte chemotactic protein 1, and two angiogenesis inhibitors, interferon-gamma and interferon-gamma induced protein 10, were identified as secreted proteins. [34,35].

It was also demonstrated that a relative small number of amniotic fluid stem cells was enough to secrete a detectable amount of proangiogenic growth factors and cytokines.

A systematic study on amniotic fluid stem cells-secreted proteins led to the conclusion that proangiogenic soluble factors from amniotic fluid stem cells can mediate the recruitment of endothelial progenitors in an ischemic rat model[36].

In a recent study, the therapeutic potential of an amniotic fluid MSCs and their secreted molecules was studied in mice with acute hepatic failure[37]. A variety of cytokines and growth factors were detected in amniotic fluid MSCs medium. Different cytokines were shown, such as interleukin 10, interleukin 27, interleukin 17 families, interleukin 12p70, interleukin-1β and interleukin-1 receptor antagonist, responsible for inducing local and systemic down-regulation of pro-inflammatory mediators. In addition, SERPINE1, MCP-1 and SDF-1 responsible for promoting tissue repair, were also detected.

AIM OF THE PROJECT

Stem cells can be found in different tissues at various developmental stages; however, the phenotype of stem cells originating from the same tissue source may vary according to the development stage at which they are isolated.

AFS cells as well as other stem cells derived from different tissues display immune modulatory properties as a common feature. AFS cells can be collected at different gestational age and their phenotype and differentiation potential seems to depend on fetal development. The differences in gene profile between 1st and 2nd trimester-AFS cells have been recently investigated by means of microarray-based transcriptome analysis. However, the assessment of immune modulatory capabilities of AFS cells at different gestational age is still lacking.

In this study, we used standardized approaches to investigate the immunological profile of AFS cells isolated at different gestational age, including phenotypic analysis, immunogenicity and immunomodulatory functions towards various immune effector cells (IECs).

METHODS

AMNIOTIC FLUID STEM CELLS

Amniotic fluid was collected at different weeks of gestation with to isolate and analyze AFS cells. AFS cells (four samples for 1st and 2nd trimester and five for 3rd trimester) were achieved from human amniocentesis carried out for diagnostic purposes (cytogenetic analysis) between 10-12 weeks of gestation for 1st trimester-AFS cells, between 16-18 for 2nd trimester-AFS cells and between 38-40 for 3rd trimester-AFS cells, after mother's informed consent.

Cells adhering to flasks were expanded in Chang B+C medium. Cluster of cells with mesenchymal-like morphology were harvested and immediately subjected to immunoselection, as previously reported[14]. Sorted c-Kit positive AFS cells were cultured in α-MEM medium (Gibco) containing heat-inactivated adult bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin (all from Gibco), supplemented with 18% Chang B and 2% Chang C (Irvine Scientific). Full characterization of AFS cells has been already described by other groups elsewhere[14].

AFS cells were detached (0.05% Trypsin-EDTA; Gibco) and harvested when 80% confluent, and then either reseeded at $10^3/cm^2$ concentration or frozen until use. All experiments were performed between passages 2 and 10.

IMMUNOPHENOTYPING

AFS cells at 80% confluence were treated or not for 40 hours with 10 ng/mL IFN-γ and 15 ng/mL TNF-α (R&D Systems). The lack of cytotoxic effects of this inflammatory cytokines was previously demonstrated[38]. To assess the specific expression of different markers, rested and primed AFS cells were labeled with the following monoclonal antibodies against human markers: IgG1k-PE, CD31-PE, CD34-PE, CD45-PE, CD73-PE, CD90-PE, CD105-PE, CD146-PE, CD107a-PE, CD54-PE, CD86-PE, CD106-PE, CD200-PE, and HLA-ABC-PE all from BD Biosciences; IgG1k-PE, CD80-PE, NG2-PE, IgG1k-FITC, and HLA-DR-FITC all from Beckman Coulter; IgG1k-PE, CD112-PE, CD155-PE, IgG2b-PE, and CD274-PE all from Biolegend; IgG1-PE, IgG2a-PE, IgG2b-PE, unconjugated IgG2a, normal goat IgG unconjugated, MICA/B-PE, ULBP-1-PE, ULBP-2-PE, CCR7-PE, CXCR3, CXCR5 and unconjugated ULBP-3 all from R&D Systems, IgG1-FITC and HLA-G from Exbio, IgG2a k-PE, IgG1-PE, TLR-4 PE, CD178_PE (FasL) from eBioscience, goat- anti-mouse-PE from DAKO and donkey-anti-goat-PE from Abcam.

10^5 AFS cells were incubated with the selected monoclonal antibody or appropriate isotype control in PBS for 15 min at room temperature. For ULBP-3 and Jagged-1 expression, PE- conjugated goat anti-mouse IgG F(ab')2 and PE-conjugated donkey F(ab')2 polyclonal secondary antibodies were added, after incubating the cells with the primary unconjugated antibody. ULBP-1 expression was validated by intracellular staining using the Cytofix/Cytoperm kit (BD Biosciences) according to manufacturer's instruction. Data were analyzed by FACSCanto II (BD Biosciences) and expressed as the ratio of geometric mean fluorescence intensity (rMFI) obtained for each marker and its isotype-matched negative control.

PROLIFERATION AND SURVIVAL ASSAYS

IECs (CD3^{pos} T cells, CD19^{pos} B cells, and CD56^{pos} NK cells) were purified from peripheral blood using appropriate negative selection kits (Miltenyi Biotech) and their purity (at least 95%) was assessed by FACS analysis.

To evaluate the immunomodulatory capabilities of AFS cells towards IEC proliferation, rested and primed AFS cells were cultured with purified T, B and NK cells at either $2x10^4$ cell concentration (high ratio, corresponding to a confluent monolayer), or $2x10^3$ cell concentration (low ratio). Following AFS cells adhesion, $2x10^5$ T cells, $2x10^4$ B cells, or $2x10^4$ NK cells previously stained with 5 mM carboxyfluorescein succinimidyl ester (Life Technologies) were added. All IECs were activated by use of specific molecules. In particular, T cells were activated with 0.5 mg/mL cross-linking anti-CD3 and anti-CD28 antibodies (Sanquin) for 6 days in Roswell Park Memorial Institute medium (RPMI) supplemented with 10% human AB serum. B cells were activated with 2 mg/mL F(ab')$_2$ anti-human IgM/IgA/IgG (Jackson Immunoresearch), 20 IU/mL rhIL-2 (Proleukin; Novartis), 50 ng/mL polyhistidine-tagged CD40 ligand, 5 mg/mL anti-polyhistidine antibody (R&D Systems), and 2.5 mg/mL CpG B (Invivogen) for 4 days in RPMI supplemented with 10%

FBS (Invitrogen Life Technologies). NK cells were activated by 100 IU/mL rhIL-2 for 6 days in Iscove modified Dulbecco medium (IMDM) supplemented with 10% human AB serum.

At the end of co-culture, cells were harvested and labeled with PerCP mouse anti-human CD45 monoclonal antibody (BD Biosciences) and TOPRO-3 Iodide (Invitrogen Life Technologies); the percentage of proliferating cells was evaluated among viable TOPRO-$3^{neg}$$CD45^{pos}$ cells by FACS analysis as the percentage of cells undergoing at least one cell division. The effect on the proliferation rate was obtained according to the following formula: (number of $CD45^{pos}$ cells in co-culturing conditions/ number of $CD45^{pos}$ cells alone) x100.

To further understand which molecules were involved in immunomodulation, the following specific inhibitors were added to co-cultured AFS and T cells: 1 mM L-N-monomethyl arginine (L-NMMA) inhibitor of inducible NO synthase (iNOS); 5 mM NS-398 (Cayman Chemicals) inhibitor of cyclooxygenase-2 (COX-2) that is necessary for PGE2 synthesis; 1mM L-1- methyl tryptophan (L-1MT) IDO inhibitor (Sigma-Aldrich); 2 mM tin-protoporphyrin (SnPP) inhibitor of HO-1 (Frontier Scientific); and 10 mg/mL purified anti-human IFN-g NA/LE mouse IgG1 (BD Biosciences).

To determine the effect of AFS cells on IEC survival, resting and primed AFS cells were cultured with IECs at the same concentration described above for the immunomodulatory assays. The analysis of IEC apoptosis was performed after either 4 days (B and NK cells) or 6 days of co-culture (T cells). Cells were detached by trypsin and stained with Allophycocyanin (APC) mouse anti-human CD45 BD Biosciences and IEC apoptosis was assessed following manufacturer's instructions (PE active caspase-3 apoptosis kit; BD Biosciences). Briefly, first the cells were fixed and permeabilized and then labeled with PE-anti-caspase-3 antibodies and cell apoptosis was assessed as percentage of active-caspase-$3^{neg}$$CD45^{pos}$ viable cells by FACS analysis.

IMMUNOGENICITY ASSAY

AFS cell immunogenicity was assessed by using a non-radioactive cytotoxicity assay, following manufacturer's instructions (Delfia Cytotoxicity kit; Perkin Elmer). NK cells were stimulated for 48 hours with 100 IU/mL rh-IL-2 and used as effector cells. AFS cells were labeled with bis-acetoxymethyl terpyridine dicarboxylate (BATDA) fluorescent dye, and used as target cells in co-culture with previously activated NK cells at different ratios (ranging from 1:1 to 50:1, corresponding to NK:AFS ratio). After 1 hour, the cytotoxicity was quantified by assessing fluorescence release in co-culture supernatants by a time-resolved fluorimeter (VictorTM X4, Perkin Elmer).

STATISTICAL ANALYSIS

Data were expressed as mean ± standard deviation, except for immunophenotype data that were expressed as mean ± standard error of the mean. Statistical analysis was performed by Prism software (GraphPad) using the Wilcoxon test to compare the effect of priming on the same AFS cells, while one-way ANOVA test was used to assess the differences among AFS derived from different trimesters. $P < 0.05$ was considered statistically significant.

RESULTS

MORPHOLOGICAL AND IMMUNOPHENOTIPIC DIFFERENCES AMONG AFS CELLS

Before focusing our attention on the immunological behaviors of AFS cells, we characterized the AFS cells in term of morphology and expression of typical specific markers. In all experiments, cells were cultured at the same condition and used at the same passages. All different types of AFS cells showed their canonical morphologies (**Figure 3A**). 1st and 2nd trimester-AFS cells displayed a fibroblast-like shape, while 3rd trimester-AFS cells were more heterogeneous, showing often an oval-round shape. The immunophenotypical analysis revealed a similar pattern of expression but with some peculiarities (**Figure 3B)**. Hematopoietic markers, CD31, CD34, and CD45 were undetectable in all AFS cells with the exception of resting 1st trimester-AFS cells, which weakly expressed CD34. In contrast, mesenchymal markers were detectable in all AFS cells. CD73 was highly expressed in 3rd trimester-AFS and modestly present in the others AFS types. All AFS cells showed a strongly expression of CD90, particularly by 1st and 3rd trimester-AFS cells, while the CD105 was weakly expressed by all AFS cell types. The expressions of neuron-glial antigen 2 (NG2) and melanoma cell adhesion molecule (CD146) were also evaluated. NG2 was highly expressed in 1st trimester-AFS cells, moderately in 3rd trimester-AFS cells and poorly in 3rd trimester-AFS cells. CD146 was slightly detected in 1st and 2nd trimester-AFS cells, while 3rd trimester-AFS cells showed the higher expression of this marker.

EFFECT OF INFLAMMATORY PRIMING ON AFS CELLS

With the aim to understand whether the gestational age could affect the immunomodulatory capabilities of AFS cells, all AFS cells were treated or not with IFN-γ and TNF-α, in order to mimic the inflammatory milieu.

First, we evaluated the expression of different molecules involved in MSC immunomodulation (**Figure 4**). HLA-ABC expression of 1st trimester AFS cells were significantly different from that observed in other AFS cells ($P<0.05$). At resting conditions, 1st trimester-AFS cells showed lower expression of HLA-ABC, while 2nd and 3rd trimester-

AFS cells displayed a moderate expression of this molecule. Interestingly, after inflammatory priming, HLA-ABC expression increased in all AFS cell types. This overexpression was reduced in 1st trimester-AFS cells (1.87-fold) as compared to 2nd and 3rd trimester-AFS cells, where the fold-change resulted significantly higher (2.23-fold, 2.15-fold respectively). Moreover, also in priming conditions, AFS cells maintained the significant differences observed at baseline ($P<0.05$). HLA-DR was never expressed at resting conditions, but following treatment with inflammatory cytokines we observed a slight overexpression of this molecule in all AFS cell types. The co-stimolatory molecules CD80 and CD86 were never detectable in all AFS cell types. In contrast, CD40 molecule was scarcely expressed only by 2nd and 3rd trimester-AFS cells, and only in the latter case the inflammatory priming significantly modulated this expression. We tested the expression of CD54 (ICAM-1), CD106 (VCAM-1), which belong to cell adhesion molecule (CAM) family and are involved in different cell mechanisms, such as migration, tethering on IEC surface and modulation of inflammation[39]. In control condition, CD54 was fairly expressed in 1st and 2nd trimester-AFS cells and less in the other AFS cells. However, following inflammatory priming, CD54 was significantly up regulated in 1st (22.17-fold), 2nd (11.53-fold) and 3rd (65.62-fold) trimester AFS cells. CD106 was not particularly expressed at baseline, but the inflammatory cytokines induced the increase of this adhesion molecule that was more evident in 2nd and 3rd trimester AFS cells. Finally, we assessed the expression of two immunosuppressive molecules, i.e. CD274 (PD-L1) and CD200, which are involved in modulation of T cell proliferation [40-42]. At baseline, CD274 was expressed in all AFS cells, especially in 2nd and 3rd trimester AFS cells. Moreover, following priming a significant increased of CD274 expression was observed in all AFS cells (4.04-fold, 3.06-fold and 3,2-fold respectively). Conversely, CD200 molecules were scarcely expressed only in 2nd trimester AFS cells, but inflammatory priming did not modulate this marker.
Further analysis of different markers were performed to better characterized the different types of AFS cells, both at resting and primed conditions, and the mean ± SEM of relative Mean Fluorescence Intensity (rMFI) are reported in **Table I**.

Then we assessed the immunomodulatory properties of AFS cells on different IECs. To this purpose, resting and primed AFS cells were co-cultured with purified T, B and NK cells at different ratios pre-stimulated or not. None of AFS cell types activated resting IECs (data not shown), in agreement to the insufficient expression of HLA class II and co-stimulatory molecules (**Figure 4**). AFS cells did not show modulatory effects on IECs at low ratio (100:1 for T cells, 10:1 for B and NK cells), except for 1st trimester-AFS cells. Conversely, at high ratio (10:1 for T cells, 1:1 for B and NK cells), all AFS cells displayed different immunomodulatory capabilities in presence of pre-stimulated IECs. Both resting and primed 1st trimester-AFS cells inhibited T cell proliferation by more than 80% at high ratio, and showed almost 30% of inhibition at low ratio (**Figure 5A**). AFS cells derived from 2nd and 3rd trimester exhibited a lower immunosuppressive effect on T cell proliferation in respect to 1st trimester-AFS cells, and only primed 3rd trimester-AFS cells showed a significant inhibition. Similarly, 1st trimester-AFS cells had the higher immunosuppressive effect on NK cell proliferation respect to other AFS cells that instead exhibited a weak suppression (**Figure 5B**). None of AFS cells affected B cell proliferation at baseline (**Figure 5C**). This effect was related to the inability of B cells to release IFN-γ, the main cytokine involved in MSC activation[43]. At baseline, 1st and 3rd trimester AFS cells had a trophic effect towards B cells, showing a slight increase of B cell proliferation. Conversely, resting 2nd trimester AFS cells failed to modulate B cell proliferation, but following priming they showed a strongly immunosuppression effect, which was never shown by other AFS cells.

MOLECULAR MECHANISMS INVOLVED IN T CELL IMMUNOSUPPRESION

Next, we explored the molecular mechanisms underlying the immunosuppressive effect of AFS cells on T cell proliferation. To this purpose, we tested the effects of different inhibitors of the main molecules involved in immunosuppression. In particular, we inhibited the function of IDO, iNOS, COX-2, HO-1 and IFN-γ by specific inhibitors and neutralizing anti-IFN-γ antibody. All the inhibitors have been previously tested on MSCs[27,44] at concentrations that do not alter T cell viability and proliferation.

The crucial role that IDO plays in T cell immune suppression has been largely demonstrated by different groups[45,46]. When L-1MT was added to AFS-T cell cultures, a complete rescue of T cell proliferation was observed with resting and primed 2nd and 3rd trimester AFS cells (**Figure 6**). Surprisingly, 1st trimester AFS cells did not exhibit the same effect, probably due to a different mechanism of suppression. Moreover, 1st trimester AFS cells, which showed the highest immunosuppressive effect on T cell proliferation, displayed another peculiarity, i.e. they strongly induced cell death. In fact, at the end of AFS/T co-culture, we observed a low percentage of TOPRO-3^{neg}CD45^{pos} cells, which instead was not altered in 2nd and 3rd trimester-AFS cells (**Figure 7A**). Although IDO activity can elicit cell death as well as immune suppression and anergy, the cytotoxic effect of 1st trimester-AFS cells was probably associated with another immunosuppression mechanism, as demonstrated by the lack effect of L-1MT. To investigated whether this results could be related to a mechanism of apoptosis, we tested the expression of Fas ligand (FasL) on AFS cells, but none of cells did show this molecule (**Figure 7B**).

The IFN-γ blocking antibody only partially rescued T cell proliferation (**Figure 6**), while the inhibitors LNMMA, NS-398 and snPP did not any effect on suppression of T cell proliferation (**Figure 8**).

IFN-γ/TNF-α-STIMULATED AFS CELLS ARE REFRACTORY TO NK CYTOTOXICITY.

With the aim to determine whether AFS cells were susceptible to NK-mediated lysis, BATDA-labeled AFS cells were used as target cells and cultured in presence of IL-2 pre-stimulated NK cells at different NK:AFS ratios, which ranged from 1:1 to 50:1 (**Figure 9A**). At baseline, AFS cells were sensitive to NK-mediated lysis in a dose-dependent manner. Nevertheless, following inflammatory priming all AFS cells acquired resistance to NK cytotoxicity. Hypothesizing that these results were related to a different expression of NK ligands on AFS surface induced by priming, we performed an immunophenotype analysis (**Figure 9B**). We tested the expression of various NK cell-activating ligands, including Nectin-2 (CD112) and Poliovirus receptor (PVR, CD155), which bind the DNAX Accessory Molecule-1 (DNAM-1), the MHC Class I-related A and B molecule (MICA/B) and UL16-binding proteins (ULBPs), which instead interact with NKG2D. CD112 and CD155 were constitutively expressed in all AFS cells. At resting condition, the expression of CD112 was higher in 3rd trimester AFS cells respect to other cells and its expression was enhanced after inflammatory priming, except for 1st trimester AFS cells. In control condition, CD115 was strongly expressed in 2nd and 3rd trimester AFS cells and modestly present in 1st trimester AFS cells; however, a strongly up-regulation was observed following inflammatory priming in all AFS cell types. Finally, we tested the expression of ULBPs and MICA/B, which are the ligand of the NKG2D receptor. None of these activating ligands was particularly expressed by all AFS cell types, except for a moderate expression of ULBP-1 in 1st trimester AFS cells and ULBP-2 and MICA/B in 3rd trimester AFS cells, but the priming did not alter significantly the levels of these proteins.

The increase in AFS cells resistance to NK-mediated lysis could be associated to the different expression of HLA-ABC, the crucial NK inhibitory ligand. It has been widely demonstrated that the overexpression of HLA class I prevails over NK activating ligands, leading to the inhibition of NK-mediated cytotoxicity[47]. It is important to point out that primed 1st trimester-AFS cells showed higher sensibility to NK cytotoxicity in respect to other AFS cells. This phenomenon is in agreement with the lower expression of HLA-ABC in 1st trimester AFS cells (**Figure 4)** and their higher expression of ULBP-1 and ULBP-2, both in resting and priming conditions.

ANTI-APOPTOTIC EFFECT OF AFS CELLS ON RESTING IECs.

We then assessed the effect of AFS cells on resting IECs. Normally, in absence of inflammatory stimuli, MSCs enhance immune cells survival[48]. Thus, to verify whether AFSs shared the same effects, resting and primed AFS cells were cultivated in presence of resting IECs at different ratios, and at the end of co-culture we evaluated their protective role towards IEC apoptosis. As previously shown for the immunosuppressive mechanism (**Figure 5A-B**), 1st trimester AFS cells displayed a different behavior towards T and NK cells (**Figure 10A-B**). Unlike the effects shown by other AFS cells, 1st trimester AFS cells did not increase T and NK cell viability. By contrast, 2nd and 3rd trimester displayed a significant supportive effect on T and NK cell viability. This trophic effect was less evident on T cell apoptosis, probably due to their greater resistance in culture alone as compared to NK and B cells; the latter lymphocyte population showed the highest percentage of caspase-3 positive cells when culture alone. However, a total rescue of B cell apoptosis was observed in presence of resting or primed AFS cells (**Figure 10C**). Finally, only in some cases we observed a dose-dependent anti-apoptotic effect, but these differences were never significant.

FIGURES

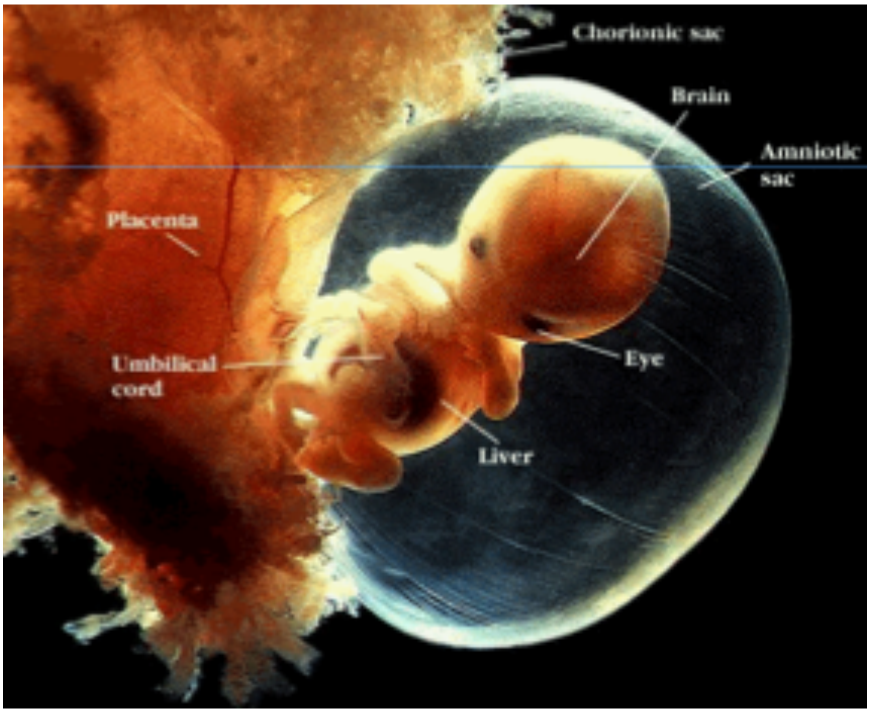
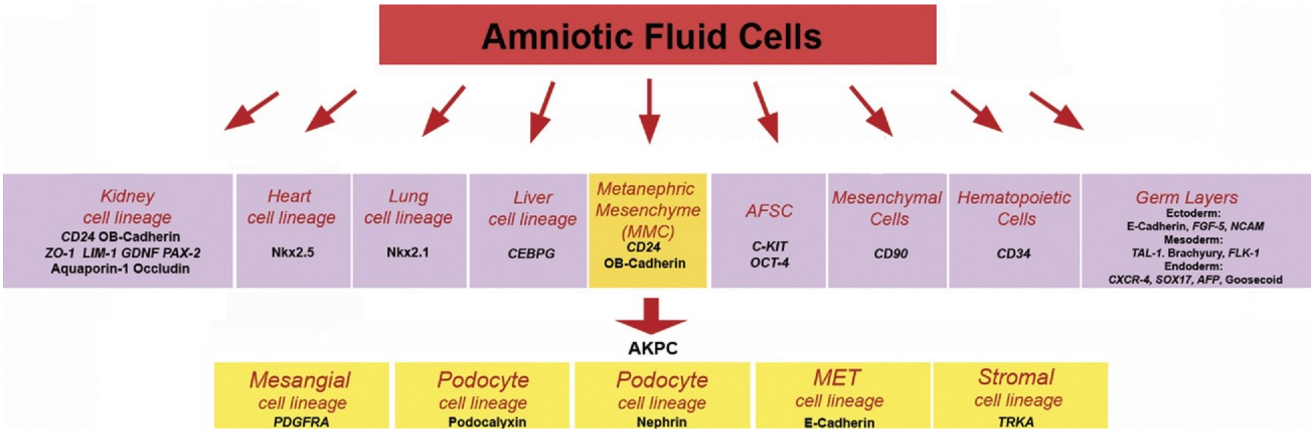
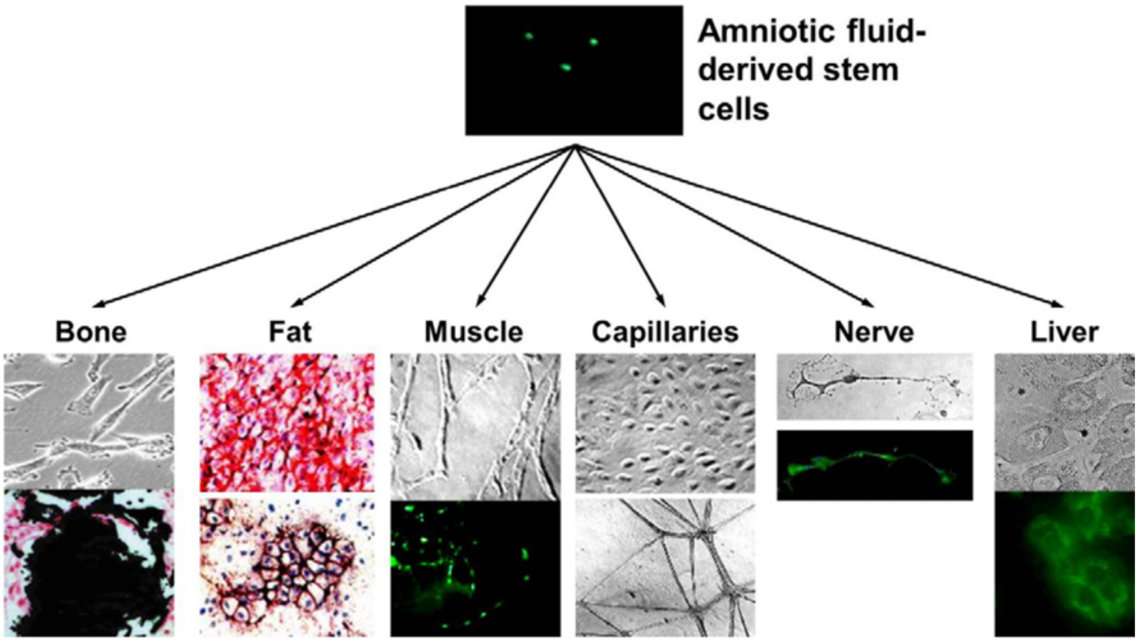


Fig.1: Gestational tissues as a source of stem cells. Cell types with therapeutic potential can be derived from gestational tissues including the placenta, umbilical cord, amniotic fluid, and fetal membranes.



A



B

Fig. 2: Cell lineages identified in amniotic fluid **(A)**; Multilineage differentiation of gestational stem cells. Stem cells from the amniotic fluid have been differentiated into a diverse range of cell types, including, but not limited to: neurons, hair and skin cells, pulmonary cells, cardiomyocytes, hepatocytes, pancreatic cells, osteocytes and adipocytes **(B).**

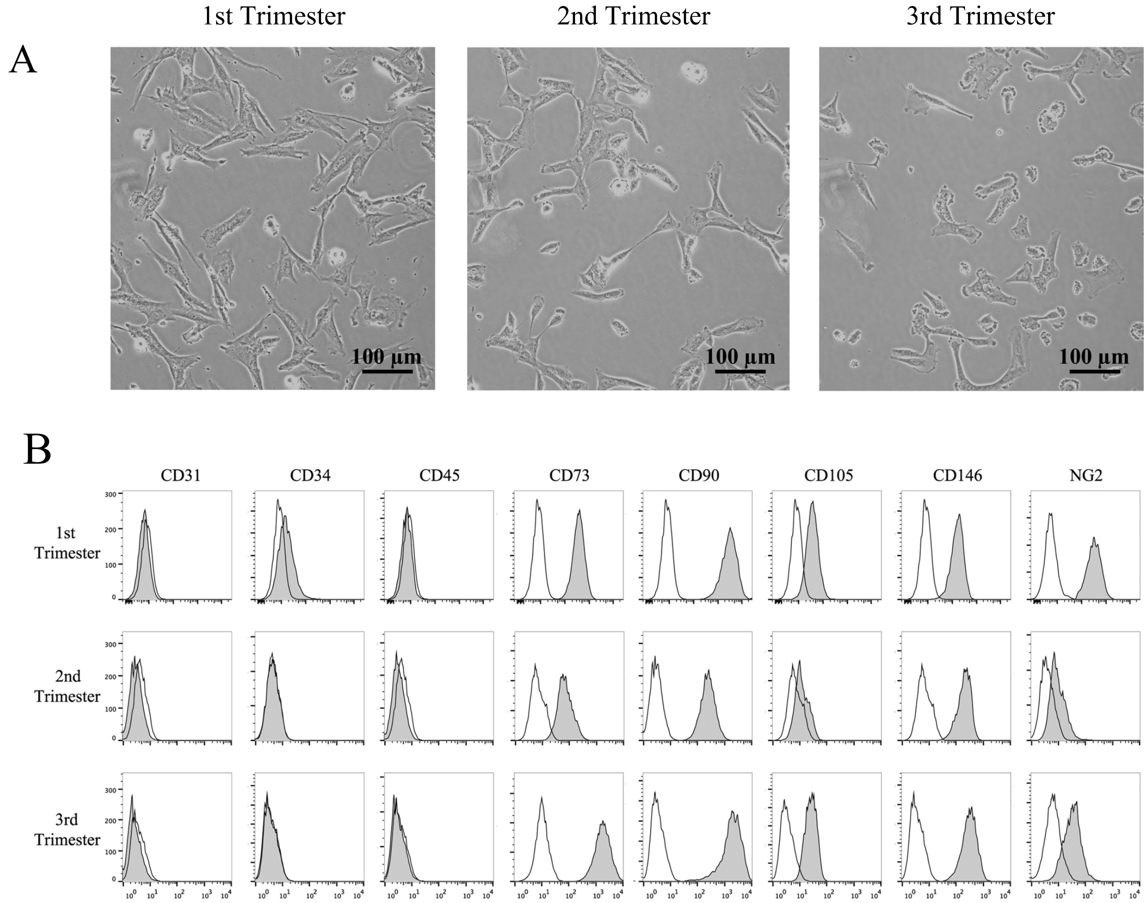


Fig. 3. *Heterogeneity of AFS cells isolated at different gestational age.* (**A**) Microscopy images showing morphologic differences among AFS cells analyzed (the scale bar indicates µm). All AFS cells were cultured in the same conditions and analyzed at the same passage. Images were obtained by Observer Z1, Zeiss. (**B**) FACS analysis of AFS cells showing the expression profile of typical hematopoietic and MSC markers. The histograms display the MFI (mean fluorescence intensity) of isotype controls (open curve) and specific markers (filled curve). Similar data were obtained in four (1^{st} and 2^{nd} trimester-AFS cells) or five (3^{rd} trimester-AFS cells) different donors. The mean ± SEM of relative MFI are reported in **Table I**.

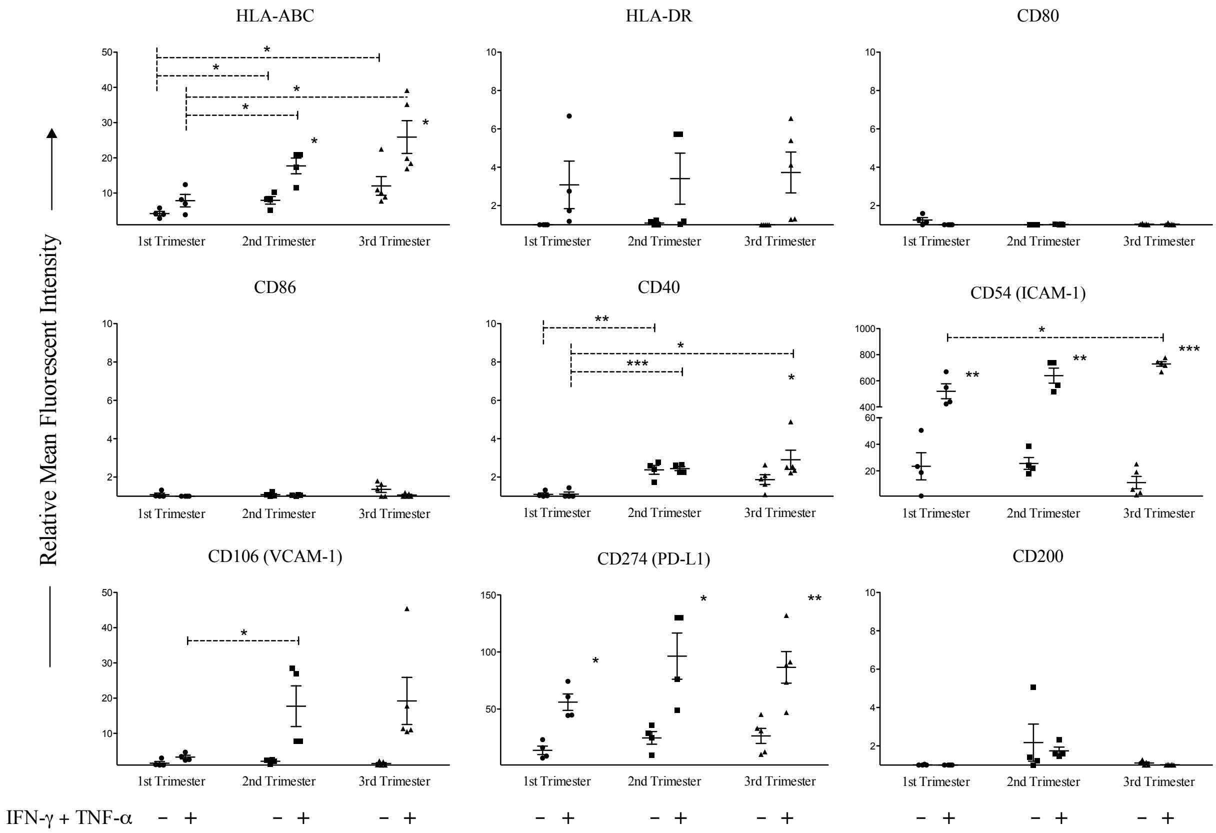


Fig. 4. *Expression of specific markers involved in immunomodulation*. AFS cells were cultured with or without inflammatory cytokines, and after 2 days the expression of CD40, CD80, CD86, HLA-ABC, HLA-DR, CD200, CD274, CD54 and CD106 were evaluated by FACS analysis. The results are expressed as relative Mean Fluorescence Intensity (rMFI) ± SEM of four (1^{st} and 2^{nd} trimester-AFS cells) or five (3^{rd} trimester-AFS cells) different experiments. **P<0.05, **P<0.01, ***P<0.001.*

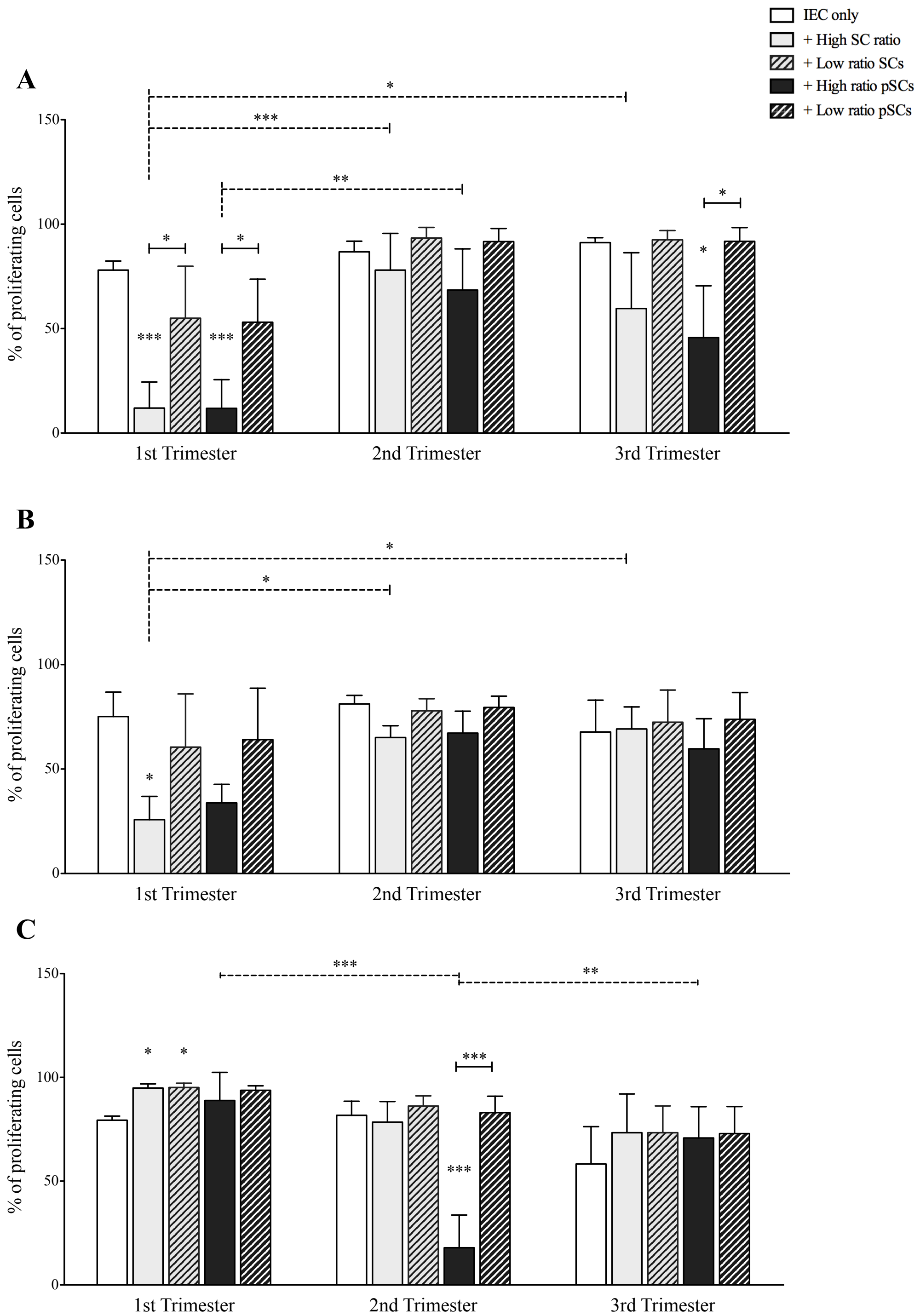


Fig. 5. *Immunomodulatory effect of different AFS cells on IEC proliferation.* CFSE-labeled IECs activated with specific stimuli were cultured alone or in presence of resting and primed AFS cells. All experiments were carried out at different IEC:AFS ratios, i.e. 10:1 and 100:1 for T cells (**A**), and 1:1 and 10:1 for both NK (**B**) and B cells (**C**) (high and low ratio, respectively). Data are represented as proliferation percentage of IECs corresponding to the mean ± SD of CFSE fluorescence of $CD45^{pos}$ $TOPRO\text{-}3^{neg}$ cells derived from 4 different experiments. **$P<0.05$*, ***$P<0.01$*, ****$P<0.001$.*

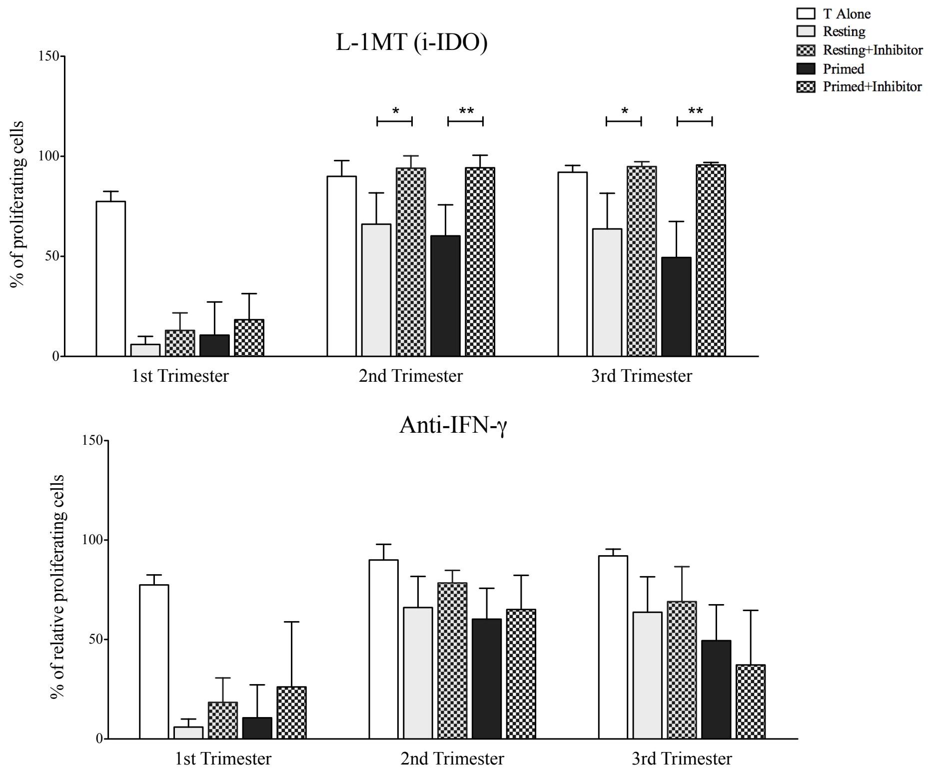


Fig. 6. *Role of IDO and IFN-γ on T cell immunosuppression.* CFSE-labeled T cells stimulated with anti-CD3 and CD28 were cultured alone or in presence of resting and primed AFS cells. L-1MT and an IFN-γ blocking antibody were added to co-cultures in order to evaluate the involvement of IDO and IFN-γ in AFS-mediated immunosuppression. At the end of co-culture (6 days) the cells were harvested and FACS analyzed the proliferation percentage. Data are represented as proliferation percentage of IECs corresponding to the mean ± SD of CFSE fluorescence of $CD45^{pos}$ TOPRO-3^{neg} cells derived from 4 different experiments. **P<0.05, **P<0.01, ***P<0.001.*

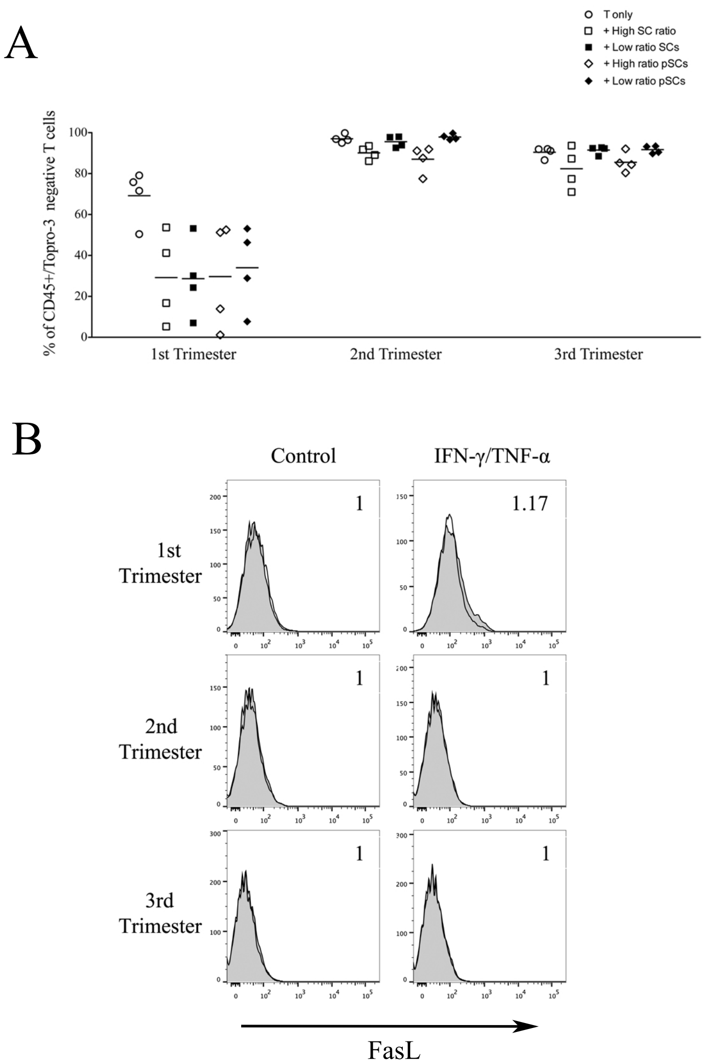


Fig 7. *Cytotoxic effect of 1st trimester-AFS cells on activated T cells.* (**A**) Data show the percentage of CD45^{pos}Topro3^{neg} derived from T cell immunosuppression assays. (**B**) Flow cytometry analysis of AFS cells showing the expression Fas ligand. The histograms display the MFI (mean fluorescence intensity) of isotopic controls (open curve), and specific markers (filled curve) and the numbers within histograms represent the rMFI.

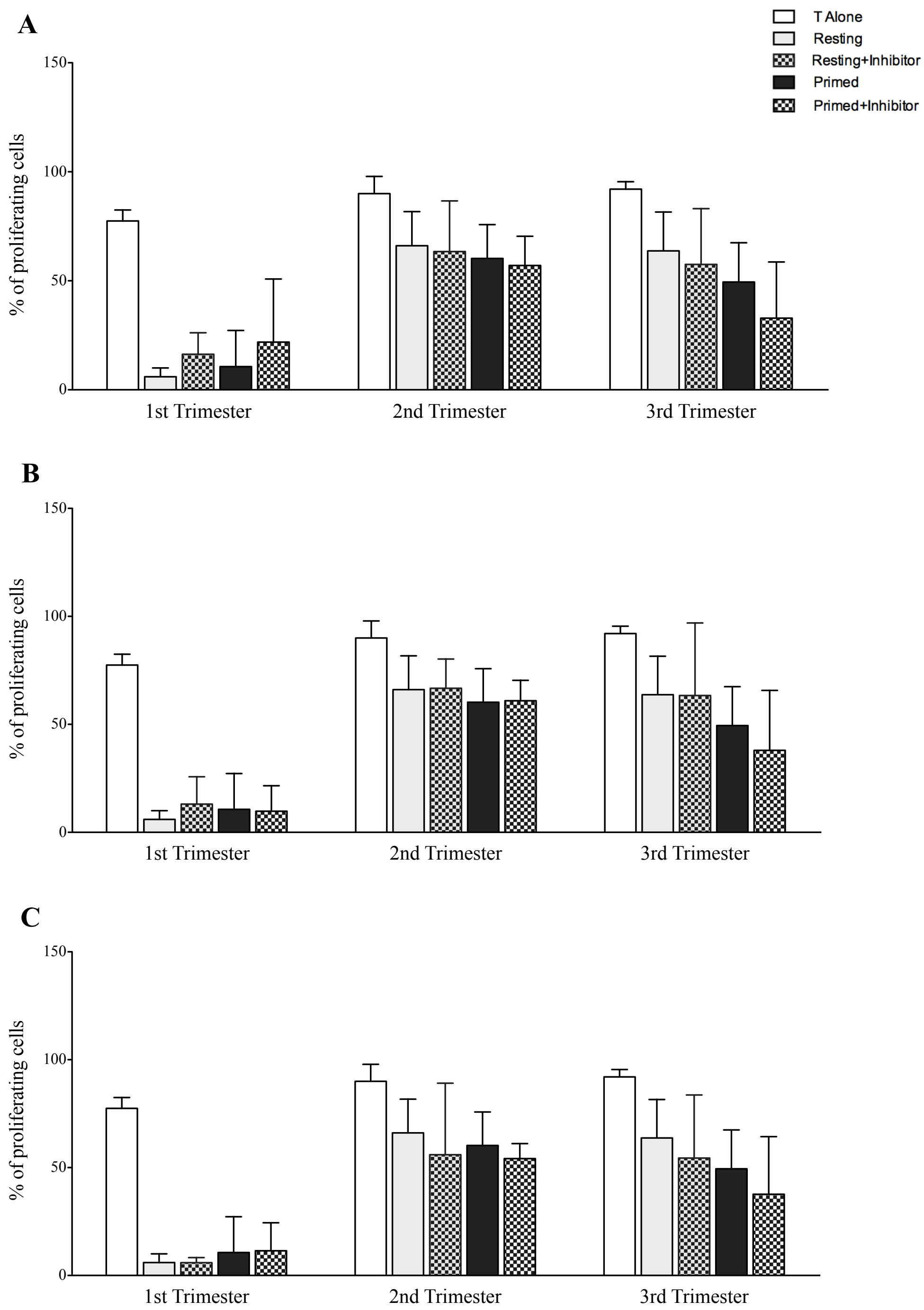


Fig. 8. *Role of NS-398, snPP and LNMMA on T cell immunosuppression.* CFSE-labeled T cells stimulated with anti-CD3 and CD28 were cultured alone or in presence of resting and primed AFS cells. The following specific inhibitors were added to T:AFS co-culture (as described in Materials and Methods): NS-398, the inhibitor of COX-2 (**A**), snPP, the inhibitor of HO-1 (**B**) and LNMMA, the inhibitor of iNOS (**C**). At the end of co-culture (6 days) cells were harvested and proliferation rate was analyzed by FACS. Data represent the mean ± SD of CFSE fluorescence of $CD45^{pos}$ TOPRO-3^{neg} cells derived from 4 different experiments.

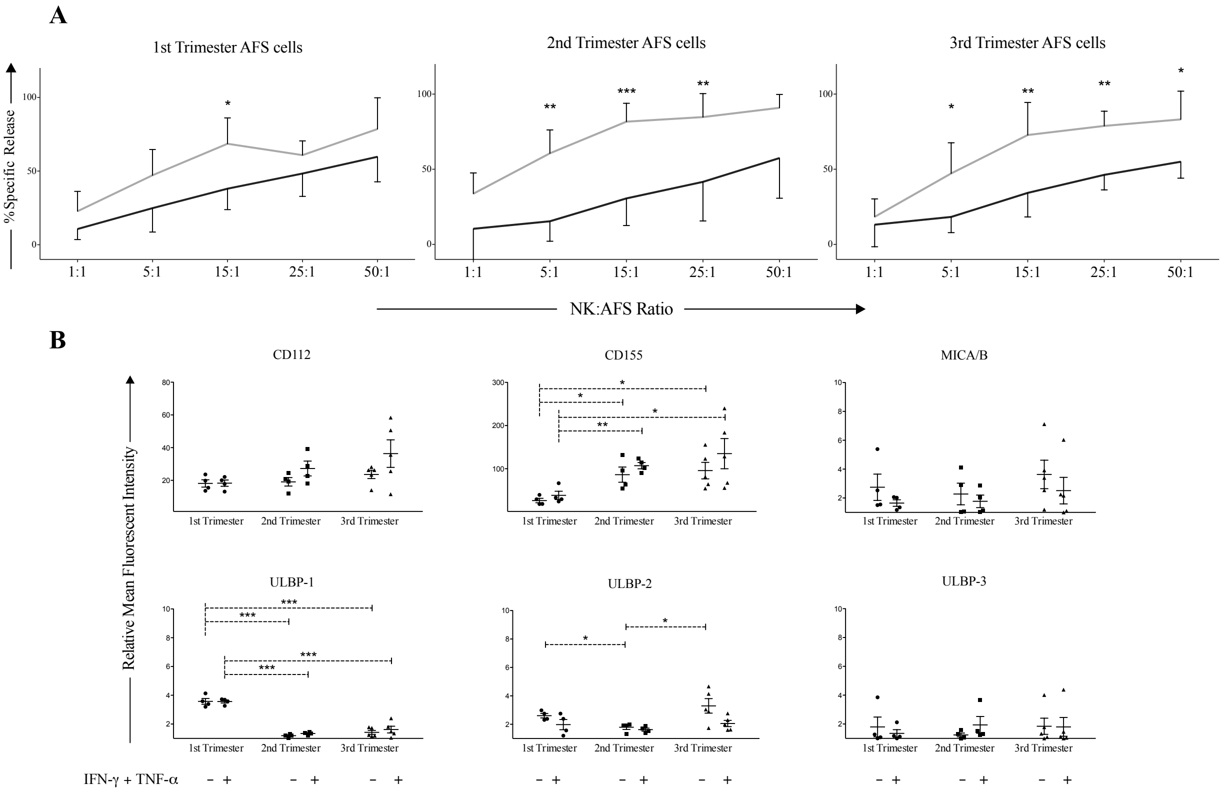


Fig. 9. *Inflammatory microenvironment reduces AFS sensitivity to NK cell-mediated lysis.* (**A**) IL-2 pre-stimulated NK cells were cultured in presence of BATDA labeled AFS cells at different ratios. AFCs were primed with IFNγ and TNFα (solid line) or not (grey line). The cytotoxic effect on resting and primed AFS cells were quantified as release of fluorescence by lysed cells and calculated by time-resolved fluorimeter (Victor X4 Multilabel Plate Reader, PerkinElmer). Data are showed as percentage of fluorescence release. Error bars represent the mean ± SD of four (1^{st} trimester-AFS cells) or five (2^{nd} and 3^{rd} trimester-AFS cells) experiments.

(**B**) AFS cells were cultured in presence or not of inflammatory cytokines. At day 2 the expression of CD112, CD155, MICA/B, and ULBPs was analyzed by FACS. The results are expressed as relative Mean Fluorescence Intensity (rMFI) ± SEM of four (1^{st} and 2^{nd} trimester-AFS cells) or five (3^{rd} trimester-AFS cells) different experiments. *$P<0.05$, **$P<0.01$, ***$P<0.001$.

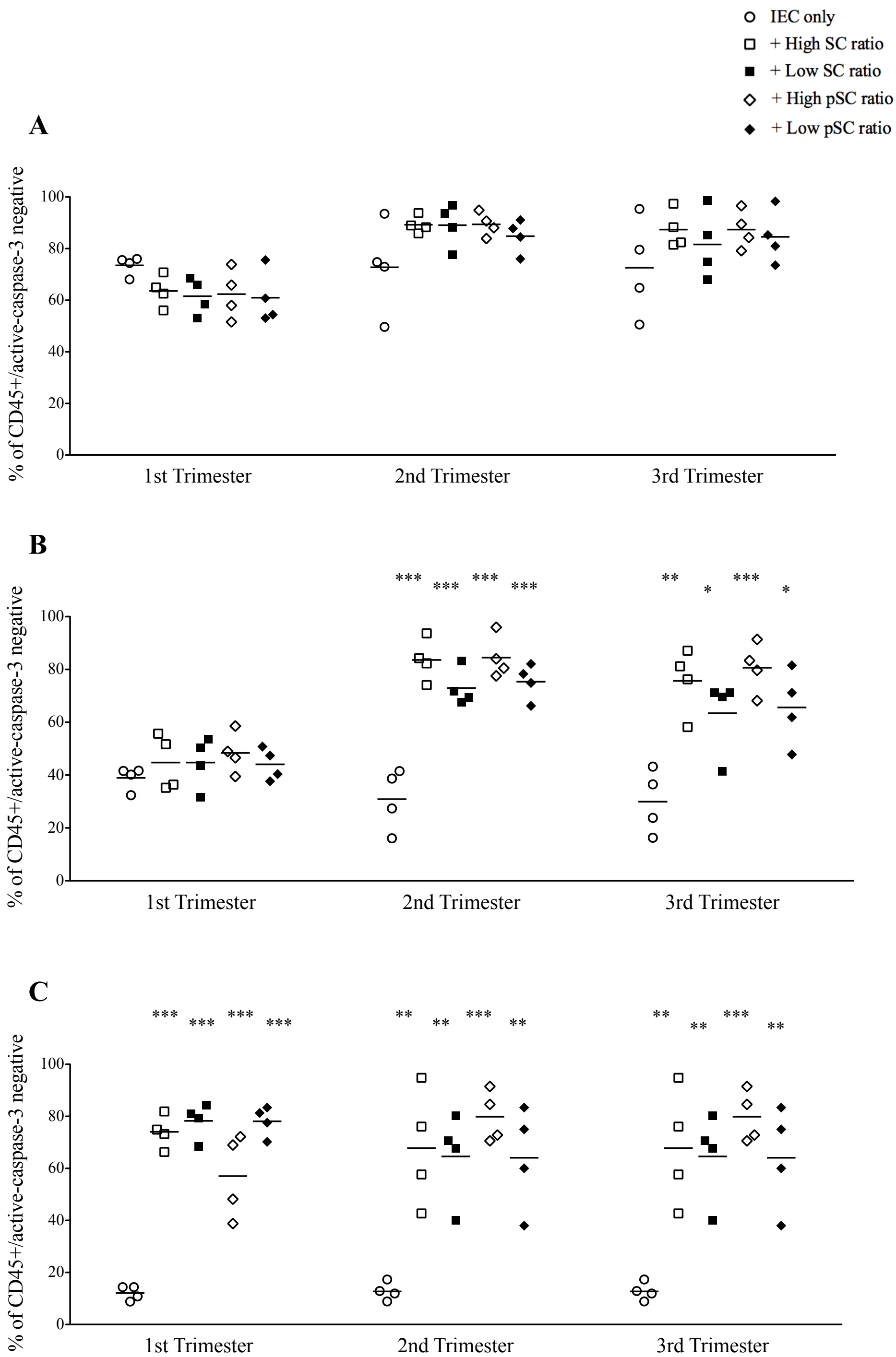


Fig. 10. *Anti-apoptotic effect of AFS cells on resting IECs.* Resting and primed AFS cells were cultured in presence of resting IEC at different ratios, i.e. 10:1 and 100:1 for T cells (**A**), and 1:1 and 10:1 for both NK (**B**) and B cells (**C**) (high and low ratio, respectively). At the end of co-culture (4 days for NK and B cells, 6 days for T cells, respectively) cells were harvested and labeled with anti-CD45 and anti-cytoplasmic caspase-3 antibodies to evaluate the IECs undergoing apoptosis. Data are expressed as percentage of caspase-$3^{neg}CD45^{pos}$ cells and analyzed by FACS. Error bars represent the mean ± SD of four different experiments for 1st, 2nd and 3rd trimester-AFS cells. **P<0.05, **P<0.01, ***P<0.001.*

NON MSC MARKERS	1st	2nd	3th
CD 31	1±0 **1±0**	1±0 **1.03±0.03**	1±0 **1±0**
CD 34	1.57±0.4 **1.07±0.07**	1.1±0.06 **1.06±0.06**	1.1±0.1 **1±0**
CD 45	1±0 **1±0**	1±0 **1.02±0.02**	1±0 **1±0**
MSCs MARKERS			
CD 73	41±5.97 **43.3±8.47**	37.5±10 **35±8.37**	86.6±30 **86.06±25.54**
CD 90	339±92 **277.55±138.3**	95±69.37 **134.35±100.5**	328.3±71.3 **247.8±45.45**
CD 105	4±0.53 **2.34±0.1**	2.82±0.4 **2.87±0.52**	4.14±1 **3.1±0.43**
COSTIMULATOTY MOLECULES			
CD40	1.1±0.1 **1.1±0.1**	2.38±0.23 **3±0.5**	1.87±0.25 **2.9±0.5**
CD 80	1.25±0.13 **1±0**	1±0 **1.02±0**	1.03±0 **1.03±0.02**
CD 86	1.1±0.1 **1±0**	1.1±0.05 **1.15±0.15**	1.36±0.16 **1.06±0.04**
ADHESION MOLECULES			
CD 54	23.45±10.22 **519.95±57.04**	25.53±8.9 **582.7±52.65**	11.1±4.63 **728.44±17.9**
CD 106	1.53±0.5 **3.26±0.5**	2.08±0.3 **28.41±8.75**	1.44±0.26 **17.15±7.56**
CD 146	22.14±3.4 **16.8±3.9**	27.3±2 **22.23±1.42**	57.9±14.43 **30.68±5.22**
CYTOKINES RECEPTOR			
CXCR3	1.37±0.1 **1.3±0.06**	1.18±0.06 **1.37±0.1**	1.38±0.1 **1.24±0.05**
CCR7	1.3±0.04 **1.47±0.17**	1.06±0.03 **1.1±0.04**	1.2±0.06 **1.17±0.1**
CXCR5	1.23±0.05 **1.07±0.04**	1.1±0.06 **1.2±0.05**	1.3±0.12 **1.33±0.05**
CD 119	2.57±0.37 **1.6±0.14**	2.91±1.17 **2.4±0.3**	4.65±0.45 **3.2±0.4**

NKG2D LIGANDS	1st	2nd	3th
ULPB-1	3.58±0.2 **3.57±0.1**	1.2±0.07 **1.34±0.07**	1.42±0.14 **1.62±0.23**
ULPB-2	2.6±0.16 **2±0.36**	1.74±0.18 **1.60±0.26**	3.3±0.5 **2.05±0.22**
ULPB-3	1.80±0.7 **1.35±0.26**	1.22±0.15 **2.1±0.7**	1.84±0.56 **1.8±0.65**
MICA/B	2.75±0.9 **1.65±0.23**	2.28±0.75 **1.77±0.43**	3.63±0.1 **2.5±0.9**
TOLL-LIKE RECEPTORS			
TRL 4	1.1±0.05 **1.44±0.2**	2.31±0.76 **2.36±0.72**	2.46±0.3 **2.14±0.1**
IMMUNOMODULATORY MOLECULES			
CD 200	1±0 **1±0**	2.17±0.97 **1.6±0.27**	1.1±0.3 **1±0**
CD 274 (PD-L1)	13.85±3.7 **56.05±7.2**	24.76±5.54 **75.85±19.2**	26.5±6.6 **86.5±13.8**
DNAM LIGANDS			
CD 112	18.14±2.24 **18.3±1.9**	19.1±2.6 **27.24±4.53**	23.63±2.52 **36.37±8.4**
CD 155	26.33±5.1 **38.3±9.6**	86.3±17.55 **107±7.2**	95.65±19.03 **135±34.8**
MHC LIGANDS			
HLA-ABC	4.18±0.6 **7.85±1.77**	7.9±1.07 **17.68±2.2**	12.04±2.67 **25.9±4.66**
HLA-DR	1±0 **3.1±1.24**	1.1±0.06 **2.30±1.14**	1±0 **3.73±1.07**
HLA-G	1±0 **1±0**	1.06±0.06 **1±0**	1±0 **1.22±0.16**
OTHER MOLECULES			
CD 107a	1.16±0.12 **1±0**	1.7±0.58 **1.5±0.4**	1.2±0.1 **1.12±0.05**
NG2	26.16±12.1 **17.45±6.1**	3.46±0.64 **12±9.16**	7.3±1.32 **12.05±6.48**
JAGGED 1	1.17±0.07 **1.12±0.12**	1.13±0.1 **1.1±0.15**	1.27±0.04 **1.12±0.07**

Table I. *Expression of different markers on AFS cells derived from different gestational age.* Resting and primed AFS cells were cultured in presence or not of IFN-γ and TNF-α for 48h. At the end of treatment, AFS cells were harvested and labeled with different antibodies (as reported in Materials and Methods). Data are shown as mean ± SEM of relative Mean Fluorescence Intensity (rMFI) of four (1st and 2nd trimester-AFS cells) or five (3rd trimester AFS cells) experiments derived from resting (upper) and inflammatory (below, bold) cells for each marker.

DISCUSSION

Adult stem cell-based approaches are promising tools for the treatment of different human inflammatory and autoimmune diseases. There are several reports regarding the beneficial roles of the immunomodulatory effects of different stem cell types (mainly MSCs) in disease models, such as graft-versus-host disease (GvHD)[49], experimental autoimmune encephalomyelitis (EAE)[50], inflammatory bowel disease (IBD)[51], and systemic lupus erythematous (SLE)[52]. Moreover, the safety and efficacy of MSC treatments have been validated in clinical trial[53-55] and many other are currently on progress.

Fetal stem cells can be isolated from different gestational tissues, such as umbilical cord blood[56], Wharton's Jelly[57], amniotic fluid (AF)[14], amnion and chorionic layers[58], and are excellent alternatives to adult stem cells.

AFS cells are a subpopulation of fetal stem cells with potential therapeutic applications. AFS cells share some features of pluripotent stem cells, as they are capable of differentiating into cells belonging to all the three germ layers under specific conditions[14] and can form embryoid bodies, which are the main step in the differentiation of pluripotent embryonic stem cells [59].

On the basis of the available data, AFS cells may have a number of advantages in comparison to other stem cells types, including adult stem cells, embryonic stem cells or iPSCs. In fact, while adult stem cells can be difficult to isolate from their niches and embryonic stem cells require embryo disaggregation, AFS cells can be easily obtained without ethical concerns from the amniotic fluid during delivery or diagnostic amniocentesis. Actually, the safety of amniocentesis has been established by several studies documenting an extremely low overall fetal loss rate (0.06-0.83%) related to this procedure[60], and AFS cells can be obtained from samples without interfering with diagnostic procedures[61]. In addition, AFS cells have a stable genome and do not form tumors where injected in vivo [61-63], while embryonic stem cells and iPSCs are tumorigenic, adult stem cells can harbor genetic alterations occurring with aging, and iPSCs may accumulate genetic alterations during expansion in culture. Finally, adult somatic cell reprogramming is a complex process that requires the introduction of exogenous factors. By contrast, first trimester AFS cells can be reprogrammed with high efficiency and stable pluripotent phenotype only by using a chemical substance, thus reducing the risks associated with the random integration of the reprogramming transgenes into the host genomes[64-66].

The effectiveness of AFS cells after transplantation reside in their abilities to reach and engraft into damage tissues and differentiate or induce resident cells into functionally-competent types, and their beneficial effects have been validated in various disease models. For instance, AFS cells integrate into skeletal muscle of *HSA-Cre,SMN*$^{F7/F7}$ mutant mice, improving the muscle functionality through the restoration of muscle cell niche[67]. Another work described the engraftment of AFS cells into nude mice with lung damage, and their expression of specific alveolar or bronchiolar epithelial markers in response to the type of lung damage[19]. Finally, recent works elucidated the regenerative potential of AFS cells into bone, kidney and neural cells, highlighting the plasticity of AFS cells to respond in different ways to various types of injury models[68,69].

Our study carried out for the first time the immunological comparison of AFS cells derived from different gestational age; so far, only comparative studies between 1st and 2nd trimester-AFS cells dealing with gene expression pattern were available. It has been demonstrated that cKit^{+} 1st trimester-AFS cells belong to the most undifferentiated portion of amniotic fluid cell population. Such AFS cell subset displayed a cell-specific expression of pluripotency molecules, such as NANOG, SSEA3, TRA-1-60 and TRA-1-81, and a higher expression of organ-specific genes, as compared to 2nd trimester-AFS cells[31]. These findings are in agreement with the higher expression by 1st trimester AFS cells, as compared to other AFS cells, of NG2 and CD34 that are two molecules typical of undifferentiated stem cell subsets. In several adult tissues, CD34^{+} cells represent a distinct cell population with enhanced self-renewal, higher colony forming unit efficiency and long-term proliferative capacity, and is also found in embryonic stem cell-derived MSCs [70].

The immunomodulatory effect of AFS cells observed in our experiments could explain some aspects of their efficacy and differentiation potential in vivo. Under inflammatory conditions, stimulation of AFS cells with IFN-γ and TNF-α induced an immunophenotypical switch, which was consistent with the immunomodulatory properties observed. As previously described in MSCs [47], the priming-dependent decrease of sensitivity to NK cell lysis of AFS cells could be explained by the overexpression of HLA-ABC and the weak expression of ULBPs and MICA/B which are, respectively, the main inhibitory and activator ligands of NK cells.

Another potential mechanism involved in the AFS cell immune properties, which we observed in the T cell proliferation assay, is the increased expression of PD-L1, ICAM-1 and VCAM-1, the latter two involved in the tethering of IECs. All these immunological features may be correlated with the lack of rejection of AFS cells once injected in vivo, but also with their engraftment capability, and could have a synergic effect in a context of injured tissues, in which these stem cells can divide and preserve the SC pool and their functionality, thus supporting tissue regeneration.

Our observations confirmed the pivotal role of inflammatory priming for the acquisition of the immunomodulatory properties of AFS cells, otherwise supportive rather than inhibitory cells, as previously shown by our group in MSCs and stem cells of different origin[27]. In particular, in absence of pro-inflammatory cytokines, such as IFN-γ, TNF-α and IL-1β, stem cells have an anti-apoptotic/pro-survival effect on IECs. On the contrary, inflammatory cytokine addition makes stem cells switch towards the immunosuppressive phenotype. At resting conditions, 2nd and 3rd trimester-AFS cells enhanced the viability of un-stimulated IECs, thus showing that their immunosuppressive effect is not a constitutive behavior of AFS cells but it is acquired upon IEC activation. By contrast, 1st trimester-AFS cells increased the survival of B cells, but not NK cells, and when these stem cells were co-cultured with resting T cells a significant increase of T cell apoptosis was induced. These differences between 1st trimester-AFS cells and AFS cells of the other two following trimesters regard also immunosuppression mechanisms. The strong inhibitory effect on T cell proliferation of 1st trimester-AFS cells is probably related to their pro-apoptotic effect towards these IECs, but also to their inability to enhance T cell viability at resting conditions. Moreover, as compared to 2nd and 3r^{d} trimester-AFS cells, 1st trimester-AFS cells possess an IDO-independent mechanism, as shown by the lack of rescue effect by using IDO inhibitors such as L1-MT. Further studies will be carried out in our lab to clarify these mechanisms.

Overall, our experiments have provided for the first time new insights on the immunological features of AFS cells isolated at different gestational age. We have highlighted the differences in term of expression of immunological markers, combining these findings with their immunological behavior towards various IECs. The efficacy of stem cells is not only related to their differentiation capacities, but also to their capability of regulating the microenvironment through the release of trophic and immunomodulatory molecules playing a fundamental role in tissue homeostasis. The activation of immune response is a crucial step in the development and progression of degenerative diseases; thus, understanding these mechanisms may help to identify novel therapeutic approaches.

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