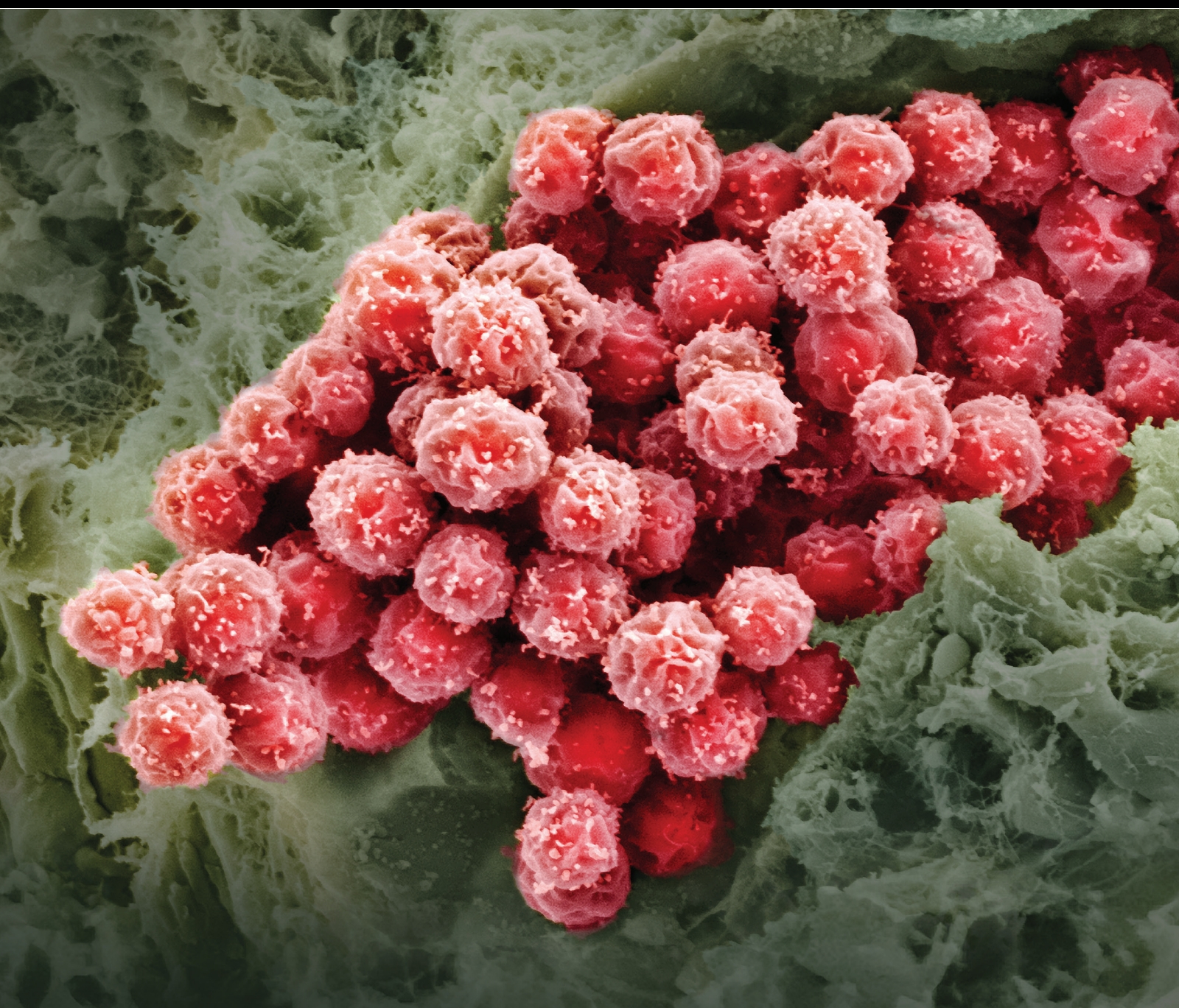
Stem Cells International

Therapeutic Use of Extraembryonic-Derived Tissues

Lead Guest Editor: Michael Uhlin
Guest Editors: Mohamed Abumaree and Essam M. Abdelalim



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Editorial Board

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Editorial

Therapeutic Use of Extraembryonic-Derived Tissues

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Instead as being seen as medical waste, umbilical cord blood (UCB), placenta-derived cells, and other extraembryonic tissue are increasingly accepted as a high-quality source of cells for therapeutic use. The best-known application is the transplantation of hematopoietic stem cells (HSCT), while UCB has become an increasingly important graft source since UCB transplantation (UCBT) has been implemented in the last 3 decades. Recently, UCB, placenta, and extraembryonic-derived cells and tissues have been also investigated as a source for adoptive cell therapy.

The nonhematopoietic stem cell types in UCB as well as placenta-derived and extraembryonic cells and tissues include several types that can be used therapeutically and are readily expanded to sufficient numbers using established methods. Most notable of these are mesenchymal stromal cells (MSCs) and endothelial-like vascular progenitors (EPCs). To complicate it further, MSCs from different sources of the placenta seem to have very different properties.

To even further potentiate the use of extraembryonic-derived tissues for therapy, the sources have to be elaborately characterized. In this special edition, the potential use for this kind of tissues in this was highlighted in several cases illustrating its role in future regenerative medicine. Examples of papers are published in this special edition.

Endometriosis is characterized by the growth of the endometrium outside the uterus, mainly in the pelvic cavity. The pathophysiology of the endometriosis is still not completely understood. Previous reports suggested that there are several factors contributing to the pathogenesis of endometriosis, such as decreased immunosurveillance in the pelvic cavity and stem cells. There is a stem cell theory assuming that because of the retrograde menstruation, mesenchymal stromal cells (MSCs) present ectopically in the pelvic cavity. In this special issue, A. Fawaz et al. published a manuscript in which they provide characterization of the functional phenotype of MSCs in ectopic and eutopic endometria isolated from women with endometriosis. They examined whether the stromal cells of endometriotic ovarian cysts (ESCcyst) and endometrium (ESCendo) have a MSC phenotype. Interestingly, they showed that stromal cells from both ESCcyst and ESCendo have MSC characteristics and were able to differentiate into other cells, such as adipocytes and osteoblasts. It has been reported that MSCs have an immunosuppressive phenotype and express immunosuppressive molecules under increased inflammatory conditions. However, under low level of inflammation, they have an immunostimulatory phenotype and express high levels of proinflammatory cytokines. A. Fawaz et al. found that ESCcyst have more immunosuppressive

characteristics than does ESCendo. Results published in this article suggested that the ESCcyst-immunosuppressive phenotype enhances immunosuppressive M2 macrophage, leading to a reduction in the immunosurveillance of ectopic lesions enhancing the growth of ESCcyst. This data supports the stem cell theory and the retrograde menstruation. In this special issue, another article has been published by the same group. In this article, they investigated the influence of allogeneic MSCs on cells isolated from endometriosis *in vitro*. Allogeneic MSCs were isolated from adipose tissue (Ad-MSC), and stromal cells were isolated from ESCendo and ESCcyst from women with endometriosis. They showed that Ad-MSCs enhance the proliferation, survival, and adhesion of ESC. Furthermore, ESCcyst migration was increased by Ad-MSCs. This article recommends that allogeneic Ad-MSCs are not suitable for therapeutic purposes of the endometriosis with ESCcyst, because they increase the growth and survival of ectopic endometrial tissue.

The placenta is rich with different types of cells and extracts that can be used for therapeutic approaches. In this special issue, O. Pogozhykh et al. published an interesting review article summarizing the types of placental derivatives and their applications in regenerative medicine. These types include cord blood cells, placental extracts, cord blood serum, isolated placental cells, amniotic and chorionic membranes, placental tissues, and amniotic fluid. The review provides information about the biobanking of placental components. Detailed information about the current clinical trial using placental derivatives is described in this review.

A Marmotti et al. described a method for isolation and expansion of UC-derived MSCs that were later successfully differentiated into chondrocytes. Their results would in the future potentially open the possibility to deliver an on-demand allogeneic population of cells for cartilage repair and bone regeneration.

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Research Article

Expansion of Gammadelta T Cells from Cord Blood: A Therapeutical Possibility

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Gammadelta ($\gamma\delta$) T cells are found in both blood and tissues and have antiviral and antitumor properties. The frequency of $\gamma\delta$ T cells in umbilical cord blood (UCB) is low, and the majority express $\delta 1$, in contrast to blood, whereas the main subset is $\delta 2\gamma 9$ T cells. UCB γδ T cells are functionally immature, which together with their scarcity complicates the development of UCB $\gamma\delta$ T cell therapies. We aimed to develop an effective expansion protocol for UCB $\gamma\delta$ T cells based on zoledronate and IL-2. We found that culture with 5 μM zoledronate and 200 IU IL-2/ml medium for 14 days promoted extensive proliferation. The majority of the cultured cells were $\gamma 9\delta 2$ T cells. The fold expansion of this, originally infrequent, subset was impressive (median and maximum fold change 253 and 1085, resp.). After culture, the cells had a polyclonal $\gamma\delta$ T cell repertoire and the main memory subset was central memory ($CD45RO^+$ $CD27^+$). The cells produced cytokines such as IL-1B, IL-2, and IL-8 and displayed significant tumor-killing capacity. These results show that development of *in vitro* expanded UCB $\gamma\delta$ T cell therapies is feasible. It could prove a valuable treatment modality for patients after umbilical cord blood transplantation.

1. Introduction

$\gamma\delta$ T cells constitute a unique minor subpopulation of T cells. Their features place them between innate and adaptive immunity [1] and include antigen recognition independent of major histocompatibility complex (MHC) presentation, cytokine production, and cytotoxicity [2–4].

In humans, there are several subsets of $\gamma\delta$ T cells, identified by the combination of specific TCR γ and δ chains. The major $\gamma\delta$ T cell population in peripheral blood (PB) expresses a TCR containing $\delta 2$ and $\gamma 9$ chains [4]. This subset, termed $\delta 2\gamma 9$ T cells, recognizes phosphoantigens, phosphorylated nonpeptidic metabolic intermediates of the isoprenoid biosynthesis [5] in both invading microbes and the body's own cells, in an MHC-unrestricted manner [4, 6]. Endogenous phosphoantigens are upregulated in cellular dysregulation, a state that can result from infection or malignant transformation, and have been implicated as a key factor in $\gamma\delta$ T cell tumor recognition [4, 7, 8]. This indicates a role for $\delta 2\gamma 9$ T cells in anticancer immunity.

Another important $\gamma\delta$ T cell subset expresses the $\delta 1$ chain and is known to be predominant in the thymus and peripheral tissues. These $\gamma\delta$ T cells are considered to recognize various stress-related antigens, most of which are uncharacterized. Known specificities include CD1 family proteins [9], MICA, and MICB [10, 11].

$\gamma\delta$ T cells constitute approximately 5% of circulating T cells in adult PB [12], but the compartment can increase substantially in certain situations [13, 14]. In umbilical cord blood (UCB), $\gamma\delta$ T cells are present at a low frequency (<1% of lymphocytes [15]) and express a naïve phenotype. The repertoire is polyclonal, with $\delta 1$ T cells being the

predominant subtype [16, 17]. The $\delta 2\gamma 9$ T cells are few in UCB [18, 19] and have been described as functionally immature: they express low frequencies of high-affinity interleukin 2 receptor (IL-2R) β chain and have reduced interferon-(IFN-) γ production [20]. However, a higher expression of the IL-2R α chain has been reported in UCB $\delta 2\gamma 9$ T cells [20], as well as a decreased expression of the common IL-2Rγ chain on UCB lymphocytes in general [21].

$\gamma\delta$ T cell immunotherapy is currently being explored. An important milestone was the discovery that bisphosphonates, drugs for osteoporosis, inhibit a downstream enzyme in the isoprenoid biosynthesis, causing accumulation of metabolites and making exposed cells $\gamma 9\delta 2$ T cell targets. Thus, *in vitro* expansion of $\gamma 9\delta 2$ T cells can be easily performed with clinically approved and readily available compounds.

Expansion of $\gamma\delta$ T cells from adult PB has been explored with considerable success [22–24], and several early clinical trials of expanded PB $\delta 2\gamma 9$ T cell immunotherapy have been performed in patients with malignancies [25]. Other trials have explored treatment of patients with bisphosphonates and IL-2 to induce *in vivo* $\gamma 9\delta 2$ T cell expansion [25]. Noteworthy results include those of a phase I/II study of *in vitro* expanded $\gamma 9\delta 2$ T cell therapy in patients with renal cell carcinoma [26], where reduction of lesions or significantly reduced tumor growth rate could be seen in 6/11 patients. A commercial product with *in vitro* expanded Vδ2Vγ9 T cell has been developed and found safe in a phase I study in patients with renal cell carcinoma [27, 28].

In vitro expansion of $\gamma\delta$ T cells from umbilical cord blood (UCB) for clinical use includes several challenges, including the low number of $\gamma\delta$ T cells present, the low percentage of $\gamma 9\delta 2$ T cells capable of responding to phosphoantigens, and their immature phenotype. UCB $\gamma\delta$ T cells have been found to be relatively unresponsive to model phosphoantigens, but to proliferate in response to bisphosphonates [15, 18].

IL-2 and IL-15 have been used in combination with bisphosphonates, and IL-15, both with IL-2 and alone, has been described to contribute to reduced apoptosis and higher cytokine and cytotoxic mediator expression upon restimulation [18]. However, *in vitro* expansion of UCB $\gamma\delta$ T cells with the bisphosphonate alendronate or zoledronate and a low dose of IL-2 has been described to preferentially induce differentiation into a cytokine production rather than a cytotoxic phenotype [15].

The development of $\gamma\delta$ T cell products for use after hematopoietic stem cell transplantation (HSCT) is an attractive prospect. The clinical significance of $\gamma\delta$ T cells in the HSCT context is clearly demonstrated in reports showing that higher frequencies of $\gamma\delta$ T cells after transplantation are associated with favorable outcome [29–31]. Importantly, reconstitution of $\gamma\delta$ T cells after HSCT depends mainly on the graft source, with poor reconstitution of $\gamma\delta$ T cells seen after umbilical cord blood transplantation (UCBT). The impact of graft source on $\gamma\delta$ T cell reconstitution can most likely be attributed to the number and quality of the $\gamma\delta$ T cells present in the graft [31], stressing the potential of $\gamma\delta$ T cell immunotherapy in UCBT recipients, preferentially with graft-derived UCB $\gamma\delta$ T cells.

The aim of the present study was to further explore the in vitro culture of UCB $\gamma\delta$ T as a potential source of cells for adoptive cell therapy (ACT), with specific focus on treatment after UCBT. The first step towards the development of a successful ACT strategy is the establishment of an efficient production protocol easily conformable to good manufacturing practice (GMP) regulations. We have here initialized the development of such a protocol, using the experiences of others, and we are continuing to explore optimal production conditions. The choice of the reagents for the protocol, zoledronate, and IL-2 was based on their availability in formulations conforming to GMP standards. We also chose to focus on the expansion of $\delta 2\gamma 9$ T cells, a subset known to be easily and reliably expanded *in vitro*, and to have antitumoral and anti-infectious properties, making it suited for use as adoptive therapy after HSCT. We found that we could successfully expand products with a high percentage of $\gamma\delta$ T cells present, of which the majority were $\delta 2\gamma 9$ T cells in spite of very small numbers of this subset at baseline. We also found that they displayed both cytokine production and cytotoxic antitumor capacity. We see considerable potential in this protocol, especially in the context of UCBT, and hope to further develop this culture protocol towards clinical application.

2. Material and Methods

2.1. Umbilical Cord Blood Units. Umbilical cord blood (UCB) was collected from healthy volunteers giving birth at the maternity ward at Karolinska University Hospital in Huddinge, Stockholm, Sweden, for the Swedish Umbilical Cord Blood Bank, and was made available for research due to insufficient cell numbers for clinical use. Collected UCB units were received without any labeling that could enable tracking of the donor and were given unique numbers according to order of collection. The UCB donors were three females and five males. Peripheral blood mononuclear cells (PBMC) were collected from healthy volunteers, two females and one male. All donors had given their informed consent prior to donation in accordance with the guidelines and regulations stipulated by the Karolinska Institute and with the declaration of Helsinki. The project was approved by the regional ethical board (2007/4:10).

2.2. Sample Preparation and Cell Culture. Mononuclear UCB and PB cells were obtained by density gradient separation (Lymphoprep, Fresenius Kabi Norge AS) and washed and cryopreserved in complete medium (as defined below) supplemented 10% di-methyl sulphoxide (DMSO, Wak-Chemie Medical GmbH, Steinbach, Germany) in liquid nitrogen. The frozen samples were thawed and washed prior to culture, and for all but the preliminary experiment, a depletion of $CD56^+$ cells was performed for all UCB cultures using a 2-step positive selection method by first staining the cells with CD56-APC antibody (BD Bioscience, Franklin Lakes, NJ, USA) and then adding MACS anti-APC beads (Miltenyi, Bergisch Gladbach, Germany) according to manufacturers' instructions. Cells were then seeded at a concentration of 1×10^6 viable mononuclear cells/ml in complete medium, defined as 1640 RPMI (Life Technologies

(Gibco)) supplemented with 10% pooled human AB-serum (Department of Transfusion Medicine at Karolinska University Hospital Huddinge), 100 IU/ml penicillin G, 100 mg/ml streptomycin, 0.25 mg/ml amphotericin B (Life Technologies (Gibco)), and 2 mM L-glutamine (Sigma Aldrich Inc, St Louis, MO). Recombinant IL-2 and the bisphosphonate zoledronate were added in varying concentrations (0–500 IU/ml and 0–10 μM, resp.) according to Table 1. Cell culture was performed at 37°C at 5% CO_2. Viable cells were counted using trypan blue exclusion every other day and replated to maintain cell concentration. Cells were replated using a medium containing IL-2 according to protocol, but zoledronate was only added on day 0. On day 7, cells were spun down by centrifugation, and the supernatant was removed in order to completely replace the culture medium. On day 14, cells were counted, harvested, and cryopreserved in complete medium supplemented with 10% DMSO.

2.3. Flow Cytometry. Cell surface staining was performed as described previously [32]. Briefly, cells were incubated at 4°C for 20 min with antibodies diluted in PBS supplemented with 1% FCS (staining buffer) and washed before FACS analysis. Acquisition was performed on a FACSCANTO flow cytometry instrument (BD) using the FACSDiva software (BD). The acquired data was analyzed with FlowJo (Tree Star Inc., Ashland, OR) and subsequently using the bh-SNE algorithm in the CYT software (Dana Pe'er Lab, ref) running on MatLab (MathWorks Inc., Natick, MA, USA). Control stained samples were used for gating according to the fluorescence minus one technique. The antibodies used in the flow cytometry panels are described in Table 1.

2.4. Spectratyping. The $\gamma\delta$ TCR repertoire with regard to the main δ families (Vδ1, Vδ2, and Vδ3) and γ families (Vγ9, Vγ10, and Vγ11) was assessed using the spectratyping method adapted from Rådestad et al. [33]. Briefly, DNA was extracted from expanded UCB cells, and amplifications of 12 δ chain subfamilies and 9 γ chain subfamilies were performed by multiplex PCR reaction using primers as described before [34] (Tables S1–3). The PCR was performed using AmpliTaq Gold 360 Master Mix (Applied Biosystems), specific primers in a final concentration of 200 or 400 nanoMoles as indicated in Table S1 and S2, and 100 ng of DNA, with a thermal cycler PCR machine (PTC-200, MJ Research, Watertown, MA). The process included the following steps: initial denaturation at 95°C for 10 minutes, followed by 35 cycles each of 94°C for 30 sec, 60°C for 45 sec, 72°C for 60 sec, and a final elongation step at 72°C for 10 minutes; and capillary electrophoresis where each PCR product was mixed with formamid (FA, HiDi Formamide) and size standard (GeneScan 400HD Rox Size std, Applied Biosystems) in 96-well MicroAmp plates (Applied Biosystems). Samples were analyzed using 3130 × 1 Genetic Analyser (Applied Biosystems). The results were analyzed using the PeakScanner software (Applied Biosystems).

2.5. Cytokine Production Assay. Relative expression of cytokine genes was determined for expanded UCB and PB $\gamma\delta$ T cells cultured with 5 μM zoledronate and 200 IU IL-2/ml medium. Briefly, cryopreserved cells were thawed and RNA was extracted from sorted $\gamma\delta$ T cells (TCR γ/δ T cell isolation kit, Miltenyi) using the PureLink™ RNA Mini Kit (Invitrogen, ThermoFisher) and converted to cDNA using SuperScript™ IV VILO™ Master Mix (ThermoFisher) according to manufacturer's instructions. Real-time (Rt) PCR was performed on 7500 fast real-time PCR instrument (Applied Biosystems), using TaqMan Gene Expression Assays for *IL-1B*, *IL-2*, *IL-6*, *IL-7*, *IL-8*, *IL-12B*, *IL-15*, and *IL-17* genes and human ACTB gene as reference gene.

2.6. Cytotoxicity Assay. $\gamma\delta$ killing was assessed using a cytometry-based assay as previously described [35]. Briefly, cryopreserved expanded UCB and PB $\gamma\delta$ T cells cultured with 5 μM zoledronate and 200 IU IL-2/ml medium were thawed and cocultured with target tumor cells from the human cholangiocarcinoma cancer cell line Hucct-1. Hucct-1 cells were labeled with the CellTrace™ Violet Cell Proliferation marker (CTV, Thermo Fisher) for easy identification and incubated with cultured $\gamma\delta$ cells at 37°C, with 5% CO_2 for 24 hours at an effector: target ratio of 10 : 1. Cell viability was then assessed by flow cytometry in CTV positive cells using annexin V. The % of target cells killed by $\gamma\delta$ T cells was calculated by the following formula: % $\gamma\delta$ T cell killing = $100 - (\%$ *viable CTV + Hucct-1 cells in cocultures with* $\gamma\delta$ *cells)/(% viable CTV + Hucct-1 cells in control culture without* $\gamma\delta$ *cells)* $\times 100$.

2.7. Statistics. Data was analyzed and displayed using Prism 6 (GraphPad, San Diego, CA). The T cells cultured under different conditions were paired according to level of IL-2 and the presence or absence of zoledronate in the culture media. Due to the limited sample size, nonparametric pair-wise comparisons were performed using the Wilcoxon rank-sum test, and in the instances where 5 or fewer measurements were available in either group, the Mann–Whitney *U* test.

The marker expression patterns of cells cultured in different culture conditions were also compared using the bh-SNE algorithm, which, briefly, converts multidimensional data into bidimensional, mapping similarities by first calculating a pairwise distance matrix for the high-dimensional space and transforming it into a similarity matrix using a varying Gaussian kernel. A random bidimensional map is then rendered, and pairwise similarities are calculated for the low-dimensional space created. The map is then optimized in iteration steps where the calculated similarity between any given two cells is rechecked to optimally redistribute them on the map. The analysis was performed using the CYT software from Dana Pe'ers lab on the Matlab platform [36]. Here, flow cytometry data was gated to include only $CD3^+$ single-cell events and exported as separate files using FlowJo. The fluorescence data was arcsin transformed with a cofactor of 150 in CYT for comparability, and subsamples of 5000 events were obtained randomly from the sample files before bh-SNE analysis.

The analysis of viability and apoptosis was performed using the SPICE software [37], which generated graphics illustrating the differences in the proportion of dead and apoptotic cells between the groups, and the statistical analysis

Table 1: Surface staining.

Target antigen	Fluorophore	Clone	Company, address
CD28	FITC	CD28.2	
CD56	FITC	NCAM16.2	
CD69	FITC	FN50	
CD94	FITC	HP-3D9	
CD95	FITC	DX2	
PD-1	FITC	MIH4	
PD-1	BV421	MIH4	
TCR$\alpha\beta$	FITC	T10B9.1A-31	
CD8	FITC	SK1	
CD8	APC Cy7	SK1	*BD Bioscience* Franklin Lakes, NJ, USA
CD8	V500	RPA-T8	
CD45RO	APC	UCHL1	
CD4	Alexa Fluor 700	RPA-T4	
CD3	PE	UCHT1	
CD3	V450	UCHT1	
CD3	BV510	UCHT1	
CD19	PE	HIB19	
CD27	PE	M-T271	
CD27	BV421	M-T271	
CCR7	PE-Cy7	3D12	
TCR$\gamma\delta$	FITC	IMMU510	*Beckman Coulter Inc.* Indianapolis, IN, USA
Cd152/CTLA-4	FITC	A3.4H2.H12	*LifeSpan Biosciences* Seattle, WA, USA
CD223/LAG-3	FITC	17B4	
CD366/TIM-3	APC	F38-2E2	
TCR Vδ2	FITC	B6	*BioLegend* San Diego, CA, USA
TCR Vγ9	FITC	B3	
TCR Vδ1	FITC	TS8.2	*Thermo Fisher Scientific* Waltham, MA, USA
TCR$\gamma\delta$	PE	REA591	*Miltenyi* Bergisch Gladbach, Germany
Apoptosis and viability			
7AAD			*BD Biosciences*
Annexin V	FITC		
Annexin V	APC		*Immunotools GmbH* Friesoythe, Germany

7-AAD: 7-aminoactinomycin; APC: allophycocyanin; CD: cluster of differentiation; FITC: fluorescein isothiocyanate; PE: phycoerythrin.

was performed using the built-in test in the software, applying 1,000,000 permutations, as described previously [37].

3. Results

3.1. The Proliferation of UCB γδ T Cells Is Influenced by the Concentrations of IL-2 and Zoleronate in the Culture Medium. UCB units were divided into five parts that were cultured in complete medium containing 50 IU IL-2/ml alone or, 50, 100, 200, or 500 IU IL-2/ml combined with 5 μM zoledronate. The selection of the experimental conditions was based on an initial experiment where a UCB unit was divided into 13 parts cultured separately with different concentrations of IL-2 (50, 100, 200, 400, or 600 IU/ml culture medium) and zoledronate (5 μM or 10 μM), or in complete medium only. Assessment on day +14 for viable cell count using trypan blue exclusion and for phenotype with flow cytometry indicated that the lower dose of zoledronate (5 μM) resulted in the best results with regard to viability, proliferation, and percentage of $\gamma\delta$ T cells. Zoledronate in the absence of IL-2 had limited effect on the proliferation of $\gamma\delta$ T cells. IL-2 was found to reinforce the zoledronate-induced proliferation of $\gamma\delta$ T cells already from a low concentration (data not shown). A sizeable number of $CD56^+$ $CD3^-$ NK cells was seen at the end of expansion, which led us to introduce bead-based depletion of $CD56^+$ cells into

the protocol. After the first experiments indicated that 500 IU IL-2/ml had limited, if any, additional effect on $\gamma\delta$ T cell proliferation compared to 200 IU IL-2/ml, the final cell cultures were performed with either 50, 100, or 200 IU IL-2/ml and 5 μM zoledronate. Lastly, for comparison purposes, peripheral blood mononuclear cells (PBMC) from healthy volunteers were cultured in medium with 200 IU IL-2/ml and 5 μM zoledronate.

Assessment of viable cell counts, using trypan blue exclusion, and the phenotype of the cultured cells was performed after 14 days of culture. The results, displayed in Figure 1, indicated that the highest number of viable UCB cells was obtained using medium containing zoledronate and 200 IU IL-2/ml (Figure 1(a)). However, the cell counts were comparatively lower in cultured UCB cells than in PBMC expansions, as might be expected based on the small numbers of $\gamma 9\delta 2$ T cells in baseline UCB. The percentage of TCR$\gamma\delta$ UCB T cells was high after culture with medium containing zoledronate with IL-2 concentrations between 50 and 200 IU/ml, with a tendency towards higher percentages $\gamma\delta$ T cells with higher IL-2 concentrations (Figures 1(b) and 1(c)). The percentage of $\gamma\delta$ T cells in PBMC cultures tended to be slightly higher (Figure 1(b)). The change from a T cell population with a small proportion of $\gamma\delta$ T cells in UCB unit at baseline sample to a postculture T cell population containing a majority of $\gamma\delta$ T cells is illustrated in Figure 1(c), where graphical "maps" generated using the bh-SNE algorithm are displayed. The analysis was performed for UCB cultures on gated $CD3^+$ T cells. The total proportion of $\gamma\delta$ T cells in each group (all included samples assessed together) indicates that, on a group basis, the highest proportion of $\gamma\delta$ T cells was achieved using 100–200 IU IL-2/ml medium (Figure 1(c), lower right panel).

The fold expansion of $\gamma\delta$ T cells was robust, with median fold changes of 14, 33, and 47 in UCB expansions cultured with medium containing zoledronate and 50, 100, and 200 IU IL-2/ml, respectively (Figure 1(d)). The fold change of PB cultures was higher, with a median of 1099.

There seemed to be no discernable difference with regard to percentage of $\gamma\delta$ T cells at baseline (Supplementary Figure 1a) or after culture, or with regard to fold expansion at the end of culture (data not shown) between UCB cultures derived from male newborns compared to ones from female newborns.

3.2. Superior Viability of Cultured UCB $\gamma\delta$ T Cells Is Achieved Using Medium Level Concentrations of IL-2. We assessed cell death and apoptosis in the UCB cells before and at the end of culture by flow cytometry using a combination of the markers 7-AAD (7-Aminoactinomycin D) and annexin V. Cells positive for both these markers were defined as dead or dying, and cells single-positive for annexin V were defined as apoptotic, as has been described before [38]. Cells negative for both these markers were defined as viable. We found that the proportion of viable, apoptotic and dead $\gamma\delta$ T cells, differed significantly after culture with 5 μM zoledronate and 100–200 IU IL-2/ml compared with 5 μM zoledronate and 50 IU IL-2/ml, with a higher proportion of viable cells and less dead and apoptotic cells with the former (Figure 2).

3.3. The Majority of Cultured UCB $\gamma\delta$ T Cells Express TCR Vδ2 and TCR Vγ9 and Have a Memory Phenotype. We further wanted to establish which subtype of $\gamma\delta$ T cells was generated by our expansion protocol, based on which TCR γ chains and δ chains made up the TCR of the cultured $\gamma\delta$ T cells. Due to technical factors regarding the flow cytometry panel setup, the analysis of pan-TCR$\gamma\delta$ and Vγ9, Vδ1 and Vδ2, respectively, in the same panel was not possible. However, using the known percentage $\gamma\delta$ T cells/$CD3^+$ T cells and comparing it with the percentage $V\gamma9^+$, $V\delta1^+$, and $V\delta2^+/CD3^+$ T cells, the proportions of γ9, δ1, and δ2 $\gamma\delta$ T cells could be estimated.

As expected, a majority expressed Vγ9 and Vδ2, respectively, in both UCB and PBMC expansions, while the percentage of $V\delta1^+$ cells was reduced from baseline (Figure 3(a)). Interestingly, we noticed that a slightly higher percentage of cells was consistently found to be positive for Vγ9 than for Vδ2 in UCB-derived cultures but not PBMC-derived cultures. At baseline, there was a nonsignificant tendency towards higher proportions of $V\delta1^+$ T cells in UCB units from male newborns than in units from females (Supplementary Figure 1b). There were no discernable differences for the other subsets at baseline (Supplementary Figure 1b) or after culture (data not shown).

The fold expansion of γ9 T cells was impressive in both PB and UCB cultures. The small numbers of cells found in UCB at baseline expanded dramatically, with median fold changes of 50, 110, and 253 in the expansions cultured with medium-containing zoledronate and 50, 100, and 200 IU IL-2/ml, respectively, and with a maximum fold change of 1085 in a culture with 200 IU IL-2/ml and 5 μM zoledronate (Figure 3(b)). The PB γ9 T cells had a median fold change of 1109. In contrast, the median fold expansion for $V\delta1^+$ T cells was moderate (Figure 3(c)). No visible difference in fold expansion could be seen between male and female UCB units.

We also studied the memory phenotype, using a standard definition based on the coexpression of CD45RO and CCR7, originally defined for conventional PB $\alpha\beta$ T cells. Naïve T cells are $CD45RO^-$ $CCR7^+$, central memory is $CD45RO^+$ $CCR7^+$, effector memory is $CD45RO^+$ $CCR7^-$, and terminally differentiated cells are defined as $CD45RO^-$ $CCR7^-$. We found that the predominant phenotype after culture corresponded with the effector memory phenotype ($CD45RO^+$ CCR7) in both UCB and PBMC expansions. However, the baseline UCB and PB $\gamma\delta$ T cell population had a high percentage of terminally differentiated cells according to this definition (Figure 3(d)). In line with several previously published articles, we then analyzed the memory phenotype using a definition where CCR7 is substituted with CD27 [15, 18, 39, 40]. According to this definition, the majority of the baseline UCB $\gamma\delta$ T cells was naïve ($CD45RO^-$ $CD27^+$), with some representation of central memory ($CD45RO^+$ $CD27^+$) and effector memory ($CD45RO^+$ $CD27^-$) cells, and almost no terminally differentiated cells ($CD45RO^-$ $CD27^-$, Figure 3(e)). Interestingly, PB $\gamma\delta$ T cells were divided almost evenly between the memory subsets at baseline, with slight predominance of the naïve and central memory phenotypes. In the cultured UCB cells, the largest subset was positive for

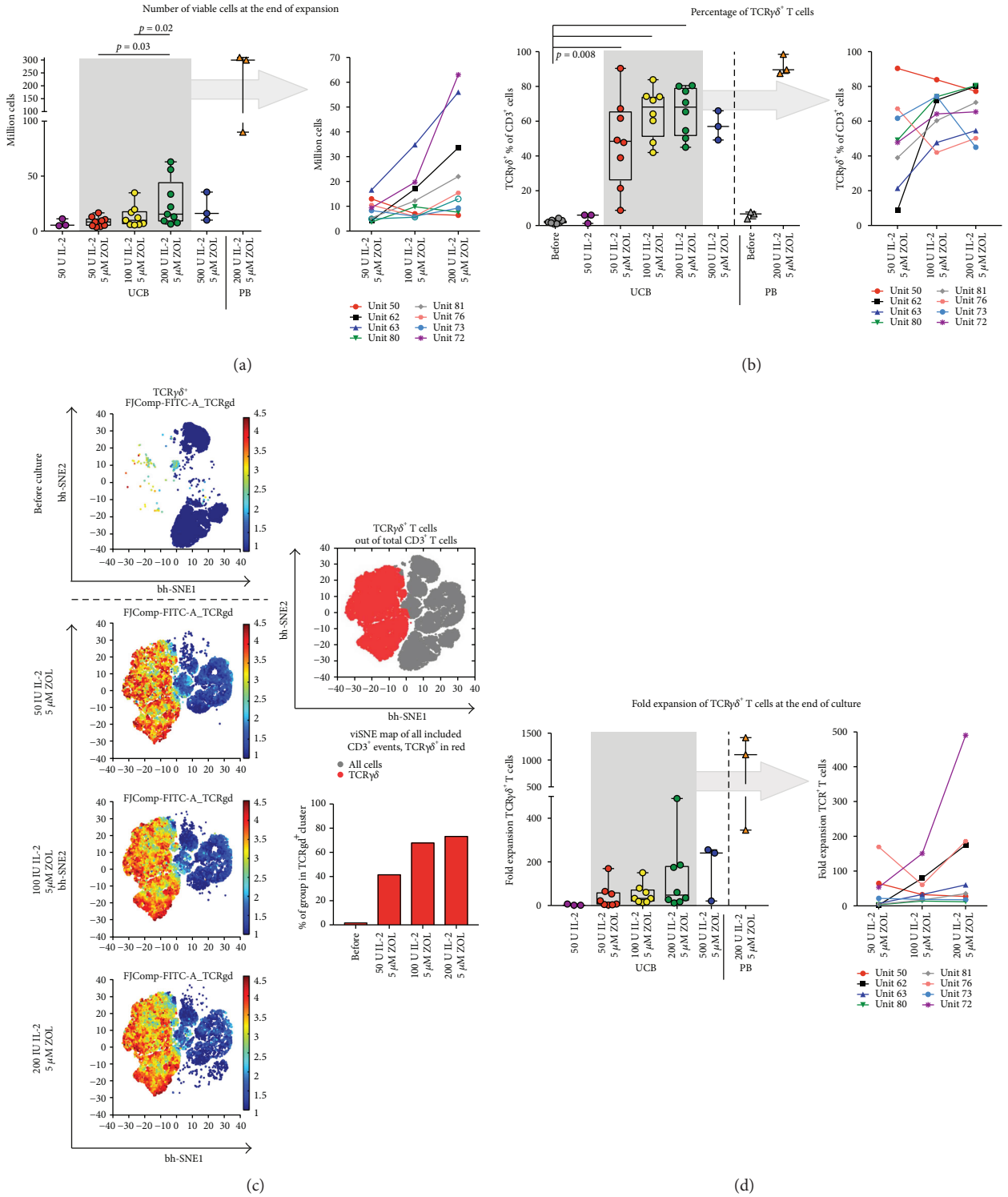

Figure 1: Expansion of $\gamma\delta$ T cells. The number of viable cells after 14 days of culture is displayed according to culture conditions in (a). Here, and in (c) and (d), the grey box in the left graph encompasses the results of UCB $\gamma\delta$ T cells cultured with 5 μM zoledronate and 50, 100, or 200 IU IL-2/ml medium, and in the in right graph, indicated by the arrow, the data inside the box is displayed separately for each cultured UB unit (each unit is indicated by a differently colored line). In (b) and (c), the proportion of $\gamma\delta$ T cells is visualized in two different manners. In (b), the percentage of TCR$\gamma\delta^+$ T cells is shown before and after culture. In (c), the same data is displayed for expansions of UCB cells using the bh-SNE algorithm to analyze and visualize the data. The panels to the left display the fluorescence intensity in the flow cytometry channel for TCR$\gamma\delta$, normalized using arcsin transformation with cofactor 150, for every cell in the displayed groups. In the top right panel, an overview of the cells in all samples is displayed and gated according to where the TCR$\gamma\delta^+$ cells are clustered. The lower right panel shows the percentage of cells in each group found in the TCR$\gamma\delta^+$ cell cluster. The fold expansion of $\gamma\delta$ T cells is displayed in (d). IL-2: interleukin 2; TCR: T cell receptor; ZOL: zoledronate.

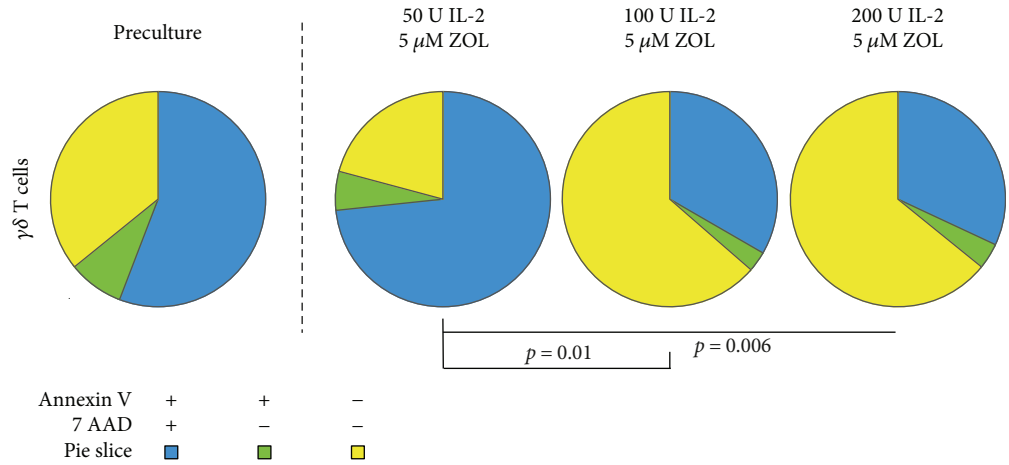

FIGURE 2: Cell death and apoptosis. Viability and apoptosis. The mean proportion of viable (annexin V^- $7AAD^-$) cells, apoptotic (annexin V^+ $7AAD^-$) cells, and dead (annexin V^+ $7AAD^+$) cells are displayed and analyzed using the SPICE software. Statistical comparisons were performed using the built-in statistical analysis in the SPICE software. IL-2: interleukin 2; ZOL: zoledronate.

CD45RO but had maintained CD27 expression, classifying them as antigen-experienced cells with a central memory phenotype, while the largest subset in the PBMC expansions had upregulated CD45RO but lost CD27 expression, indicating an effector memory phenotype with this definition.

3.4. Spectratyping Showed a Polyclonal Pattern, Indicating a Postexpansion Cell Product Containing a Variety of TCR γδ Specificities. Spectratyping analysis was performed in order to assess the clonality of the repertoire of the expanded UCB γδ T cells. The subfamilies of γ and δ chains can be classified as polyclonal (here defined as >6 peaks), oligoclonal (defined as 3–6 peaks), or monoclonal (defined as <3 peaks).

The results indicated that the expansion protocol resulted in similar TCR γδ repertoires in all tested culture conditions (5 μM zoledronate combined with either 50, 100, or 200 IU IL-2/ml culture medium). The cultured cell populations had mainly polyclonal repertoires in the Vδ1-Jδ1, Vδ2-Jδ1, and Vδ3-Jδ1 subfamilies, the Vδ1-Jδ2 and Vδ2-Jδ2 subfamilies, the Vδ1-Jδ3 and Vδ2-Jδ2 subfamilies, and for the majority of the Vγ9, Vγ10, Vγ11, and Vγfl subfamilies (Figure 4(a)). This indicates that the culture procedure caused independent expansion of a multiplicity of different clones, leading to a final cell population with a broad TCR repertoire.

Several subfamilies had a predominantly oligoclonal or monoclonal repertoire or were missing peaks in a several analyzed samples, however. These included the Vδ3-Jδ2 subfamily and all the Vδ1-Jδ4, Vδ2-Jδ4, and Vδ3-Jδ2 subfamilies. Samples with missing peaks in the Vδ1-Jδ4 and Vδ3-Jδ4 subfamilies were slightly more common in the cultures exposed to 50 IU IL-2/ml culture medium (4/5, in both) than in the expansions cultured with 100 or 200 IU IL-2/ml (2/5 and 1/5, respectively, and 1/5 and 4/5, respectively, Figure 4).

3.5. Cytokine Production Differed between Cultured Umbilical Cord Blood and Peripheral Blood γδ T Cells. Cytokine gene expression was measured by Rt-PCR in expanded UCB and PBMC γδ T cells cultured with 5 μM zoledronate and 200 IU IL-2/ml medium (selected based on superior fold expansion of UCB γδ T cells, especially of γ9 T cells). IL-1β, IL-2, IL-6, IL-12β, IL-15, and IL-17 were examined, and, interestingly, the expression of IL-1β, IL-2, and IL-8 was significantly higher in UCB γδ T cells (Figure 5(a)). This was especially striking in the case of IL-1β, as the PB γδ T cells expressed IL-1β at a very small extent. The gene expression with regard to IL-17 was low in both UCB and PB γδ T cells (Figure 5(a)).

3.6. The Cultured UCB γδ T Cells Display Cytotoxic Capacity. A cytotoxicity assay using coculture of γδ T cells and CTV-labeled cells from the Hucct-1 tumor cell line was performed to test the killing capacity of the cultured γδ T cells. We selected expansions that had been cultured with 5 μM zoledronate and 200 IU IL-2/ml medium for this assay. Target cell killing, measured as percentage annexin V positivity in tumor cells, ranged from 33.9–72%, with a median of 49.8% in the 5 tested UCB culture products (Figure 5(b)). The two PBMC cultures tested for comparison showed killing rates of 38.8 and 40.9 (Figure 5(b)).

3.7. The Expression of Phenotypical T Cell Markers Is Affected by the Culture Process. We wanted to further elucidate the kinetics of activation markers and cosignaling receptors on the cultured cells and thus analyze the costimulation marker CD28, the activation marker CD69, the activation/proapoptosis marker CD95, and the coinhibitory receptors CTLA-4, PD-1, LAG-3, and TIM-3. Due to technical considerations in the setup of flow cytometry panels, these receptors were assessed on total $CD3^+$ T cells. We could see that significant downregulation of CD28 and upregulation of CD95 and CD69 was induced during the culture process compared to the baseline values in both UCB- and PBMC-derived culture products (Figures 6(a) and 6(b)). There was a significantly higher proportion of $CD69^+$ UCB T cells in expansions cultured in medium with 200 IU IL-2/ml compared to with 50 IU IL-2 /ml,

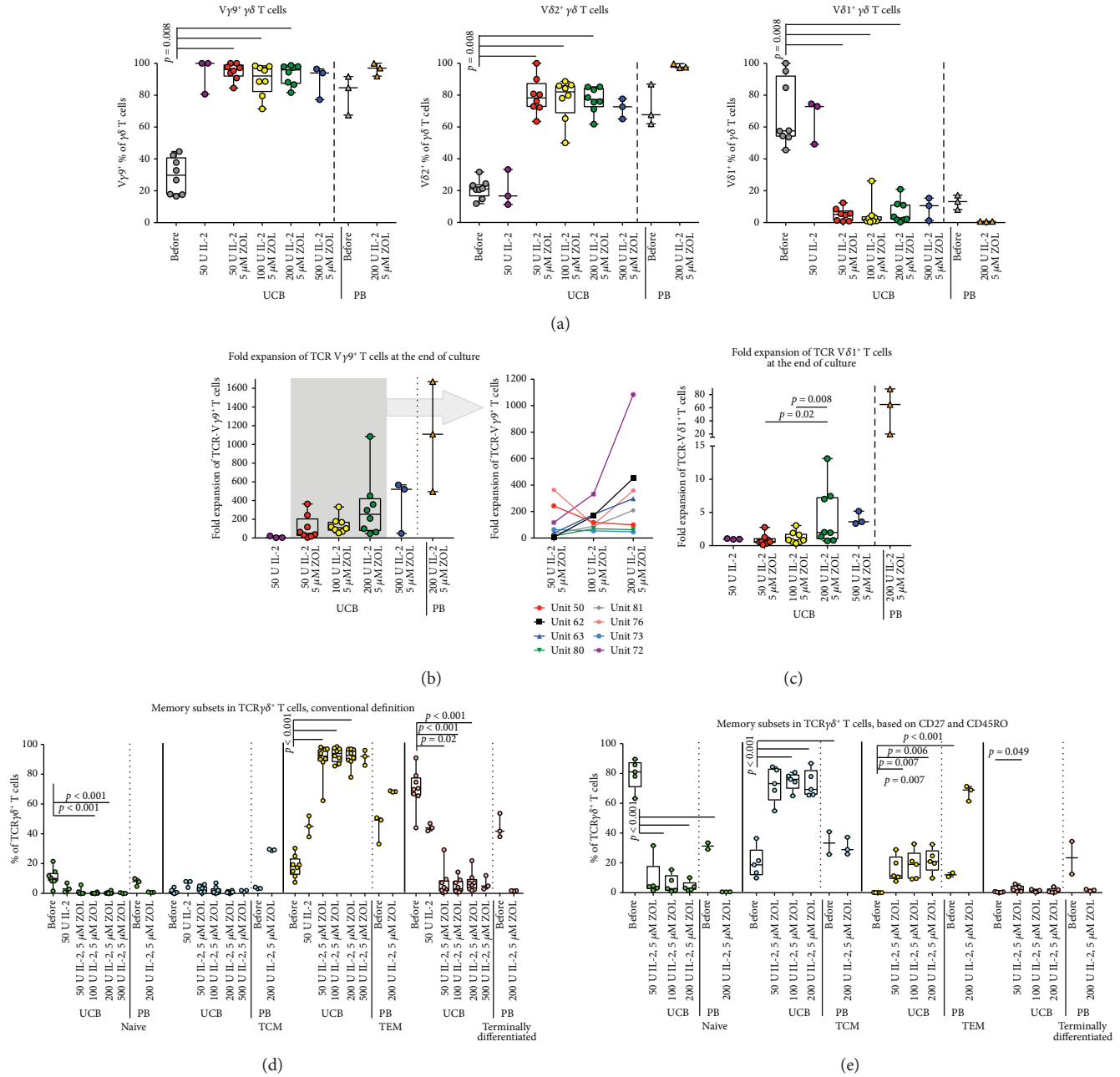

FIGURE 3: γδ chain usage and memory phenotype in cultured γδ T cells. Phenotype of the cultured γδ T cells. The phenotype of the γδ T cells with regard to the expression of memory markers and specific γ and δ chains was assessed by flow cytometry. In (a), the percentage of γδ T cells positive for Vγ9 (left panel), Vδ2 (middle panel), and Vδ1 (right panel) is shown. The fold expansion of Vγ9⁺ T cells and Vδ1⁺ T cells is displayed in (b) and (c), respectively. In (b), the grey box in the left graph contains the UCB γδ T cells cultured with 5 μM zoledronate and 50, 100, or 200 IU IL-2/ml medium, and in the right graph, indicated by the arrow, the data inside the box is displayed separately for each cultured UB unit (each unit is indicated by a differently colored line). The memory phenotype of the γδ T cells before and after culture, using a definition based on the expression of CD45RO and CCR7, is displayed in (d). Naïve T cells were defined as $CD45RO^-$ $CCR7^+$, central memory as $CD45RO^+$ $CCR7^+$, effector memory as $CD45RO^+$ $CCR7^-$, and terminally differentiated cells were defined as $CD45RO^-$ $CCR7^-$. The memory phenotype, defined by the expression of CD45RO and CD27, is displayed in (e). Naïve T cells were defined as $CD45RO^-$ $CD27^+$, central memory T cells as $CD45RO^+$ $CD27^+$, effector memory T cells as $CD45RO^+$ $CD27^-$, and terminally differentiated T cells as $CD45RO^-$ $CD27^-$. IL-2: interleukin 2; TCM: central memory T cells; TCR: T cell receptor; TEM: effector memory T cells; ZOL: zoledronate.

with a nonsignificant trend towards the same for CD95 (Figure 6(b)). The expression of co-inhibitory receptors was also affected: PD-1 showed a general increase in expression in UCB but not PB γδ T cells after culture. For TIM-3, there was a nonsignificant trend toward the same (only studied in UCB expansions). The percentage of

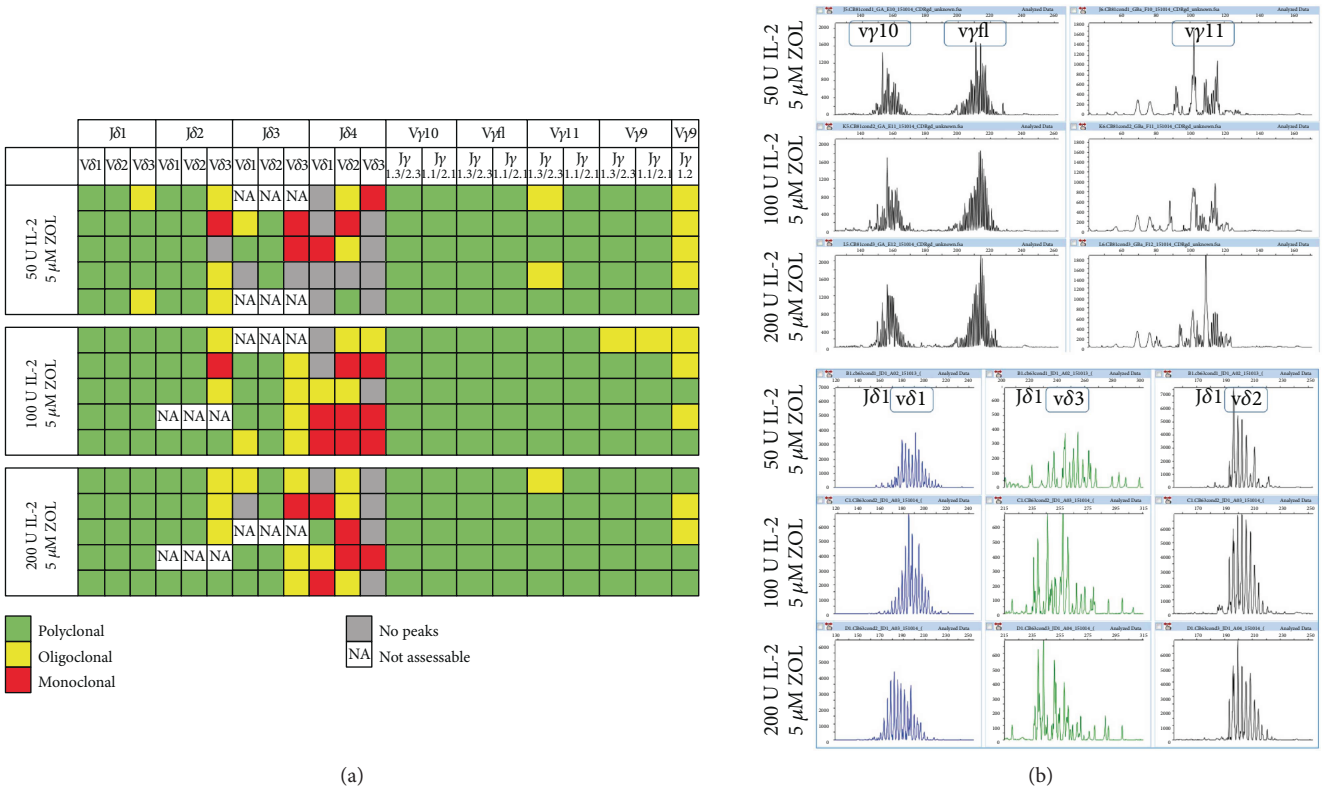

Figure 4: Spectratyping results. TCR repertoire of the cultured γδ T cells. Spectratyping results with the expression of γ and δ chains in the TCR of the expanded γδ T cells are displayed, with regard to the clonality of each subfamily, for the postculture sample, in (a). Each row displays the results for one cultured umbilical cord blood unit. Polyclonal populations were defined as having >6 peaks (green squares), oligoclonal populations had 3–6 peaks (yellow squares), and monoclonal populations were defined as having <3 peaks (red squares). Grey squares indicate that no peaks were detected for that subfamily. NA, an abbreviation of not assessable, indicates that data could not be obtained due to technical reasons, such as insufficient DNA material in the test. In (b), representative examples of data analyzed with the peak scanner software is displayed for the Vγ10 Jγ1.3/2.3, Vγfl Jγ1.3/2.3, Vγ11 Jγ1.3/2.3, and Vδ1 Jδ1, Vδ3 Jδ1, and Vδ2 Jδ1 subfamilies.IL-2: interleukin 2; ZOL: zoledronate.

CTLA-4$^+$, in UCB and PB cultures, LAG-3$^+$ (only studied in UCB cultures), and T cells was significantly increased preferentially in UCB T cells cultured with 50 IU IL-2/ml compared to baseline and to UCB T cells cultured with medium containing 200 IU IL-2/ml (Figure 6(c)).

3.8. The Representation of Other Lymphocyte Subsets in the Final Culture Is Dominated by CD56$^+$ NK$^-$ and NKT Cells. Even the UCB and PB cultures with a predominant γδ T cell population had a remaining proportion of other lymphocytes, containing a mixture of αβ T cells and NK cells (Figure 6(d), left and middle panel). We also noticed a percentage of CD56$^+$ CD3$^+$ cells (Figure 6(d), middle panel). The remaining percentage of TCRαβ$^+$ T cells was significantly reduced in cultures exposed to zoledronate and IL-2 in a manner corresponding to the increased percentage of TCRγδ$^+$ T cells (Figure 6(d), left panel). At baseline, a non-significant trend towards higher percentages of NK cells was seen in UCB units from male newborns compared to female donors (Supplementary Figure 1a).

No B-cells could be detected in the cultures on day +14 (Figure 6(d), right panel, only tested for UCB cultures).

4. Discussion

In vitro expansion of UCB γδ T cells is a challenge. This is due to several factors, such as the scarcity of γδ T cells in UCB [15] and the very low frequency of the γδ T cell subset known to be easily expanded *in vitro*: the γ9δ2$^+$ T cells [18, 19]. Also, the functional immaturity of UCB γδ T cells [15], including a reduced capacity to respond to IL-2 [20, 21], and the previous observations that indicate a tendency towards development of cytokine production rather than cytotoxicity during *in vitro* culture [15] contribute to the difficulties. This is especially true for the development of a culture protocol robust and reproducible enough for the production of adoptive cell therapies for clinical use, as the goal here is to manufacture cell products that are consistent in quality and that contain the desired cellular phenotype with antitumoral and anti-infectious properties. This study was performed with the aim of exploring the possibility of translating the protocols used for expansion of peripheral blood γδ T cells to the UCB setting, with the long-term goal to explore the possibility of adoptive γδ T cell therapy after

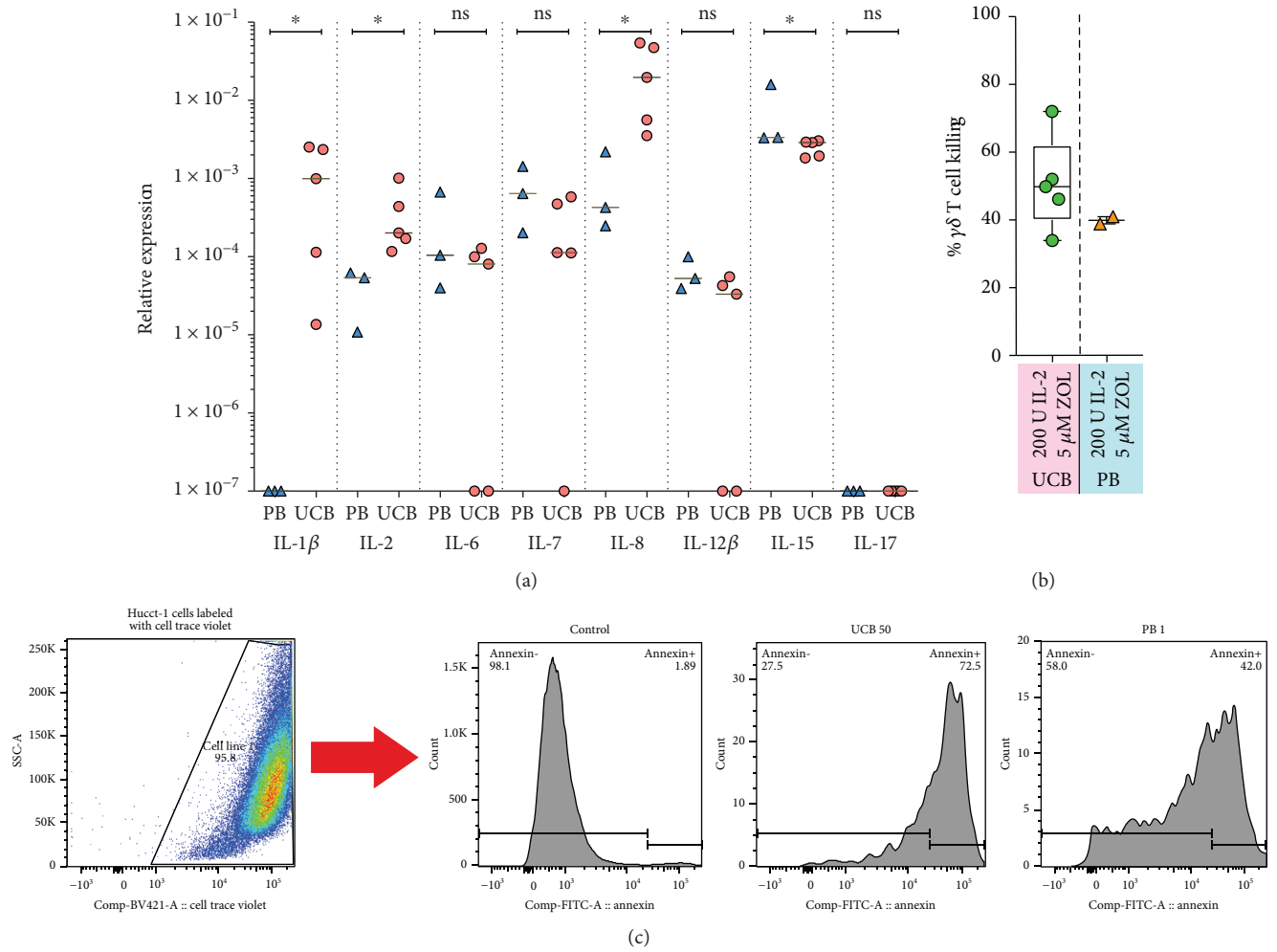

Figure 5: γδ T cell cytokine production and cytotoxicity. The results of real-time PCR analysis of the gene expression for the cytokines IL-1, IL-2, IL-6, IL-7, IL-8, IL-12, IL-15, and IL-17 in UCB and PB γδ T cells cultured with 5 μM zoledronate and 200 IU IL-2/ml medium displayed in (a). In (b) and (c), the results of a cytotoxicity assay based on coculture of Celltrace Violet® - (CTV-) labeled cholangiocarcinoma cells from the Hucct-1 cell line and thawed UCB and PB γδ T cells previously cryopreserved after culture with 5 μM zoledronate and 200 IU IL-2/ml medium. (b) displays the results for all tested units, and (c) shows representative FACS plots of the gating strategy for CTV-labeled tumor cells (left) and histogram plots of annexin V^+ gated cells from a control culture (no γδ T cells added), and from cocultures with UCB and PB γδ T cells, respectively (three plots to the right). $^*p < 0.05$.

UCBT. γδ T cell reconstitution is known to be important for HSCT outcome [29–31], perhaps due to their MHC-unrestricted antigen recognition and antitumoral and anti-infectious properties. The reconstitution of γδ T cells after UCBT is especially poor, and thus there is a need for innovative strategies, such as expanded γδ T cells from an aliquot of the original graft. We see the results presented here as a stepping-stone toward the development of such therapies.

We based the choice of zoledronate and IL-2 for the culture protocol on their easy availability in GMP-compliant preparations as clinically approved drugs and on the extensive published experience showing that they can be used to reliably expand γ9δ2 T cells. The choice of zoledronate was made based on results showing better proliferative response in UCB γδ T cells to zoledronate compared to pamidronate [15]. We did consider IL-15, as this cytokine has been described to confer less apoptotic γδ T cells after culture, and higher cytokine and cytotoxic mediator expression upon restimulation [18]. However, the final choice of IL-2 was based on this cytokine having, in addition to easy availability in GMP-preparations, the largest documented experience of *in vitro* as well as *in vivo* use.

We found that the protocol here used, combining zoledronate and IL-2 at different concentrations, led to a substantial proliferation of UCB γδ T cells. The resulting culture contained a majority of $CD3^+$ T cells, out of which a varying proportion expressed a γδ TCR. The proportion of γδ T cells in UCB cultures seemed to increase with the concentration of IL-2 in the culture medium up to 200 IU/ml and the combination of 5 μM zoledronate, and 100–200 IU IL-2/ml resulted in the highest percentages of UCB γδ T cells (Figure 1(b)). No significant differences could be noted in fold expansion of γδ T cells or the percentage of γδ TCR^+ in UCB cultures

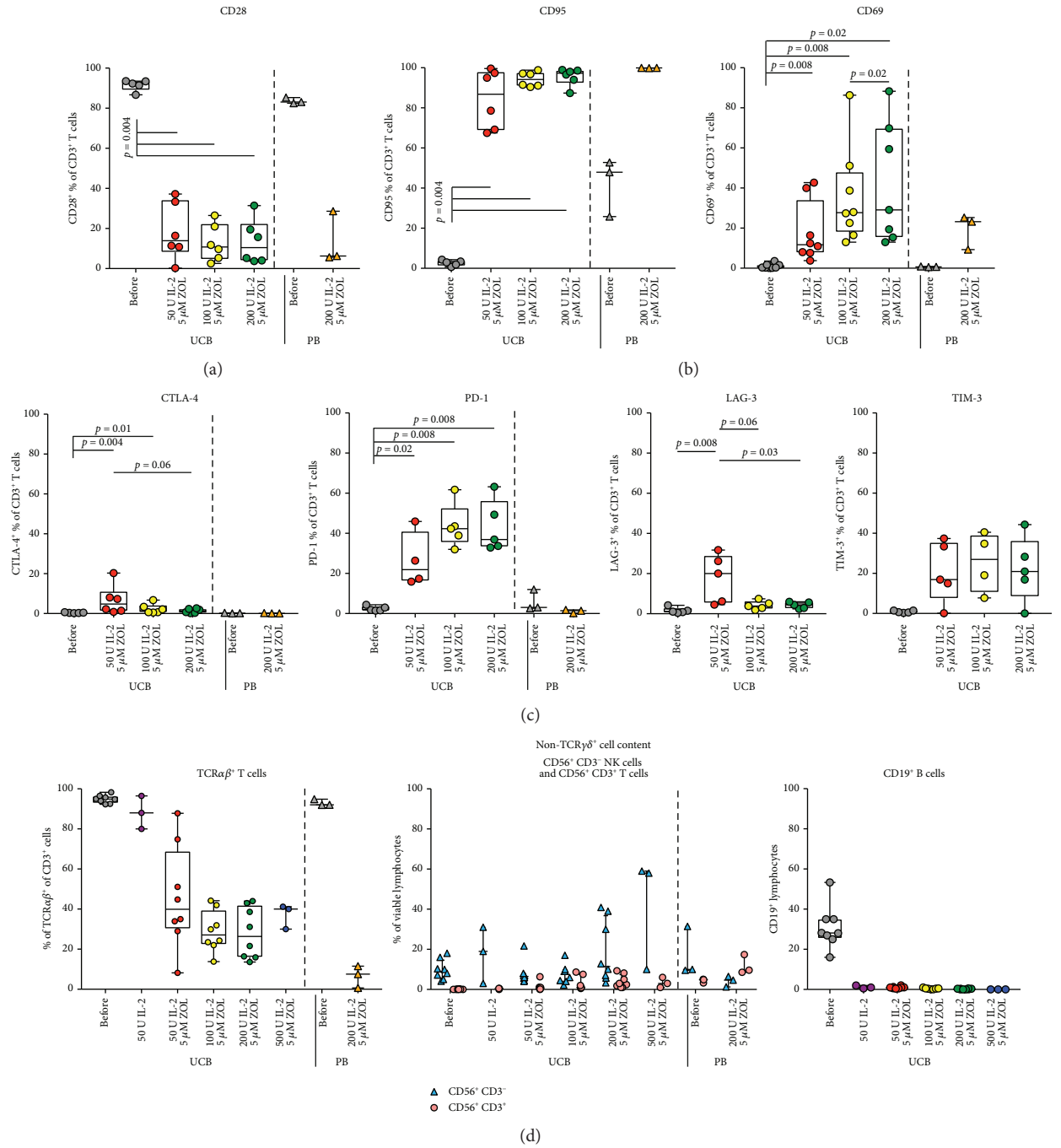

Figure 6: Marker expression in total T cells. Cosignaling marker expression. The phenotype of the cultured T cells from UCB and PB with regard to the expression of markers of activation and costimulatory and inhibitory receptors was assessed by flow cytometry. The percentage of cells expressing the costimulatory marker CD28 (a), the activation markers CD95 and CD69 (b), and the coinhibitory markers CTLA-4, PD-1, LAG-3, and TIM-3 (c) are displayed. In (d), the percentage of TCR$\alpha\beta^+$ T cells before and after expansion is displayed in the left panel. The percentages of $CD56^+$ $CD3^-$ and $CD56^+$ $CD3^+$/viable cells are displayed in the middle panel of (d), and the right panel shows the percentage of $CD19^+$ lymphocytes before and after culture. IL-2: interleukin 2; TCR: T cell receptor; ZOL: zoledronate.

treated with 100 IU or 200 IU IL-2/ml. The only difference was that the highest IL-2 concentrations were associated with a tendency towards more $CD56^+$ NK cells at the end of the culture (Figure 6(d)). Another important aspect was that a concentration of IL-2 of 100–200 conferred a significantly higher percentage of viable UCB cells at the end of culture than a concentration of 50 IU IL-2/ml (Figure 2).

The majority of the cultured $\gamma\delta$ T cells expressed Vγ9, and to a slightly lesser extent, Vδ2. This was not unexpected, as $\gamma\delta$ T cells with a TCR made up by a γ9 chain and a δ2 chain are known to be activated *in vitro* by the presence of bisphosphonates, such as zoledronate, in the culture medium [4, 6, 22, 23, 28]. Two interesting aspects of this finding are worthy of notice, however. The first is the very substantial

fold expansion of $\gamma 9$ T cells. The median fold expansion and maximum fold expansion of subset with the expansion conditions with the highest yield, treated with 5 μM zoledronate and 200 IU IL-2/ml medium, were 253 and 1085, respectively (Figure 3(b)). Compared to PB cultures treated with 5 μM zoledronate and 200 IU IL-2/ml, the fold expansion of total $\gamma\delta$ T cells was considerably lower in UCB cultures, but the difference with regard to $\gamma 9$ T cell expansion was noticeably less (Figures 1(d) and 3(b)). This indicates that UCB $\gamma 9$ T cells have an impressive proliferation potential [13, 14]. This is a prerequisite for the development of potential cell therapy schemes using bisphosphonates, as the percentage of this subset is small in baseline UCB.

The other interesting feature seen in the UCB cultures, but not in the PBMC-derived expansions, is the discrepancy in the percentages of positive cells between V$\gamma 9$ and V$\delta 2$, with a somewhat smaller proportion of the cultured $\gamma\delta$ T cells staining positive for V$\delta 2$. This could indicate that the cultured cell population contains a smaller subset of V$\gamma 9^+$ cells with TCRs made up with another Vδ chain, such as $\delta 1$. The TCRs of V$\delta 1^+$ T cells are known to include various Vγ chains [41–43], and thus the low proportion of V$\delta 1^+$ T cells found to persist after culture could indicate that a population of $\gamma 9\delta 1$ T cells is maintained through the culture process. UCB contains higher levels of this cell subset [18, 44]. High frequencies of $\gamma 9\delta 1$ T cells have been described in a patient with a recurrent fever syndrome [45], indicating that this subset can be induced to extensive proliferation in specific situations. The ligands recognized by V$\delta 1$ T cells (with different Vγ chains) remain largely uncharacterized but include CD1 family proteins [9, 43] and MICA/B through activation via the TCR and the NK cell receptor NKG2D [10, 11]. MICA and MICB expression has been described in a variety of tumors and has been associated with an increased tumor infiltration by V$\delta 1^+$ T cells [11]. During HIV infection, V$\delta 1^+$ T cell numbers are increased, thus suggesting the potential involvement of V$\delta 1^+$ T cells in antiviral immunity [43].The dual antiviral and antitumor potential of this subset, analogous with the recognition of phopshoantigens in both cellular stress and in infection by $\gamma 9\delta 2$ T cells [4, 8], suggests that the *in vitro* expansion of a parallel smaller population of V$\delta 1$ T cells might bring additional value to the cultured product.

The published studies on both *in vivo* and *in vitro* expansion of $\gamma\delta$ T cells have, to the knowledge of the authors of this paper, almost exclusively focused on the $\gamma 9\delta 2$ T cell subset, with one exception. In this study, *in vitro* expansion of $\gamma\delta$ T cells was induced with anti-$\gamma\delta$ TCR antibodies, and the resulting cell product contained both V$\delta 2^+$ and V$\delta 1^+$ T cells [46]. As the majority of the UCB $\gamma\delta$ T cells at baseline are V$\delta 1^+$ [16, 17], this approach may be interesting to explore also in the UCB setting in the future.

The spectratyping analysis indicated that the expanded cell populations cultured with zoledronate and varying concentrations of IL-2 (50–200 IU/ml) were mainly polyclonal (Figure 4). This indicates that the expanded $\gamma\delta$ T cells, the majority of which expressed V$\gamma 9^+$ and V$\delta 2^+$, had proliferated in a nonclonal, non-TCR specificity-dependent manner. This is in accordance with known data regarding the activation of V$\delta 2^+$ T cells by phosphoantigens: a complex process independent of MHC molecules, including the binding of the CD277 receptor and resulting in nonclonal proliferation [47]. The wide range of TCR specificities in the expanded cells could increase the chance of efficiency of adoptive therapy schemes based on the current protocol.

The memory phenotype of the UCB $\gamma\delta$ T cells was of interest to us, as it has been described that certain T cell subsets are more effective when used as adoptive therapy than others [48]. We initially utilized the most common T cell memory definition based on CCR7 and CD45RO. However, the baseline UCB and PB $\gamma\delta$ T cell population had high percentages of terminally differentiated cells according to this definition (Figure 3(d)). Both human and animal model-based studies of skin $\gamma\delta$ T cells have shown that this subset expresses low levels of CCR7 and that their recirculation between lymph nodes and skin is less dependent on CCR7 expression than the recirculation of $\alpha\beta$ T cells [49, 50]. V$\delta 2^+$ T cells have been described to express comparatively higher levels of CCR7 [51]. This might indicate that the standard definition for memory subsets is less suited to UCB $\gamma\delta$ T cells. We then chose to further study a memory phenotype definition based on CD27 and CD45RO. This decision was based on the previous use of this definition in several published articles [39, 40]. The results with regard to the memory phenotype UCB $\gamma\delta$ T cells before and after culture were then more in accordance with expectation: the majority of the UCB $\gamma\delta$ T cells at baseline was naïve (CD45RO$^-$ CD27$^+$) and the largest subset after culture expressed a central memory phenotype (CD45RO$^+$ CD27$^+$, Figure 3(e)). The latter finding is reassuring, as this subset has been described to have the highest efficiency in vivo in a study of adoptive cell therapy with $\alpha\beta$ T cells in primates [48]. In contrast, PB $\gamma\delta$ T cells expressed almost equal proportions of the CD27$^-$ and CD45RO-based memory phenotypes, with slightly more naïve and central memory cells at baseline, while the dominant phenotype after culture was a CD27$^-$ CD45RO$^+$ effector memory phenotype [48].

Exploration of the cytokine gene expression at the end of culture indicated, interestingly, that there were differences in the expression patterns of UCB and PB $\gamma\delta$ T cells. There was with significantly higher expression of IL-1β, IL-2, and IL-8 in UCB-derived cells (Figure 5(a). This might indicate a preferential development of a cytokine-producing phenotype, as has been previously reported [15]. IL-1β is a proinflammatory cytokine, which is released following activation of inflammosomes in response to viral infection [52], suggesting that UCB $\gamma\delta$ T cells might have superior properties for mounting antiviral responses. IL-8 is known to cause chemotaxis and degranulation in neutrophil granulocytes [53], indicating a general proinflammatory capacity. The cytokine-producing phenotype did, however, not exclude cytotoxic ability. The cytotoxicity assay we performed indicated that the capacity for tumor cell killing was comparable in UCB and PB $\gamma\delta$ T cells (Figures 5(b) and 5(c)). This is reassuring, as it indicates antitumor potential in UCB $\gamma\delta$ T cell products cultured in this manner.

We saw downregulation of CD28 and upregulation of CD95, CD69 in cultured UCB and PB T cells, and upregulation of PD-1 in UCB T cells only, compared to baseline values (Figures 6(a) and 6(b)). This probably reflects activation [54, 55], indicating that the exposure to zoledronate and IL-2 led to successful antigen recognition-based stimulation. The fact that the percentage of CTLA-4^+ and LAG-3^+ T cells was significantly increased preferentially in UCB cells cultured with 50 IU IL-2/ml compared to baseline and to UCB T cells cultured with higher IL-2 concentrations (Figure 6(c)) may be due to expression kinetics or to the fact that the percentage of $\gamma\delta$ T cells in cultures expanded with 50 IU IL-2/ml varied substantially between the UCB units used, and thus results might partly reflect marker expression in $\alpha\beta$ T cells.

The cultured cell populations contained non-$\gamma\delta$ T cells, consisting of $\alpha\beta$ T cells and NK cells (Figure 6(d), left and middle panel). This might indicate that a final selection step could be introduced into the protocol, to ensure a pure $\gamma\delta$ T cell product.

Interestingly, there was also a small fraction of $CD56^+$ T cells present in after culture in the majority of expansions. As $\gamma\delta$ T cells are known to express both CD56 and the NK cell-associated markers CD16 and NKG2D, these cells could be part of the expanded $\gamma\delta$ T cell population. CD56 has been associated with cytotoxicity in previous studies of *in vitro* $\gamma\delta$ T cell expansions [18].

In summary, the protocol for *in vitro* PB $\gamma\delta$ T cell expansion here adapted to UCB induced substantial proliferation of UCB $\gamma\delta$ T cells. Culture for 14 days in the preferred culture conditions, with 200 IU IL-2/ml combined with 5 μM zoledronate in the culture medium, resulted in cell products with a high proportion of $\gamma\delta$ T cells, mainly expressing γ9 chains and δ2 chains, in both UCB- and PBMC-derived cultures, and with a smaller population of remaining δ1 T cells possibly also expressing the γ9 chain, in UCB-derived cultures. The cultured UCB $\gamma\delta$ T cells expressed a central memory phenotype. We could demonstrate that the cultured UCB $\gamma\delta$ T cells were based on gene expression and efficient cytokine producers, especially with regard to IL-1β, IL-2, IL-8, and IL-15, and had a capacity for killing tumor cells comparable to cultured PB $\gamma\delta$ T cells. Together, these results indicate that the development of *in vitro* expanded UCB $\gamma\delta$ T cell therapies with this protocol is feasible and holds considerable potential, especially for ACT after UCBT.

Conflicts of Interest

The authors declare no conflicts of interest pertaining to this work.

Acknowledgments

The authors would like to express their gratitude to the midwives working for the Swedish Umbilical Cord Blood Bank for their assistance with obtaining the UCB units used in this study.

Supplementary Materials

The supplementary material includes 3 tables, Tables S1–3, and one figure, Supplementary Figure 1. The supplementary tables detail the setup of the spectratyping analysis with regard to analyzed δ chain subfamilies in Table S1, with regard to assessed γ subfamilies in Table S2, and for the primers used in Table S3. Supplementary Figure 1 displays the baseline proportions of $\gamma\delta$ T cells, of $\gamma\delta$ T cells positive for Vγ9, Vδ2, and Vδ1, and of NK cells divided according to UCB donor sex. Cell subsets at baseline in UCB from male and female newborns. The proportions of key cell subsets at baseline are displayed separately for UCB units obtained from male and female donors. In A, the proportions of $\gamma\delta$ T cells and NK cells are displayed, and in B, the percentages of $\gamma\delta$ T cells positive for Vγ9, Vδ2, and Vδ1 are shown. *(Supplementary Materials)*

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Research Article

Mesenchymal Stromal Cells Support Endometriotic Stromal Cells *In Vitro*

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Endometriosis is an inflammatory disease marked by ectopic growth of endometrial cells. Mesenchymal stromal cells (MSC) have immunosuppressive properties that have been suggested as a treatment for inflammatory diseases. Therefore, the aim herein was to examine effects of allogeneic MSC on endometriosis-derived cells *in vitro* as a potential therapy for endometriosis. MSC from allogeneic adipose tissue (Ad-MSC) and stromal cells from endometrium (ESC_{endo}) and endometriotic ovarian cysts (ESC_{cyst}) from women with endometriosis were isolated. The effects of Ad-MSC on ESC_{endo} and ESC_{cyst} were investigated using *in vitro* proliferation, apoptosis, adhesion, tube formation, migration, and invasion assays. Ad-MSC significantly increased proliferation of ESC compared to untreated controls. Moreover, Ad-MSC significantly decreased apoptosis and increased survival of ESC. Ad-MSC significantly increased adhesion of ESC_{endo} and not ESC_{cyst} on fibronectin. Conditioned medium from cocultures of Ad-MSC and ESC significantly increased tube formation of human umbilical vein endothelial cells on matrigel. Ad-MSC may significantly increase migration of ESC_{cyst} and did not increase invasion of both cell types. The data suggest that allogeneic Ad-MSC should not be considered as a potential therapy for endometriosis, because they may support the pathology by maintaining and increasing growth of ectopic endometrial tissue.

1. Introduction

Endometriosis affects approximately 10% of women of reproductive age, is marked with ectopically growing endometrial cells, and exhibits increased local inflammation leading to chronic pelvic pain and infertility [1]. Despite medical and surgical treatments to reduce inflammation and remove ectopic lesions, recurrence or therapy resistance is very common [2]. Therefore, there is an urgent need of new therapies for endometriosis.

Despite active research, the pathogenesis of endometriosis remains largely unclear. The most commonly accepted theory is that endometriosis develops from reflux of menstrual debris into the pelvic cavity during menstruation, which then implants resulting in endometriosis [3]. Although almost all women exhibit retrograde menstruation, only approximately 10% develop endometriosis [4]. This conundrum must be explained by other factors playing a role in disease development [3, 4]. For example, the endometrium of women with endometriosis displays resistance to apoptosis with a subsequent increase in cell proliferation, migration, adhesion, and invasion of the mesothelial lining of the pelvic cavity and increased ability to induce angiogenesis to cause endometriosis [5].

The immunosuppressive properties of mesenchymal stromal cells, also called mesenchymal stem cells (MSC), have made them a potential treatment for inflammatory and autoimmune diseases such as graft versus host disease (GvHD), multiple sclerosis (MS), and Crohn's disease [6]. It has been suggested that the immunosuppressive properties of MSC are due to their ability to sense the changing levels of inflammation in their microenvironment and respond accordingly [7]. Therefore, MSC may be a potential therapy for the inflammatory component of endometriosis. More specifically, previously, it has been reported that allogeneic MSC derived from adipose tissue (Ad-MSC) have immunosuppressive properties with potential to treat inflammatory diseases such as GvHD and MS [8, 9]. Previously, it has been found that autologous MSC are altered by the pathology of endometriosis [10]. In addition, we found that MSC from the ectopic (ESC_{cyst}) endometrium were phenotypically and functionally different from MSC from the eutopic (ESC_{endo}) endometrium in women with endometriosis suggesting that autologous MSC may be altered by the pathology [11]. Therefore, in the present study, we aimed to investigate the effects of allogeneic Ad-MSC on endometriosis-derived cells *in vitro* as the first step of a long-term goal of developing a potential therapy for endometriosis. The effects of Ad-MSC on ESC_{cyst} and ESC_{endo} were examined using proliferation, apoptosis, adhesion, tube formation (*in vitro* angiogenesis), migration, and invasion assays, which are the aforementioned parameters that are perturbed in endometriosis. It was found that allogeneic Ad-MSC may promote ESC_{cyst} proliferation, survival, and migration and support ESC_{cyst} to promote tube formation of human umbilical vein endothelial cells (HUVEC) but did not affect adhesion or invasion of ESC_{cyst} *in vitro*. The data suggest that allogeneic Ad-MSC should not be considered as a potential therapy for endometriosis because they may support the pathology by maintaining and increasing growth of ectopic endometrial tissue. Moreover, since MSC are present in ectopic lesions in endometriosis as confirmed by us [11] and others [12], this indicates that MSC are likely involved in the pathogenesis of endometriosis.

2. Materials and Methods

2.1. Human Tissue Samples. The inclusion criteria for the study were female women of fertile age suffering from endometriosis that have not undergone hormonal treatment for three to six months before undergoing laparoscopic surgery for confirmation of diagnosis and treatment. Three types of tissues were collected: (i) endometriotic ovarian cysts (ectopic endometrium) and (ii) endometrium (eutopic endometrium), which were both from women with endometriosis who underwent surgery for removal of endometriotic ovarian cysts, and (iii) adipose tissue from healthy women undergoing elective caesarean section at term. The endometriotic ovarian cysts and endometrium were collected from women aged 31 to 42 (36.3 ± 5.8 years (mean ± SD), $n = 4$) undergoing laparoscopic surgery for confirmation or treatment of endometriosis. All women were histologically confirmed to have endometriosis by a pathologist. Only one woman underwent hormonal treatment. Moreover, two of the biopsies were from the proliferative phase, one was unknown, and one had amenorrhea. The adipose tissue was collected from women aged 34 to 39 (36.5 ± 3.54 years (mean ± SD), $n = 2$). Informed oral and written consent was obtained from each participant, and ethical approval was obtained from The Regional Ethical Review Board in Stockholm (2013/1094-31/2, 2017/1017-32).

2.2. Isolation of Stromal Cells from Eutopic and Ectopic Endometrium. Human endometrial and endometriotic ovarian cyst tissues were digested to single cell suspension using 1 mg/mL collagenase type I (Sigma, Missouri, United States) diluted in Hank's Balanced Salt Solution (Life Technologies, Paisley, UK) (90 min for endometriotic tissue and 30 min for endometrial tissue) at 37°C with shaking every 10 min. The tissue digests were filtered twice through 100 μm cell strainers (Corning, New York, United States), and eventually, the stromal cells were filtered through a 40 μm cell strainer (Corning), with undigested tissue and epithelial cells being removed at each of the steps. The cell suspension was washed twice with phosphate-buffered saline (PBS) (Life technologies) by centrifugation at 500 ×g for 10 min. Finally, the cell pellet was resuspended in complete growth medium containing Dulbecco modified essential medium low glucose (DMEM-LG) (Life technologies) + 10% MSC certified fetal calf serum (FCS) (Life technologies) + 1% antibiotic and antimycotic (Life technologies). Viable cells were counted in 1% eosin (Merck KGaA, Darmstadt, Germany) and cultured at 4000 cells/cm^2 in tissue culture flasks at 37°C with 5% CO_2. After two days, the growth medium was changed and thereafter every three to four days. When the cells reached 70–90% confluency, they were trypsinised using 0.05% trypsin/EDTA (Life technologies) and cultured as described above. At passage 2, the stromal cells were cryopreserved in 10% dimethyl sulfoxide (DMSO) (Sigma) in complete growth medium. Flow cytometry showed that they were positive for stromal markers, such as CD73, CD90, and CD105 (data not shown). To ensure that we were working with a pure population of cells, ESC_{endo} and ESC_{cyst} were used at passages three to six, as earlier passages may be contaminated with other cell types.

2.3. Isolation of Allogeneic Ad-MSC from Adipose Tissue. Human adipose tissue was obtained from healthy pregnant women undergoing elective caesarean section. The tissue was digested as described above but for 60 min. The tissue digest was centrifuged at 500 ×g for 10 min at 4°C. Following centrifugation, the top layer of fat and middle layer of blood were carefully removed, with the resulting cell pellet resuspended in complete growth medium as described above. The stromal cells were cultured and cryopreserved as described above. These Ad-MSC were characterized by flow cytometry for CD73, CD90, CD105, HLA classes I and II, CD14, CD45, and CD31; formation of colonies in colony-forming units-fibroblasts; and differentiation assays into the osteogenic and adipogenic mesenchymal lineages and were found to be MSC [13]. To ensure that we were working with a pure population of cells, Ad-MSC was used at passages

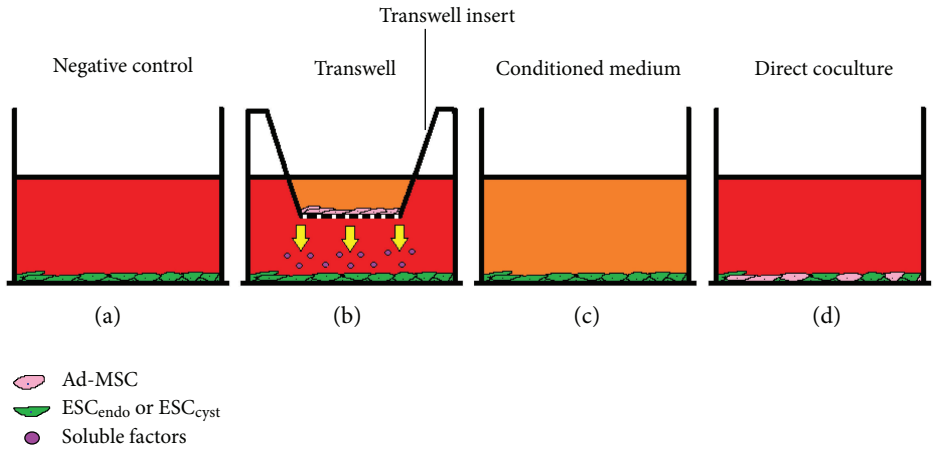

FIGURE 1: Schematic image showing the various cell coculture systems employed in the study. ESC$_{endo}$ and ESC$_{cyst}$ were cultured alone as a negative control (Neg C), with Ad-MSC using 0.4 μm pore inserts as a transwell system in conditioned medium (Cond) derived from Ad-MSC, or with Ad-MSC in direct cell coculture (Direct).

three to six, as earlier passages may be contaminated with other cell types.

2.4. Cell Coculture Setup. Ad-MSC were investigated for their effects on ESC$_{endo}$ and ECS$_{cyst}$ using cell proliferation, apoptosis, adhesion, migration, and invasion assays. Based on optimization experiments a 1 : 1 ratio of Ad-MSC to ESC$_{endo}$ and ESC$_{cyst}$ was selected for the transwell and direct cell coculture experiments (data not shown); the number of cells used were optimized to be within the optimum capacity of the inserts (an insert can hold up to 1.12×10^5 cells) and the bottom wells (a well can hold up to 3.8×10^5 cells) according to Corning. The different cell coculture systems that were employed in the study are shown in Figure 1; each of the cell coculture systems mimic the effects MSC may potentially be causing *in vivo*, and therefore, they are all representative and hence essential to give an overall picture of the potential effects of MSC.

In the eosin exclusion assay, unprimed Ad-MSC and Ad-MSC primed with interferon gamma (IFN-γ) (100 U/mL, Sigma) or IFN-γ (100 U/mL) + tumor necrosis factor alpha (TNF-α) (10 ng/mL, PeproTech, London, UK) and their conditioned medium were used. MSC can be primed using pro-inflammatory cytokines such as IFN-γ and TNF-α, to make them more immunosuppressive and secrete more immunosuppressive factors, which means that they may be more therapeutically effective for a disease with an inflammatory basis such as endometriosis [14, 15]. Therefore, we examined if priming Ad-MSC to become more immunosuppressive would give an effect that would be different to unprimed MSC. For the carboxyfluorescein succinimidyl ester (CFSE), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), apoptosis, adhesion, tube formation, migration, and invasion assays, only unprimed Ad-MSC were used. This is because primed Ad-MSC had no effect on the proliferation of ESC$_{endo}$ and ESC$_{cyst}$ compared to unprimed Ad-MSC.

The following cell coculture setup was used, with slight modifications for each of the assays. When Ad-MSC were ~70% confluent, the growth medium was removed, the cells were washed twice with PBS and growth medium alone, or growth medium with IFN-γ, or growth medium with IFN-γ + TNF-α were added. After three days, the conditioned medium was collected, Ad-MSC harvested, and were irradiated at 20 Gy to inhibit their proliferation. The conditioned medium was centrifuged at 500 ×g for 10 min to remove cellular debris, aliquoted, and frozen at −80°C. ESC$_{endo}$ and ESC$_{cyst}$ were harvested and added to 12-well plates at 6000 cells/cm^2. An equal amount of irradiated Ad-MSC was added to transwell inserts with a 0.4 μm pore size (Corning) and placed in the wells with ESC$_{endo}$ or ESC$_{cyst}$ for direct cell coculture. Conditioned medium from Ad-MSC was also used, and untreated ESC$_{endo}$ or ESC$_{cyst}$ were used as controls. After 3 days of cell culture, the proliferation, apoptosis, adhesion, migration, or invasion of ESC$_{endo}$ and ESC$_{cyst}$ were quantified as described below.

2.5. Cell Proliferation Assays. Cell proliferation was measured using three different methods in order to confirm the data: the manual eosin exclusion, CFSE, and MTT assays. For the eosin exclusion assay, the total number of cells were counted using 1% eosin.

For the CFSE assay, ESC$_{endo}$ and ESC$_{cyst}$ were stained with 1 μM CFSE (Life Technologies) for 10 min at 37°C with 5% CO_2 as described previously [16], before they were added in the 12-well plates as above. The cells were incubated with 5 mL of complete growth medium for 10 min at 37°C with 5% CO_2 to quench the reaction and to remove the remaining free dye. The cells were washed three times, resuspended in complete growth medium, and kept for 10 min at 37°C with 5% CO_2 to allow the CFSE stain to undergo acetate hydrolysis. On day 0, CFSE-stained ESC$_{endo}$ and ESC$_{cyst}$ were used to set voltages on the BD FACSCalibur (Becton-Dickinson, New Jersey, United States) to ensure the cells were on the far right of the CFSE histograms. After 3 days of cell culture, ESC$_{endo}$ and ESC$_{cyst}$ were harvested and analyzed on a BD FACSCalibur. As described previously, the data was analyzed using the median fluorescence intensity (MFI) with the software FlowJo (Tree Star version 10.1r5 Inc., Ashland, United States), with a lower MFI representing greater cell

proliferation and dilution of the CFSE dye [17]. For direct cell coculture, gating was only on the CFSE-positive ESC_{endo} and ESC_{cyst}. The results are shown relative to the untreated controls (ESC alone).

For the MTT assay, after 3 days of cell culture, the growth medium was removed, centrifuged at 500 ×g for 10 min to remove cellular debris, aliquoted, and frozen at −80°C, for later use in the tube formation assay (see below). Then, ESC_{endo} and ESC_{cyst} were stained with 0.5 mg/mL MTT reagent (Life Technologies) for 4 hours at 37°C with 5% CO_2. Afterwards, the MTT reagent was removed, the MTT crystals were solubilized in dimethyl sulfoxide (DMSO), and the plates were kept at 37°C with 5% CO_2 for 10 min. Then, the absorbance was measured at 540 nm using the infinite F200 Pro Tecan spectrophotometer (Tecan, Mannedorf, Switzerland), with DMSO used as a blank. The absorbance for irradiated Ad-MSC cultured alone was subtracted from the absorbance of the direct cell coculture wells to account for the absorbance of ESC_{endo} and ESC_{cyst} only.

2.6. Analysis of Apoptosis with Annexin V Assay. The annexin V assay was used to analyze apoptosis, as previously described [18], of ESC_{endo} and ESC_{cyst}, which were stained with CFSE as described above before they were added to 12-well plates and cultured with irradiated Ad-MSC. After 3 days of cell culture, ESC_{endo} and ESC_{cyst} were harvested and resuspended in 100 μL annexin V binding buffer (10 mM of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Life technologies) + 140 mM of sodium chloride (Sigma) + 2.5 mM of calcium chloride (Sigma)). Then, the cells were stained with annexin V PE antibody (BioLegend, California, United States) and 7-AAD (BD Biosciences, Stockholm, Sweden) for 15 min at room temperature (RT) in the dark. Then, 400 μL annexin V binding buffer was added and the CFSE positive cells were analyzed on a BD LSR Fortessa (Becton-Dickinson). For direct cell coculture, gating was only on the CFSE-positive ESC_{endo} and ESC_{cyst}. The data was analyzed using the software FlowJo.

2.7. Cell Adhesion Assay. Tissue culture-treated 48-well plates (Corning) were prepared by coating overnight with 10 μg/mL human fibronectin (BD Biosciences) at 4°C, and the cell adhesion assay was carried out as previously described [19]. The remaining sites were blocked with 0.1% bovine serum albumin (Sigma) for 2 hours at RT and washed once with PBS. The 48-well plates were dried and wrapped in parafilm and stored at 4°C until use for the cell adhesion assay.

After 3 days of cell culture as described above, ESC_{endo} and ESC_{cyst} were harvested and counted. The fibronectin-coated plates were brought to RT for at least 10 min. ESC_{endo} and ESC_{cyst} were resuspended in serum-free DMDM-LG medium and seeded at 21000 cells/cm^2. Following 2 hours of cell adhesion at 37°C with 5% CO_2, the medium was gently removed, and the wells were washed with PBS containing 2 mM calcium chloride and 2 mM magnesium chloride. The remaining adherent cells were quantified using the MTT assay as described above.

2.8. Tube Formation Assay. The tube formation assay was carried out as previously described [20]. HUVEC were kindly provided by Dr. Nina Heldring (Karolinska Institutet). They were isolated ($n = 2$) as previously described [21] and expanded on 0.1% gelatin- (Kodak) coated surfaces in complete HUVEC growth medium containing human endothelial serum-free basal medium (Fisher Scientific, Göteborg, Sweden) + 10% FCS (Life technologies) + 1% penicillin and streptomycin (Life technologies). They were used for experiments at passages two to five. For the tube formation assay, all pipette tips and plates were prechilled at −20°C. Matrigel (Corning) was thawed overnight at 4°C on ice, aliquoted at 50 μL per well in 96-well plates, and kept at 37°C with 5% CO_2 for 1 hour in order for the matrigel to gel. HUVEC were harvested and counted with eosin as described above, then 20×10^3 were resuspended in 50% complete HUVEC growth medium + 50% conditioned medium collected from the MTT assay (see above) and added gently on the matrigel. The cells were kept at 37°C with 5% CO_2 for 17-18 hours, then they were visualized using the Olympus CKX41 inverted microscope (Olympus, Tokyo, Japan), and images were taken at 4x magnification capturing the whole well in the 96-well plates. The number of tubes formed per well were quantified by using the angiogenesis analyzer plugin on ImageJ (Version 1.48, National Institutes of Health, Bethesda, United States) as described previously [22].

2.9. Cell Migration Assay. The transwell cell migration assay was carried out as previously described [23]. After 3 days of cell culture as described above, ESC_{endo} and ESC_{cyst} were harvested and counted. ESC_{endo} and ESC_{cyst} were added at 25×10^3 per transwell insert (8 μm in pore diameter, Corning) in serum-free DMEM-LG growth medium in 24-well plates and were allowed to migrate towards 10% FCS in DMEM-LG growth medium at 37°C with 5% CO_2. The negative controls were untreated ESC_{endo} and ESC_{cyst}. After 20 hours, the nonmigrated cells on top of the inserts were removed using a wet cotton swab, the inserts washed with PBS, and the cells fixed with ice cold methanol for 5 minutes. The inserts were again washed with PBS, before being stained with 1% eosin for 1 hour. Finally, the inserts were washed in milliQ water and dried and 5 random fields per insert of the migrated cells were captured at 10x magnification. The number of cells that migrated were analyzed and counted using ImageJ.

2.10. Cell Invasion Assay. The transwell cell invasion assay was carried out as previously described [23]. After 3 days of cell culture as described above, ESC_{endo} and ESC_{cyst} were harvested and counted. On the same day, transwell inserts (8 μm in pore diameter, Corning) fitting 24-well plates were coated with 0.1 mg/mL matrigel for two hours. ESC_{endo} and ESC_{cyst} were added at 25×10^3 per insert in serum-free DMEM-LG growth medium, placed in the 24-well plates and allowed to invade through matrigel towards 10% FCS in DMEM-LG growth medium at 37°C with 5% CO_2. The negative controls were untreated ESC_{endo} or ESC_{cyst}. After 20 hours, the invaded cells were stained and quantified as described above for the migration assay.

2.11. Statistical Analysis. All statistical analyses were performed using GraphPad prism 6. When data was normally

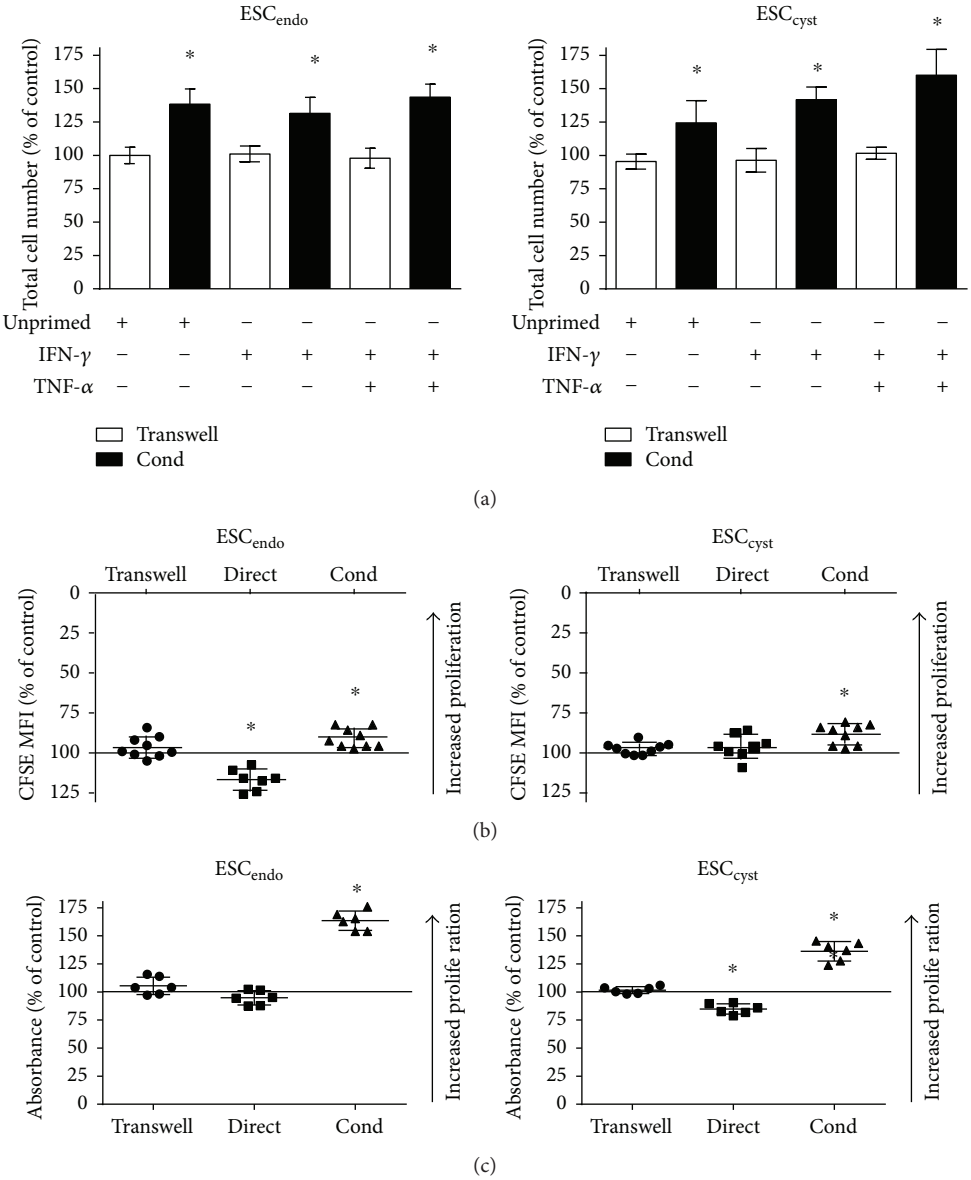

Figure 2: Ad-MSC increased proliferation of ESC$_{endo}$ and ESC$_{cyst}$. A transwell system using Ad-MSC and conditioned medium (Cond) from allogeneic unprimed and primed (100 U/mL IFN-γ or 100 U/mL IFN-γ + 10 ng/mL TNF-α) Ad-MSC were used in coculture with ESC$_{endo}$ or ESC$_{cyst}$. Direct cell coculture of unprimed Ad-MSC and ESC$_{endo}$ or ESC$_{cyst}$ was also carried out. Proliferation of ESC$_{endo}$ and ESC$_{cyst}$ was determined using the eosin exclusion (a), CFSE (b), and MTT (c) assays. Conditioned medium increased proliferation of ESC$_{endo}$ and ESC$_{cyst}$ ($^*P < 0.05$), and the transwell system had no effect on the proliferation of both cell types. The effect of direct cell coculture was more ambiguous. The data was normalized to untreated controls for each cell type. Thirty-six independent experiments (n = 3-4 biological replicates) were carried out in duplicates (mean ± SD).

distributed, the means were analyzed with Student's t-test, and when it was not normally distributed, the medians were analyzed with the Mann–Whitney test. All values are shown as the mean ± standard deviations (SD). For the study, n refers to the number of biological replicates. Results were considered to be statistically significant if $P < 0.05$.

3. Results

3.1. Ad-MSC Increased Proliferation of Stromal Cells. To study the effects of Ad-MSC on stromal cell proliferation, Ad-MSC were cocultured with ESC directly or in a transwell system, or the effects of conditioned medium from Ad-MSC (either primed or unprimed with IFN-γ or IFN-γ + TNF-α) on stromal cells was examined. Cell proliferation was measured by the manual cell count, CFSE, and MTT assays.

Manual cell count showed that both unprimed and primed conditioned medium from Ad-MSC increased proliferation significantly ($P < 0.05$) for ESC$_{endo}$ and ESC$_{cyst}$ compared to the untreated controls (Figure 2(a)). Moreover, the CFSE and MTT assays also showed that conditioned medium from unprimed Ad-MSC increased proliferation significantly ($P < 0.05$) for ESC$_{endo}$ and ESC$_{cyst}$ compared to the untreated controls (Figures 2(b) and 2(c)). However, in the transwell

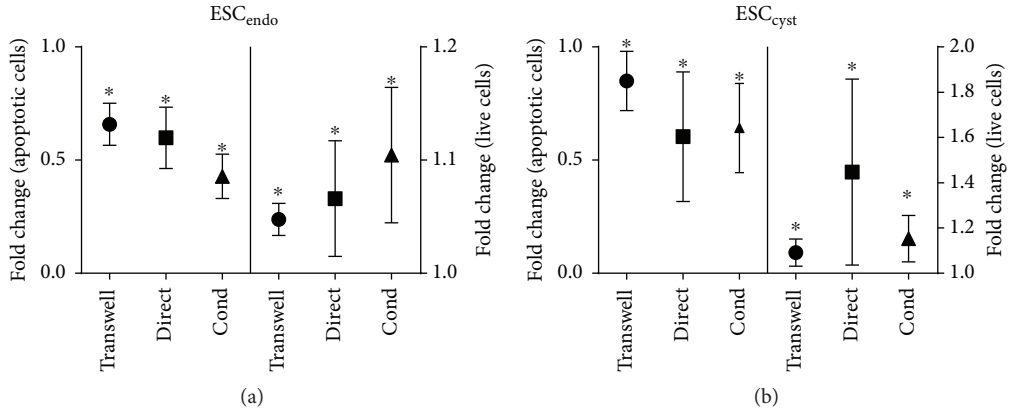

Figure 3: Ad-MSC reduced apoptosis and increased survival of ESC_{endo} and ESC_{cyst}. A transwell system using Ad-MSC and conditioned medium (Cond) from allogeneic unprimed Ad-MSC were used in coculture with ESC_{endo} or ESC_{cyst}. Direct cell coculture of unprimed Ad-MSC and ESC_{endo} or ESC_{cyst} was also carried out. Apoptosis and survival of ESC_{endo} and ESC_{cyst} was determined using the annexin V assay by flow cytometry. All three systems reduced apoptosis and increased survival of ESC_{endo} and ESC_{cyst} ($^*P < 0.05$). The data was normalized to untreated controls for each cell type. Four independent experiments ($n = 4$ biological replicates) were carried out in triplicates (mean ± SD).

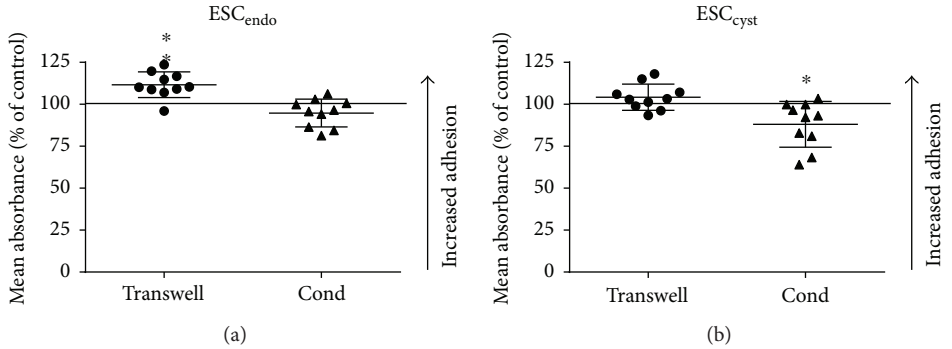

Figure 4: Ad-MSC did not increase adhesion of ESC_{cyst}. A transwell system using Ad-MSC and conditioned medium (Cond) from allogeneic Ad-MSC were used in coculture with ESC_{endo} or ESC_{cyst}. Adhesion of ESC_{endo} and ESC_{cyst} on fibronectin was determined by quantifying the number of adherent cells using the MTT assay. The transwell system increased adhesion of ESC_{endo} ($^*P < 0.05$), and conditioned medium reduced adhesion of ESC_{cyst} ($^*P < 0.05$). The data was normalized to untreated controls for each cell type. Five independent experiments ($n = 4$ biological replicates) were carried out in duplicates (mean ± SD).

system, there was no effect on the proliferation of ESC_{endo} and ESC_{cyst} using manual cell count, CFSE, or the MTT assays (Figure 2). As measured by the CFSE assay, direct cell coculture decreased proliferation significantly ($P < 0.05$) for ESC_{endo} but had no effect on ESC_{cyst} (Figure 2(b)). On the contrary, using the MTT assay, the direct cell coculture system significantly ($P < 0.05$) decreased proliferation for ESC_{cyst} but had no effect on ESC_{endo} (Figure 2(c)). Priming Ad-MSC with the proinflammatory cytokines IFN-γ and TNF-α resulted in no difference on cell proliferation compared to unprimed Ad-MSC, and therefore, priming was discontinued for the rest of the study. Taken together, the results showed that conditioned medium from Ad-MSC increased proliferation of ESC, and the transwell system had no effect on the proliferation of ESC. The effect of direct cell coculture was more ambiguous.

3.2. Ad-MSC Increased Survival of Stromal Cells. To examine the effects of Ad-MSC on the survival and apoptosis of ESC_{endo} and ESC_{cyst}, the annexin V assay using flow cytometry was used. The transwell system, conditioned medium, and direct cell coculture significantly ($P < 0.05$) reduced apoptosis and increased survival for both ESC_{endo} and ESC_{cyst} compared to the untreated controls (Figure 3).

3.3. Ad-MSC Did Not Increase Adhesion of ESC_{cyst}. To examine the effects of Ad-MSC on the adhesion of ESC_{endo} and ESC_{cyst}, a fibronectin adhesion assay was employed. The transwell system increased adhesion significantly ($P < 0.05$) for ESC_{endo} but had no effect on ESC_{cyst} compared to the untreated controls (Figure 4(a)). In contrast, the conditioned medium system decreased adhesion significantly ($P < 0.05$) for ESC_{cyst} but had no effect on ESC_{endo} compared to the untreated controls (Figure 4(b)). Therefore, it can be concluded that the adhesion of ESC_{cyst} is not increased following treatment with Ad-MSC. Moreover, although the conditioned medium reduced the adhesion of ESC_{cyst}, the transwell system maintained the adhesion of ESC_{cyst}, which does

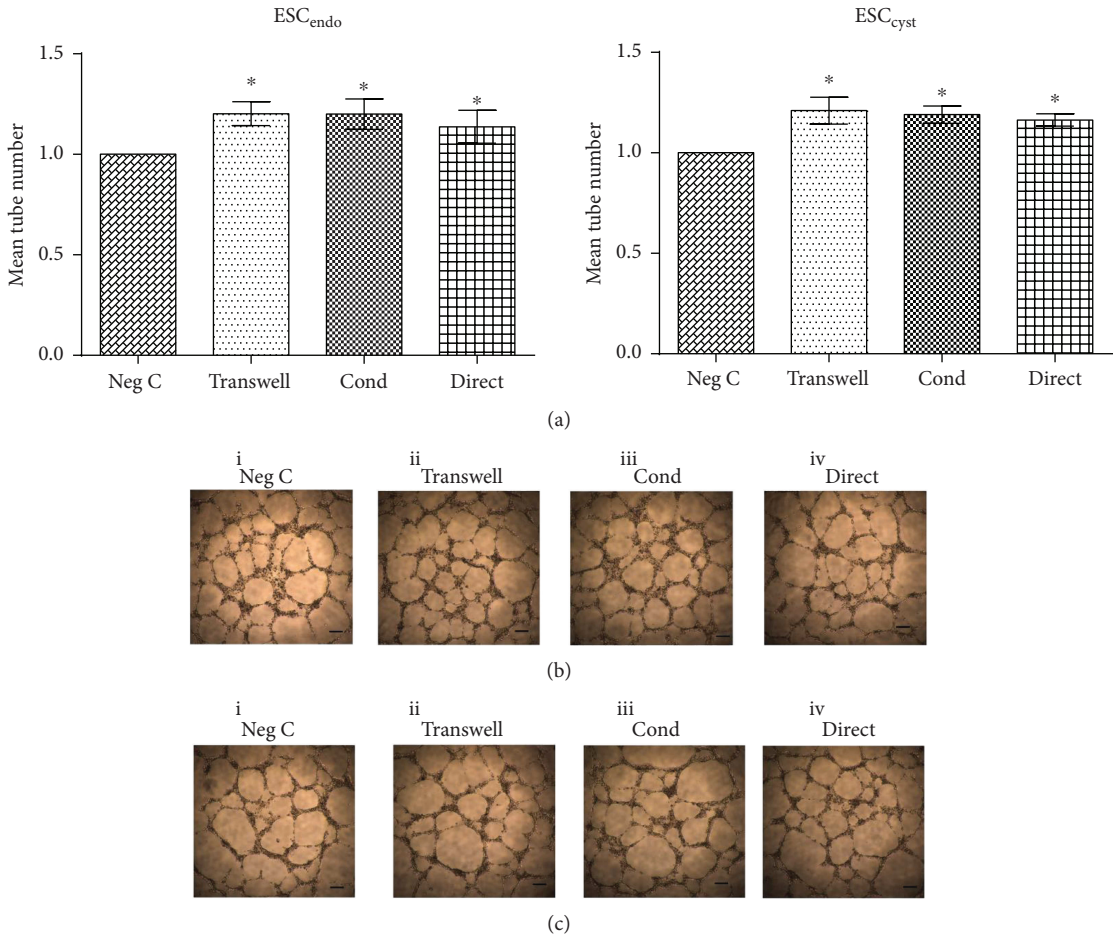

Figure 5: Conditioned medium from Ad-MSC/ESC$_{endo}$ and Ad-MSC/ESC$_{cyst}$ cocultures increased tube formation of HUVEC on matrigel. HUVEC were cultured on matrigel for 17-18 hours in conditioned medium (Cond) from cell cocultures of allogeneic Ad-MSC/ESC$_{endo}$ or Ad-MSC/ESC$_{cyst}$, and tube formation was determined. All conditions induced tube formation (a) ($^*P < 0.05$). Representative images showing connections between HUVEC forming ring or tube structures for each condition for ESC$_{endo}$ (b) and for ESC$_{cyst}$ (c), respectively. The images are at 4x magnification to image and quantify the whole 96-plate well. The data was normalized to untreated controls for each cell type. Four independent experiments ($n = 4$ biological replicates) were carried out in duplicates (mean ± SD). Scale bars represent 50 μm at 4x magnification.

not support our assumption that Ad-MSC may be therapeutically useful for endometriosis.

3.4. Ad-MSC Increased Tube Formation. To evaluate the influence of Ad-MSC on tube formation of HUVEC, the effects of conditioned medium collected from the Ad-MSC/ESC$_{endo}$ and Ad-MSC/ESC$_{cyst}$ cocultures on HUVEC tube formation was studied. Conditioned medium from all three systems induced a significant ($P < 0.05$) increase in tube formation compared to the untreated controls for both ESC$_{endo}$ and ESC$_{cyst}$, respectively (Figure 5(a)). This shows that conditioned medium derived from cocultures of Ad-MSC and ESC can support tube formation of HUVEC *in vitro*.

3.5. Ad-MSC May Promote Migration of ESC$_{cyst}$. To investigate the migratory activity of ESC, they were allowed to migrate towards 10% FCS through 8 μm pore filters after being treated with conditioned medium or in a transwell system with Ad-MSC. The transwell system increased ESC$_{cyst}$ migration significantly ($P < 0.05$) but had no effect on ESC$_{endo}$ compared to the untreated controls (Figure 6(a), i). Moreover, the conditioned medium system reduced migration of both ESC$_{endo}$ and ESC$_{cyst}$ significantly ($P < 0.05$) compared to the untreated controls (Figure 6(a), ii). The results on the effects of Ad-MSC on ESC$_{cyst}$ migration were conflicting; however, it may be concluded that Ad-MSC may promote migration of ESC$_{cyst}$.

3.6. Ad-MSC Did Not Increase Invasion of ESC$_{endo}$ and ESC$_{cyst}$. To determine the invasive capacity of ESC$_{endo}$ and ESC$_{cyst}$, invasion was analyzed using a matrigel transwell assay. The transwell system had no effect on the invasive capacity of ESC$_{endo}$ and ESC$_{cyst}$ compared to the untreated controls (Figure 7(a), i). Contrary to this, conditioned medium reduced invasion of both ESC$_{endo}$ and ESC$_{cyst}$ significantly ($P < 0.05$) compared to the untreated controls (Figure 7(a), ii). Therefore, it can be concluded that the invasion of ESC$_{endo}$ and ESC$_{cyst}$ is not increased following treatment with Ad-MSC. Moreover, although the conditioned medium reduced the invasion of ESC$_{endo}$ and ESC$_{cyst}$, the

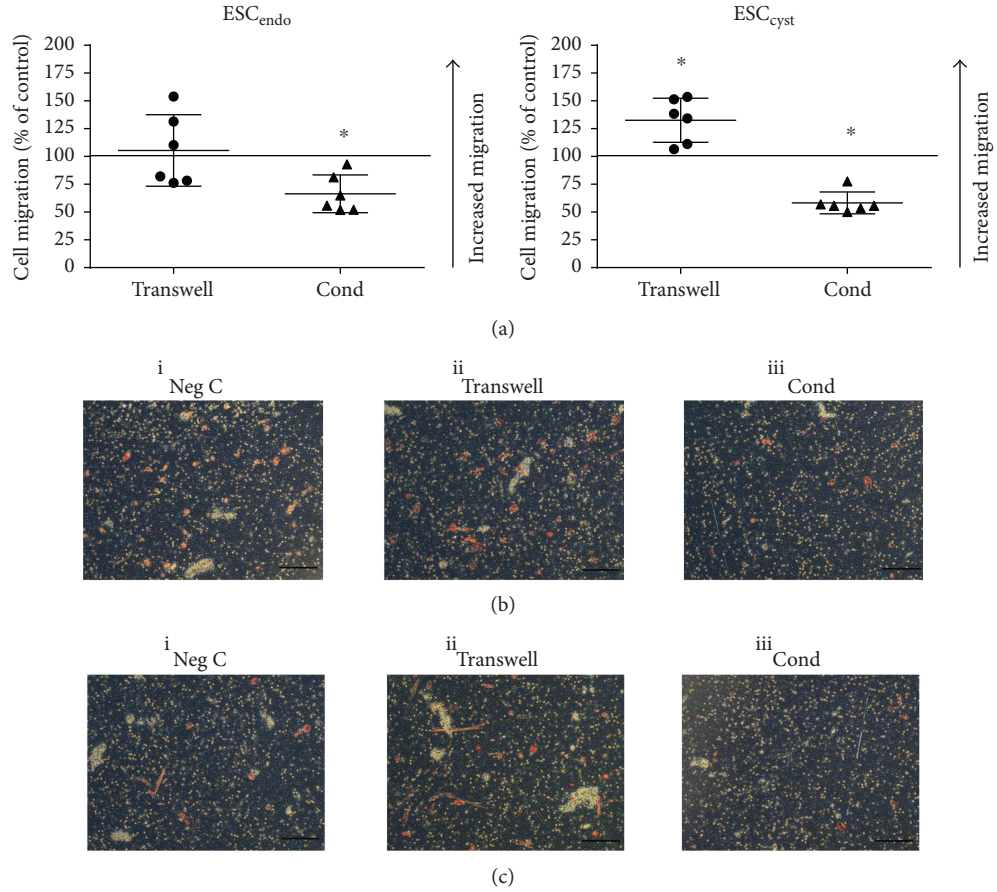

Figure 6: Ad-MSC may promote migration of ESC_{cyst}. A transwell system using Ad-MSC and conditioned medium (Cond) from allogeneic Ad-MSC were used in coculture with ESC_{endo} or ESC_{cyst}. Following cell coculture, migration of ESC_{endo} and ESC_{cyst} was determined after 20 hours using the transwell migration assay. Conditioned medium reduced migration of ESC_{endo} ($^*P < 0.05$). The transwell system increased migration, and conditioned medium reduced migration of ESC_{cyst} ($^*P < 0.05$). Representative images of migration for each condition for ESC_{endo} (b) and ESC_{cyst} (c). The 0.4 μm pores of the inserts are the white dots, and the migrated cells are light/dark red in color (10x magnification). The data was normalized to untreated controls for each cell type. Three independent experiments ($n = 3$ biological replicates) were carried out in duplicates (mean ± SD).

transwell system maintained the invasion of both cell types, which does not support our assumption that Ad-MSC may be therapeutically useful for endometriosis.

4. Discussion

In this study, we show that allogeneic Ad-MSC may promote ESC_{cyst} proliferation, survival, and migration, and may support ESC_{cyst} to increase tube formation of HUVEC but did not increase adhesion or invasion of ESC_{cyst} *in vitro*. The effects of Ad-MSC on ESC_{cyst} shown here suggest that they should not be considered as a potential therapy for endometriosis, because they may support the pathology of endometriosis by maintaining and increasing growth of ectopic endometrial tissue. Moreover, since MSC are present in ectopic lesions in endometriosis as confirmed by us [11] and others [12], this indicates that MSC are likely involved in the pathogenesis of endometriosis.

Li et al. studied the effect of conditioned medium from MSC on ESC_{endo} and ESC_{cyst}, and similar to our data, they found that MSC induce a significant increase in ESC proliferation [24]. Also, MSC isolated from Wharton's jelly induced a significant increase in ESC_{endo} proliferation in a transwell system [25]. In contrast, Xu et al. reported that umbilical cord-MSC (UC-MSC) significantly reduce proliferation of ESC_{cyst} [26]. These discrepancies between the studies may be explained by the different tissue sources for derivation of MSC, which has previously been described [27]. There are no other studies examining the effects of MSC on ESC in terms of proliferation. However, MSC have been previously shown to increase the cell proliferation of other cell types through their release of cytokines, and growth factors [28–30]. In our study, conditioned medium induced proliferation significantly of both ESC_{endo} and ESC_{cyst}. In contrast, the transwell system had no effect on the proliferation of ESC_{endo} and ESC_{cyst}. In the transwell system, there is a paracrine effect between ESC and Ad-MSC; Ad-MSC may become modulated by factors secreted by ESC, subsequently hindering their growth-promoting effects on ESC. Paracrine signaling is the local cell-to-cell communication through the paracrine factors (e.g., cytokines, hormones, and microvesicles) that are secreted into the extracellular environment

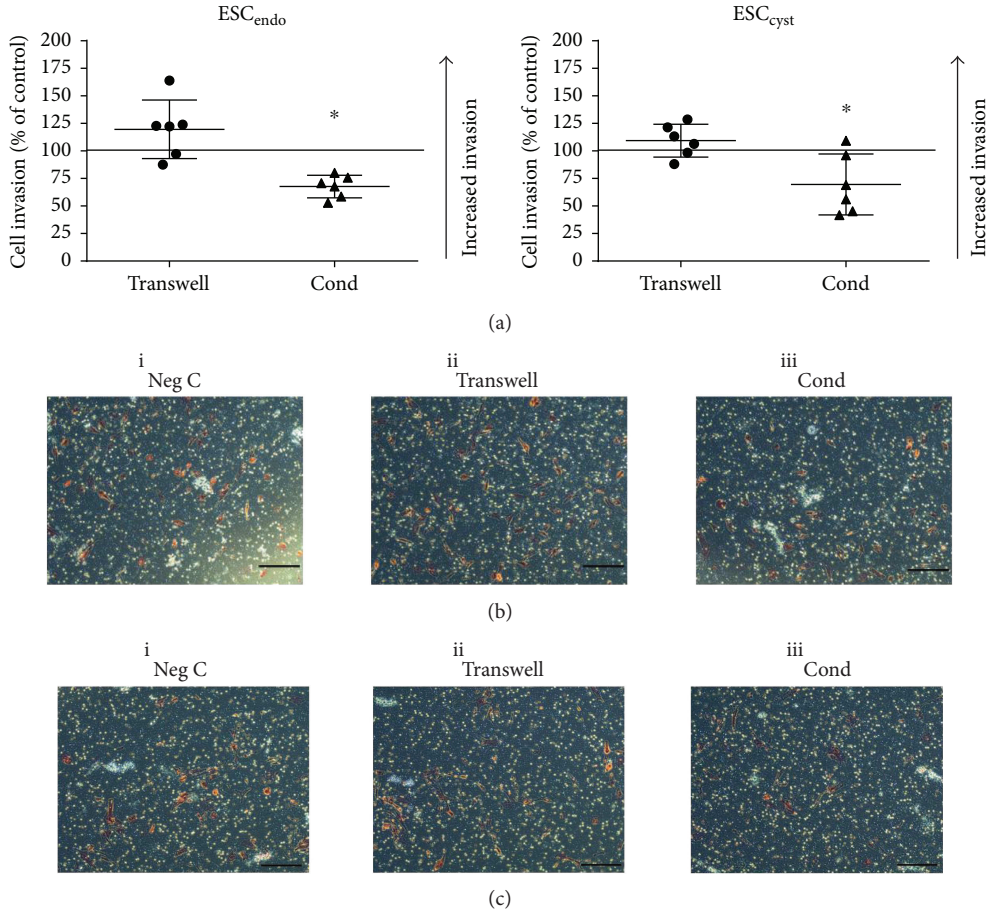

FIGURE 7: Ad-MSC did not increase invasion of ESC$_{endo}$ and ESC$_{cyst}$. A transwell system using Ad-MSC and conditioned medium (Cond) from allogeneic Ad-MSC were used in coculture with ESC$_{endo}$ or ESC$_{cyst}$. Following cell coculture, invasion of ESC$_{endo}$ and ESC$_{cyst}$ was determined using the transwell invasion assay after 20 hours. Conditioned medium reduced invasion of ESC$_{endo}$ and ESC$_{cyst}$ ($^*P < 0.05$). Representative images of invasion for each condition for ESC$_{endo}$ (b) and ESC$_{cyst}$ (c). The 0.4 μm pores of the inserts are the white dots, and the invaded cells are light/dark red in color (10x magnification). The data was normalized to untreated controls for each cell type. Three independent experiments ($n = 3$ biological replicates) were carried out in duplicates (mean ± SD).

[31]. This feedback effect is absent in the conditioned medium, which contains factors secreted by unmodulated Ad-MSC. This may explain the different results from the transwell, and the conditioned medium systems in this study. Moreover, it is unlikely that the conditioned medium effect was nonspecific; instead, it is most likely specific for Ad-MSC since the effects with conditioned medium differ from the untreated controls. The direct cell coculture system had no effect, or caused a significant decrease in the proliferation of ESC$_{endo}$ and ESC$_{cyst}$. The decrease in cell proliferation could be due to the growth inhibitory effects of MSC through direct contact. As previously shown, MSC mediate their greatest inhibition on cell proliferation *in vitro* through direct contact compared to the transwell system [32].

In this study, Ad-MSC significantly decreased apoptosis, and increased survival of ESC$_{endo}$ and ESC$_{cyst}$, which is in keeping with studies in other cell types [33–35]. These results suggest that Ad-MSC may decrease apoptosis, increase survival of ESC$_{endo}$ and ESC$_{cyst}$, and may support endometriosis. In contrast, UC-MSC have been reported to induce apoptosis of ESC$_{cyst}$ in a transwell system by a mechanism involving the tensin homologue gene (PTEN), an important housekeeping gene in endometrial tissue [26]. This discrepancy to our study may be due to the use of a different source of MSC [27].

Cell adhesion is crucial in the development of endometriosis to allow the attachment of endometrial tissue onto the mesothelial lining in the pelvic cavity following retrograde menstruation. Treatment via the transwell system was found to cause a significant increase in the adhesion of ESC$_{endo}$, but it had no effect on the adhesion of ESC$_{cyst}$. Conditioned medium caused a significant decrease in the adhesion of ESC$_{cyst}$, but it had no effect on the adhesion of ESC$_{endo}$. These results can be understood in light of the results of cell proliferation, since it is known that cells that divide rapidly are likely to be less adherent [36]. Moreover, the results may be explained by differences between the transwell and the conditioned medium systems; there is a paracrine effect in the transwell system that is absent in the conditioned medium as described above. To our knowledge, there are no other studies in the literature that have examined the effects of MSC on the adhesion of ESC$_{endo}$ and ESC$_{cyst}$.

The tube formation assay is commonly used to quantify the effects of various treatments on the ability of HUVEC to form tubes on a gelled membrane matrix as an *in vitro* model of angiogenesis [20]. Conditioned medium collected from Ad-MSC/ESC_{endo} and Ad-MSC/ESC_{cyst} cell cocultures significantly induced tube formation of HUVEC compared to untreated controls. There are no previous studies that have examined the outcome of MSC-treated ESC_{endo} and ESC_{cyst} on HUVEC tube formation. Our results are in line with previous reports showing that MSC have the ability to increase tube formation [37–39].

The transwell system significantly increased the migration of ESC_{cyst} compared to the untreated control but had no effect on ESC_{endo}. Moreover, the transwell system had no effect on the invasion of either cell type. Meanwhile, the conditioned medium system significantly decreased the migration and invasion of both ESC_{endo} and ESC_{cyst}. Migration and invasion require initial cell adherence, and therefore, these results are in line with the cell adhesion data and may be explained by differences between the transwell and the conditioned medium systems [40]. The transwell system has a paracrine effect that is absent in the conditioned medium, as described above. Only one previous study has examined the effect of MSC conditioned medium on the migration and invasion of ESC_{endo} and ESC_{cyst} [24]. In contrast to our results, they found that migration and invasion of both cell types were significantly increased compared to untreated controls [24]. Again, this discrepancy could be explained by the different sources of MSC [27]. Their study used MSC isolated from endometriotic ovarian cysts and the endometrium of women with endometriosis, and we used allogeneic Ad-MSC [24].

The limited number of donors and the donor hormonal status did not affect the consistency of data acquired from the *in vitro* cell experiments. Statistically significant results were observed and meaningful conclusions could still be drawn. Moreover, a similar number of patients have been used in other studies [10, 41]. Also, the use of Ad-MSC isolated from the adipose tissue of pregnant women may not be optimal; however, it has been previously shown that pregnancy has no detrimental effects on the nature of the isolated Ad-MSC [42]. Nevertheless, it must be remembered that this is an *in vitro* study, and additional *in vitro* and *in vivo* studies are needed to validate the findings of the current study. The present study suggests that allogeneic Ad-MSC should not be used as a potential therapy for endometriosis, because they may support the pathology of endometriosis by maintaining and increasing growth of ectopic endometrial tissue. In addition, since MSC are present in ectopic lesions in endometriosis, this means they are likely involved in the pathogenesis of endometriosis. This is the most extensive *in vitro* study showing that this may indeed be true, and will be significant in further understanding the pathogenesis of endometriosis to potentially find new therapeutics by targeting MSC.

5. Conclusion

In conclusion, Ad-MSC should not be considered as a potential therapy for endometriosis with endometriotic ovarian cysts, because they may promote proliferation, survival, and migration of ESC_{cyst} and support ESC_{cyst} to promote angiogenesis of endothelial cells to worsen the pathology. However, further studies examining other sources of MSC are needed to confirm if MSC are indeed an ineffective therapy for endometriosis.

Conflicts of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contributions

Fawaz Abomaray, Sebastian Gidlöf, and Cecilia Götherström contributed to the idea and design of the paper. Fawaz Abomaray contributed to the data acquisition. Fawaz Abomaray, Sebastian Gidlöf, and Cecilia Götherström contributed to the data analysis and interpretation. Fawaz Abomaray performed the laboratory assays. Fawaz Abomaray, Sebastian Gidlöf, and Cecilia Götherström wrote the paper and revised it critically. Fawaz Abomaray, Sebastian Gidlöf, Bartosz Bezubik, Mikael Engman, and Cecilia Götherström proofread the manuscript. All the authors read and approved the final paper.

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Review Article

Placenta and Placental Derivatives in Regenerative Therapies: Experimental Studies, History, and Prospects

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Placental structures, capable to persist in a genetically foreign organism, are a natural model of allogeneic engraftment carrying a number of distinctive properties. In this review, the main features of the placenta and its derivatives such as structure, cellular composition, immunological and endocrine aspects, and the ability to invasion and deportation are discussed. These features are considered from a perspective that determines the placental material as a unique source for regenerative cell therapies and a lesson for immunological tolerance. A historical overview of clinical applications of placental extracts, cells, and tissue components is described. Empirically accumulated data are summarized and compared with modern research. Furthermore, we define scopes and outlooks of application of placental cells and tissues in the rapidly progressing field of regenerative medicine.

1. Background

The human placenta is a unique temporary organ which ensures mutual coexistence of the organisms of mother and fetus, determining growth and development of the latter [1]. Initially, it was believed that the fetus and placenta are closely related genetically to the mother; but with the development of assisted reproductive technology of the egg donation, it became clear that their genotypes could be completely foreign [2], which can be regarded as a natural model of engraftment after allogeneic transplantation. The main functions of the placenta are ensuring the supply, growth, and development of the fetus, as well as removing metabolic products and preventing immune rejection [1]. Since the placenta is a provisional organ, it becomes a salvage material after delivery [3]. For decades, clinicians and researchers work on the application of the placenta for therapeutic purposes, initially in the form of extracts and cell or tissue transplants, thus accumulating substantial empirical experience [4, 5]. However, at the same time, a large amount of research was little systemized and not always correlated with conventional pharmaceutical and other methods of treatment. Recent developments of cell therapy approaches along with opportunities for autobanking significantly increased the interest in the placenta as a source of biological material. Novel studies revealed a number of typical features of placental-derived cells, which define the direction of clinical use [6]. The major aim of this review was to identify and systemize general properties specific to various biological products of placental origin and characterize the most promising directions for their clinical application based on the analysis of data available in the scientific literature. Since placental structures have been used in a broad range of therapies, in our analysis, we have only considered the data, which have been confirmed repeatedly by several independent groups at various time points.

2. Structure and Properties of the Placenta and Fetal Membranes

2.1. Development of the Placenta. During pregnancy provides the key to understand its structural and functional

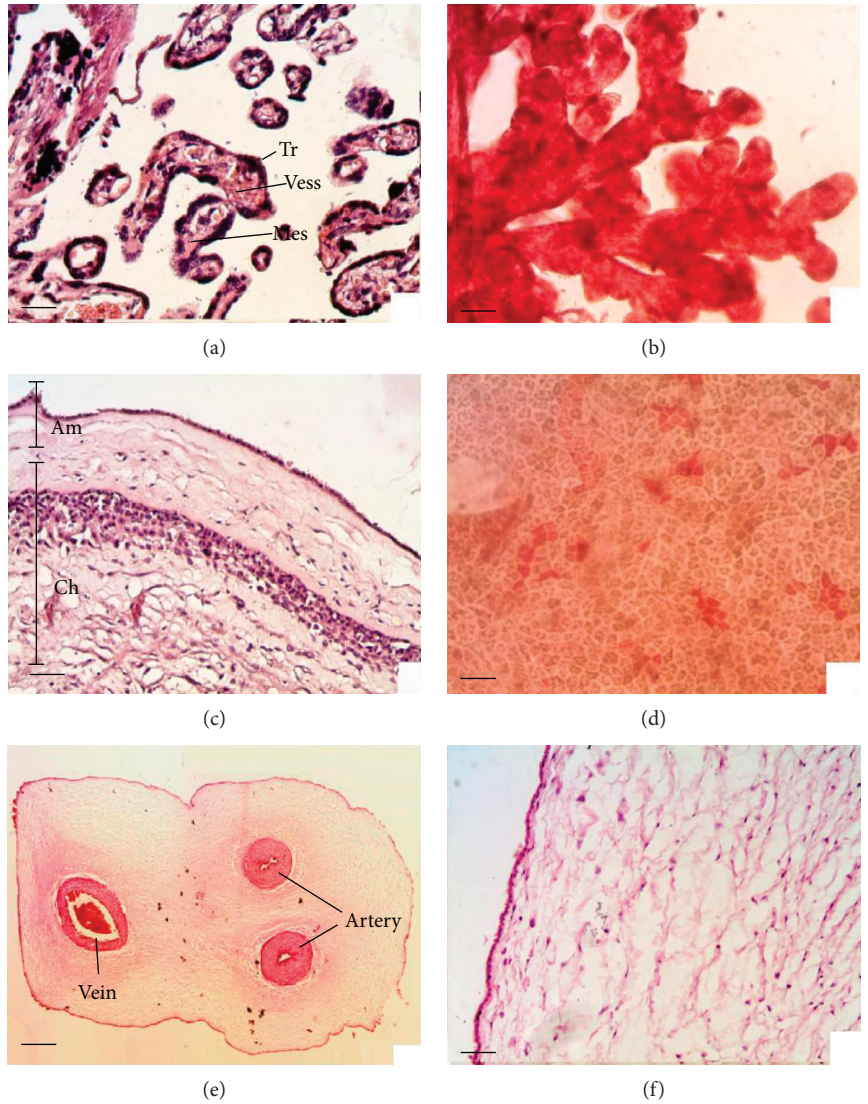


Figure 1: Morphology of placental components: (a, b) placental tissue (villi): Tr: trophoblast; Vess: vessels; Mes: mesenchyme; (c) fetal membranes: Am: amniotic membrane; Ch: chorionic membrane; (d) surface cells of amniotic epithelium; (e) cross-section of the umbilical cord; (f) umbilical cord tissue. Staining: (a, c, e, and f) hematoxylin-eosin, sections; (b, d) neutral red, native preparation. Scale bars: (a, b, c, d, and f) 50 μm; (e) 1000 μm.

peculiarities. At the stage of 8 blastomeres, the blastocyst divides into embryoblast and trophoblast. Trophoblast forms villi and first primary, containing only the trophoblast, then secondary, containing the stroma (embryonic mesenchyme), and later tertiary, containing the vessels (Figures 1(a) and 1(b)). At the same time, division of the trophoblast into syncytium and cytotrophoblast takes place. Implantation processes and trophoblast invasion occur through the enzymatic destruction of the endometrium and decidua of the uterus and layering of the resulting lacunae with trophoblast cells, replacing the choroid of the spiral arteries with trophoblast, which prevents thrombosis and makes the arteries refractory to vasopressor agents. After 6–8 weeks of pregnancy, the villi remain only on the placental site. The rest of the villi become atrophied and the smooth chorion, containing significant amounts of the trophoblast elements, is being formed [6, 7].

2.2. Morphology. Postpartum placenta has a disk-shaped form 16–20 cm in diameter, weighing 500 g on average. Trophoblast cells, mesenchymal cells, and endothelial cells of vessels are the main cell types of the placenta.

Since the use of "early placenta" (from the first two trimesters of pregnancy) encounters a number of ethical issues, the majority of the researchers are focused on the third-trimester placenta, also known as "mature placenta" (38–40 weeks of pregnancy) [3, 8]. Mature placenta consists of fetal and maternal parts. The fetal part includes the chorionic plate, amnion, and umbilical cord. Fetal membranes (Figure 1(c)), amniotic and chorionic, are formed on the basis of the smooth chorion and can be easily separated at the intermediate layer. Thin, transparent, and smooth amniotic membrane is composed of a single layered epithelium and the amniotic mesenchyme, an avascular connective

tissue. Chorionic membrane is composed of fibroblasts and a large number of trophoblast cells. The chorionic plate represents the fetal surface of the placenta, which is covered by the amnion. The umbilical cord enters the chorionic plate and connects the fetus to placenta. Umbilical cord is 50–70 cm in length and 1-2 cm in thickness. It is covered by the amniotic epithelium and contains two arteries and one vein that are immersed in the Wharton's jelly (which contains a large amount of fibroblast cells and has an intercellular substance rich in hyaluronic acid) (Figures 1(d)–1(f)) [1, 6]. The maternal part, or so-called basal plate, is comprised of bed and walls of lacunae, formed by decidual endometrial tissue. Additionally, the maternal part contains NK cells, macrophages, and other immune cells. Therefore, postpartum placental cells possess mainly the fetal genotype with a certain amount of maternal cells [1, 7].

2.3. Immunological Features. The structure of the placenta has several features that determine its function as well as the possibility of effective application in clinics and in biotechnology. Trophoblast cells are protected from the maternal immune system, due to reduced expression of the major histocompatibility complex (MHC), apoptosis-inducing mechanisms, and the influence of hormones and growth factors on the cells of the immune system [9]. According to majority of the authors as well as to online gene annotation portals and databases (e.g., http://biogps.org/), trophoblast has virtually no expression and other cells of placenta express very low amounts of classical MHC (HLA-A, HLA-B, and HLA-C), thus making it difficult for the immune system to recognize these cells [10, 11]. Furthermore, trophoblast expresses nonclassical MHC, which is inherent to pregnancy and includes HLA-E, HLA-F, and HLA-G [2]. In particular, according to some authors, HLA-G inhibits the migration of natural killer cells (NK cells) and proliferation of T-lymphocytes by interacting with the NKR2B4 receptor. Similar properties were described for the HLA-E expressed in the trophoblast [2, 7, 12].

Other mechanisms of protection of trophoblast from the maternal immune aggression involve apoptosis-inducing ligands FasL and TRAIL, which have the influence on immunocompetent cells [13]. During pregnancy, an increase in the number of Th2 lymphocytes occurs, which secrete anti-inflammatory interleukins IL4, IL5, IL9, IL10, and IL13, and a decrease in Th1 lymphocytes, which secrete proinflammatory IFNγ. This phenomenon is determined by the action of progesterone, as well as the capability of placental cells to secrete a certain variety of cytokines. The reduction in NK cells is also detected, though it is compensated by the activation of nonspecific immunity [2]. In this regard, it is observed that pregnancy is often accompanied by remission of a number of autoimmune diseases, such as multiple sclerosis and rheumatoid arthritis [2, 14].

2.4. Endocrine Function. Trophoblast cells synthesize a number of hormones, such as estradiol, progesterone, and chorionic gonadotropin, which regulate growth and development of the fetus as well as changes in the organism of a mother during pregnancy [15]. These hormones have the impact primarily on the reproductive and immune systems, which explains the therapeutic efficiency of the components of the placenta in the treatment of respective pathologies. Estradiol causes proliferation of the endometrium and mammary glands, causes calcium retention, and possesses feminizing and antisclerotic effect, as well as it affects sexual behavior. The main function of progesterone is in providing occurrence and preservation of pregnancy. Chorionic gonadotropin is an analogue of gonadotropin hormones, with the properties of luteinizing and follicle-stimulating hormones. It possesses the trophic corticotropic function and supports the development of pregnancy as well as enhanced resistance to stress. Exogenous administration of human chorionic gonadotropin stimulates ovulation, synthesis of ovarian estrogen in females, and androgen synthesis and spermatogenesis in males [15, 16].

2.5. Trophoblast Deportation. Among the most important physiological properties of placental cells is the capability of deportation and long-term existence in the mother's organism outside of the placenta itself. Fragments of different sizes, from multinuclear symplasts to exosomes, are emitted from the villi and fall into the uterine veins; a part of them are embolized by pulmonary capillaries and smaller particles fall in the systemic circulation. Settling in the mother's tissues, trophoblast cells can remain viable for some time and can be traced up to three–four days on average, with certain cases of reported detection in up to two weeks postpartum [17]. Elimination of deported trophoblast cells is achieved through nonspecific immunity and lytic factors. To date, there is no consensus about the role of trophoblast deportation in the course of pregnancy. Most researchers believe that this is a physiological phenomenon and assumes that the trophoblast in such manner participates in the formation of tolerance [17, 18]. In the case of preeclampsia and placental dysfunction, the amount of necrotic and apoptotic-altered trophoblast cells increases. In modern medicine, diagnostic methods based on the isolation of fetal cells from the maternal blood are actively developing. At the moment, this is primarily a diagnosis of hereditary diseases and risk of preeclampsia. Therefore, not only the placenta is a natural model of organ transplantation in the organism of a mother during pregnancy but also the trophoblast deportation is a natural model of cell transplantation [12].

3. History of Clinical Application of Placental Components

Multicomponent cellular and biochemical composition of the placenta, along with its ability to perform a wide variety of functions and availability of a large amount of material, has always attracted close attention of clinicians and researchers. Substantial experience in the application of the placenta in experimental and clinical practice has accumulated over the past 100 years. Various groups of researchers at different time points often observed similar therapeutic effects and patterns even by applying different techniques

and using different dosages and forms (extracts, tissue fragments, cells, serum, etc.) [4, 8, 16, 19]. Unfortunately, this immense amount of work and clinical data is little systemized and many successful approaches received no follow-up. We believe that analysis of these data will contribute to finding ways of further development of therapies using the placental material.

The first application of the amniotic membrane in ophthalmology was published by Davis, J. (1910) [8]. Results of the first studies on the effect of placental extracts and tissues on the reproductive system appear in the literature in the early 20th century (Aschner B., 1912, Hirose T., 1919) [16]. Majority of the world's research on devitalized preparations was published in the 1930s to 1980s. The first successful transplantation of cord blood cells in Fanconi anemia in 1982 resulted in enhanced interest of researchers in cord blood stem cells [20]. Since the end of the 20th century, components of the placenta (placenta, umbilical cord, and membranes) are increasingly seen as a source of stem cells; cryopreserved viable placental preparations are successfully applied [6, 21].

Due to a range of historical reasons, a significant amount of the studies is unfortunately not included into modern international databases, such as PubMed. For example, most of the works of academician Vladimir P. Filatov [22], founder of the Institute of Tissue Therapy and Eye Diseases (USSR), in the 1930s to 1960s were not even translated to English, highly limiting the audience of this valuable publications. At the same time, his fundamental work on the use of tissue therapy methods has been published in over 3000 scientific works; thousands of patients received effective treatment with devitalized placental medications; and dozens of departments and institutes on tissue therapy were established on this basis [23–25]. Also, currently, more information on the centuries of experience of application of placental derivatives in traditional Chinese medicine becomes available from the recent publications [8].

The researchers have used in their studies the fragments of placental tissue, amniotic and chorionic membranes, umbilical cord, amniotic fluid, placental extracts and lyophilizates, and cord blood serum, as well as various types of differentiated cells and stem cells (Figure 2). Such material has been used in native form as well as after chemical and thermal processing, after cryopreservation and sublimation. Methods of application widely vary such as subcutaneous, intramuscular, intravenous, intraorperative, as biocovers, and substitutive material, as well as via oral administration [26–32].

The views on the mechanism of action of placental preparations have been changing along with the development of biology and medicine. The researchers hypothesized and attempted to explain the patterns of therapeutic effects by vitamin, mineral, protein, and peptide composition, by the presence of "resistance factors," "preservation factors," cytokines, hormones, and stem cells [4, 21, 23]. Such approach could explain common features of placental derivatives and shed light on the mechanism aspect and unique biological activity. At the same time, very often, the studies of the beginning of 21st century repeat the works from the mid-twentieth century, explaining obtained results in a different manner, while observing similar effects under the same pathological conditions [8, 19].

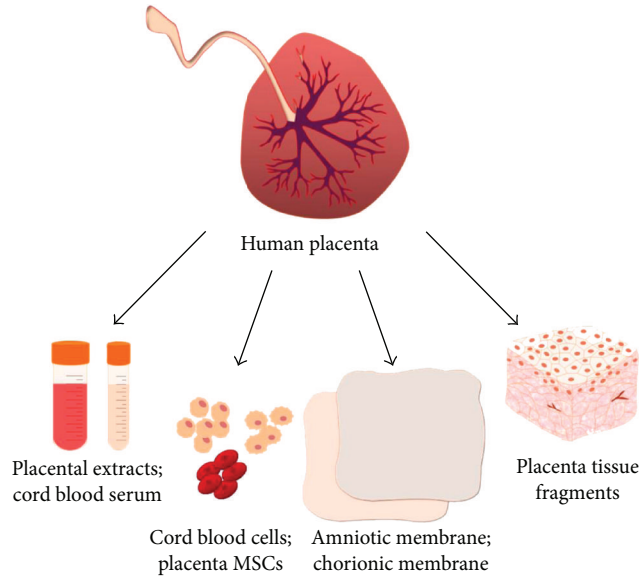


Figure 2: Conventional forms of application of placenta-derived biomaterial in clinics: placental extracts and lyophilizates, cord blood serum, various types of differentiated and stem cells, amniotic and chorionic membranes, and fragments of placental tissue.

4. Cord Blood Cells

The greatest attention of researchers and clinicians is traditionally attracted by cord blood cells. Umbilical cord blood is an easily accessible rich source of hematopoietic stem cells which is an alternative to the bone marrow [33–35]. High rates of success were achieved with allogenic transplantation of umbilical cord blood cells for the treatment of the patients with hematologic and metabolic pathologies [20, 36]. Application of cord blood stem cells is described to be quite effective in the treatment of erectile dysfunction and diabetes mellitus, resulting in improved erectile function and reduction in fasting blood sugar [37]. In former years, mainly due to the limited dosage of cord blood cells available for each transfusion, the application was limited to child patients [38]. However, recent advances in cord blood cell expansion methods, isolation of particular units with the following dosage adaptation, and availability of HLA matching techniques allowing pooling of the units from the different donors resulted in significant improvement of related therapies [20, 39–42].

Besides the transplantation of hematopoietic cord blood cells, a special interest of the researchers is attracted by erythrocytes and platelets of the cord blood [43–45]. The main distinction of cord blood erythrocytes from the erythrocytes of an adult is the content of fetal hemoglobin. The content of fetal hemoglobin in cord blood erythrocytes is over 90%, while in adult blood it is less than 10% [46]. Fetal hemoglobin has a significantly higher affinity to oxygen, which enables

the transfer of oxygen through the placenta from the mother's blood [47]. Despite the possibility to obtain only 50–100 ml of cord blood postpartum, transfusion of the cord blood erythrocytes is advantageous for the treatment of infants, especially premature infants, as well as for the intrauterine blood transfusions, when the recipient's blood mainly contains the fetal hemoglobin. Such approach allows reducing the number of transfusions and significantly accelerates recovery [48, 49]. Transfusion of the cord blood erythrocytes to adult patients results in a more rapid recovery of neutrophils and slower recovery of platelets in comparison to transfusion of adult erythrocytes [42]. Transfusion of the cord blood is also mentioned as a perspective for utilization in pediatric practice in the countries where the availability of donated blood is limited [33].

5. Placental Extracts

High number of publications is related to the studies of placental extracts. Such extracts are obtained by lysing human placental tissues collected at full-term delivery. Therefore, the extracts do not contain cells but are rich in a wide range of proteins, minerals, amino acids, and steroid hormones [4]. According to the data of various research groups, such extracts possess anti-inflammatory, analgesic [26], antioxidant [50, 51], cyto- and radioprotective [52], and anti-allergic properties and express hormonal activity [16, 53, 54], as well as stimulate proliferation and reparative processes [14, 55, 56].

Placental extracts were shown to enhance the proliferation of fibroblasts and cord blood cells *in vitro*. At the same time, it was noted that extracts isolated from the late gestation placenta possess the highest biological activity [53, 56]. Cytoprotective and antioxidant properties of the extracts are usually associated with the protein components; in particular, they are correlated with the concentration of alpha-fetoprotein [50, 51]. Animal model studies showed that prophylactic administration of the extracts increases the resistance of animals to oxidative stress [57]. Placental extracts reduce the concentration of free radicals, inflammatory cytokines IL6, TNF, and IL1, at the same time increasing the colony formation of progenitor cells *in vitro* and reducing oxidative and radiation damage of the cells [52, 57]. Analysis of biosafety of placental extracts revealed the absence of toxic or mutagenic influence on cell cultures and adult animal models; however, fetotoxicity in animals at early gestation was reported [58].

Placental extracts have been applied for the treatment of a wide variety of pathological conditions—most commonly in surgery, neurology, gynecology, and dermatology. Pronounced positive effects were received in the treatment of wounds, nonhealing ulcers, and burns: rate of epithelialization was significantly increased and a decrease of infiltration and reduction of the pain syndrome were observed [27, 59]. The extracts accelerate the wound healing in animals with the diabetes model, which can be interpreted as a treatment for diabetic neuropathy and angiopathy [28]. The mechanism of action of placental extracts in the wound healing is associated with the increase of TGF-β in the early phase of regeneration and VEGF in the late phase, as well as with the presence of FGF, amplification of angiogenesis, and the increase of expression of CD31 [28, 60]. Application of placental extracts in menopausal disorders allowed reducing the number and severity of hot flushes, irritability, and normalize hormonal profile [54, 61]; the amount of estrogen receptors in the experiment was increased, and the effects of vaginal atrophy were reduced, while the activity of osteoblasts was improved [53]. Experimental studies on the effect of placental extracts on behavior and physical condition in the animal model showed decrease in symptoms of fatigue and increased resistance to physical stress [53, 62]. This phenomenon was explained by the rise of the level of intracellular calcium, activation of splenocytes and T cells, and reduction of synthesis of proinflammatory cytokines associated with fatigue (IL6, TNF, and IFNγ) [53, 62]. Similar results were obtained in preclinical studies [29].

Placental extracts showed expressed efficiency in neurology by supporting regeneration of the nerve tissue in experimental treatment of the nerve damage and facial spasm. The authors explain the resulting effect with the increased synthesis of GAP-43 and Cdc2 regenerative factors [63, 64]. Placental extracts were effective in the treatment of rheumatoid arthritis [65] and experimental renal failure [66]. A certain amount of practical experience in the application of placental extracts is also accumulated in veterinary medicine. Here, the extracts were applied for stimulation of mammogenesis, lactogenesis, and galactopoiesis [67].

6. Cord Blood Serum

Cord blood serum has been used in ophthalmology in treatment of chemical and thermal damage of the cornea and corneal erosion, as well as recovery after laser operations. A more complete and rapid epithelialization of the cornea after application of the preparations containing cord blood serum in comparison to conventional pharmacological therapies was demonstrated [30, 68]. Besides, positive effects in the treatment of neurotrophic keratitis were described [69].

The ability of cord blood serum to stimulate pancreas cells for insulin synthesis along with the formation of pancreatic islets has been demonstrated *in vitro* [70]. Application of cord blood serum in obstetrical antiphospholipid syndrome improves the readiness of preimplantation endometrium and reduces the number of antiphospholipid antibodies [71]. Besides, application of cord blood serum has shown efficiency in wound healing [72, 73]. These effects are explained by the presence of EGF, FGF, HGH, fibronectin, NGF, and IGF-1 in the composition of cord blood serum [68].

7. Isolated Placental Cells

By now, cells from the amniotic and chorionic membranes, placental villi, and umbilical cord have been successfully isolated, phenotyped, and characterized [74, 75]. Protocols based on trypsin, collagenase, or dispase digestion are used to isolate the cells from the fetal membranes. Isolation of the cells from placental villi requires application of DNase, and isolation of the cells from umbilical cord requires

hyaluronidase or application of the explant technique [6]. Among the variety of cell types, which can be isolated from the placenta and placental derivatives, mesenchymal stromal cells (MSCs) receive the highest attention in research and clinical trials [21]. According to the majority of researchers, MSCs obtained from all placental sources express CD105, CD90, CD73, CD29, CD13, CD10, and to a minor extent HLA-A, B, and C, while not expressing CD14, CD34, CD45, and HLA-DR [21]. Importantly, the cells obtained by the mentioned protocols retain the ability to synthesize chorionic gonadotropin and express cytokeratin-7 and CD200 [14]. Moreover, placental MSCs possess the capability of differentiation not only into three classical mesodermal lineages (adipogenic, osteogenic, and chondrogenic) but were also shown to be able to differentiate in myogenic, angiogenic, pancreatic, cardiogenic, and neurogenic cell types [6, 21].

In vivo studies in the mouse model showed that placenta-derived cells inhibit the delayed hypersensitivity reaction, improve the course of experimental encephalomyelitis, and induce tolerogenic immune response due to differentiation of dendritic cells and inhibition of Th1 response in favor of the Th2. Inhibition of the antigen-specific proliferation of T cells was observed *in vitro* [8, 14]. A large number of *in vitro* and *in vivo* studies is devoted to comparative analysis of the influence of mesenchymal stromal cells from various sources on the cells of the immune system. It was shown that the cells derived from the placenta possess more pronounced immunomodulating effect in comparison to the cells of the adipose tissue and bone marrow [76, 77]. Moreover, placental MSCs possess higher proliferation rates in comparison to the cells from the other sources [78, 79], which can be highly valuable in reconstructive therapies (e.g., tissue engineering) [80].

MSCs of amniotic membrane were efficient in the treatment of premature depletion of ovarian function after chemotherapy. Direct administration to the murine ovary resulted in recovery of the estrous cycle and sexual function, as well as certain other reproductive parameters [81].

It was shown that mesenchymal stromal cells isolated from the amniotic membrane can differentiate into hepatocyte-like cells [21, 82]. Intravenous application of placental cells in experimental models of intoxication with carbon trichloride enhanced liver regeneration and accelerated recovery of the animals [83, 84]. Transplantation of placental cells in the heart muscle resulted in their differentiation into cardiomyocytes [85], which improved the regeneration after experimental acute myocardial infarction [31, 86]. Efficiency in the heart failure treatment is attributed to paracrine interactions [87].

Expression of a range of neuronal markers was found in placental cells, possibly due to the involvement of the placenta in the metabolism of neurotransmitters [88]. Neuroprotective effect of placental MSCs has been proven after direct transplantation to the central nervous system as well as after intravenous administration; the course of experimental Parkinson's disease and spinal cord injury has been improved, and acceleration of rehabilitation after experimental ischemic stroke in the animal model was observed [6, 89, 90]. Efficiency of placental cells in the treatment of ischemic stroke is explained by the influence of VEGF, HGF, and neurotrophic factors [91]. Moreover, reduction of beta-amyloid plaques and anti-inflammatory effect with increasing TGF-β and IL10 was observed in the treatment of Alzheimer's disease [92].

Treatment of experimental diabetes with placental cells (intravenous administration) resulted in normalization of the weight of the laboratory animals and restoration of normal glucose levels. The authors detected no teratoma formation, at least within the few months of the follow-up observations [93]. Moreover, treatment with placental cells had positive effects on complications of diabetes. Namely, it accelerated wound healing in a diabetic animal model [94].

Antiaging effect of transplantation of placental cells has been reported in a mouse model [95]. Intravenous application of placental cells demonstrated efficiency in the treatment of rare diseases in the experiment, in particular, pulmonary fibrosis, osteogenesis imperfecta, and muscular dystrophy [6, 21, 96].

Besides the cells isolated from the term placental material, immature placental cells from amniocentesis and chorionic villus sampling have been successfully applied in prospective studies [97, 98]. Since the fetus is not sacrificed in these cases, the ethical issues surrounding discarded early gestation placental tissue are avoided.

Cells obtained from prebirth tissues, such as the umbilical cord blood, amniotic fluid, and chorionic villi, have great potential in cardiovascular tissue engineering for the fabrication of heart valves, prevascularization of *in vitro* engineered tissue constructs, or *in vitro* endothelialization of synthetic blood vessel replacements [99, 100]. Combined with the use of cell banking technology, this approach may be also applied for postnatal applications [101].

8. Amniotic and Chorionic Membranes

Amniotic membrane has been used in clinical practice to a higher extent in comparison to the other components of the placental complex. Mainly, it is used in surgery as a biological coating [102, 103]. The first reported treatment with the amniotic membrane was performed in 1910 by J. Devis for the closing of skin defects; later, it was applied as the plastic material in different fields of surgery [32]. Most often, the amniotic membrane is used in ophthalmology for the closure of corneal pathology defects. Such application is determined by a range of unique properties of the amniotic membrane, such as transparency and the ability to stimulate proliferation and migration of stem cells from the limbus area, as well as the ability to suppress vascularization [103, 104]. The method is widely applied in clinics with expressed long-term effect [105, 106]. Application of amniotic and chorionic membranes is also convenient for the treatment of nonhealing trophic ulcers, vaginal reconstruction surgery, enterocutaneous fistula, prevention of adhesions, orthopedic pathology, replacement of the pelvic peritoneum, and so on [8, 19, 107, 108]. The mechanism of action of placental membranes is explained by the effect

of biological dressings, activation of epithelialization and neovascularization, suppression of inflammation, and scarring, as well as by antimicrobial properties [19, 109].

9. Placental Tissues

References on the application of placental fragments are rarely found in the international scientific literature databases. Successful application of the placental tissue in extracorporeal detoxification was reported [110]. The placental tissue has been used as a biosorbent for the treatment of chronic inflammatory diseases (peritonitis, suppurative cholangitis, mastitis, pancreatic necrosis, and phlegmon). The treatment resulted in improved general condition, reduction of bacteria in blood of the patients, lowering of the laboratory indicators of endotoxemia (bilirubin by 56%, transaminases by 55%, and creatine by 25%), positive dynamics in parameters of the immune system, strengthening of the central and peripheral blood flow, and, importantly, the absence of traumatization of cells in comparison to other conventional sorbents [110]. The efficiency of application of cryopreserved placental fragments was shown in experimental atherosclerosis: acceleration of atherosclerosis regression and neoangiogenesis of myocardium were observed [111]. Utilization of placental fragments in male infertility allowed increasing the quality of sperm as well as the number of pregnancies in couples [112].

10. Amniotic Fluid

Amniotic fluid is rarely used in experimental and clinical studies compared to the other placental material due to a smaller number of cells and the active compounds, as well as due to the difficulty of obtaining under sterile conditions. Nevertheless, certain researchers reported the efficiency of the amniotic fluid in bone healing [113], regeneration of nerve tissue [114], and prevention of epidural fibrosis [115]. Recently, an interest in amniotic fluid as a source of stem cells has increased due to a potential in the correction of pathologies of the nervous, musculoskeletal, reproductive, and cardiovascular systems [99, 116, 117]. Preclinical studies in the ovine animal model showed the possibility of prenatal implantation of tissue-engineered cell-based heart valves, composed of biodegradable matrixes with autologous amniotic fluid cells. Such valves possessed *in vivo* functionality with intact valvular integrity and absence of thrombosis [118]. Moreover, amniotic fluid cells have proven high efficiency in neurology: successful spina bifida treatment was performed in both rodent and ovine animal models [97, 119].

11. Current Research and Clinical Trials

The areas of research and the number of studies and clinical trials utilizing placental derivatives were analysed on the basis of open sources of the US National Library of Medicine (https://www.ncbi.nlm.nih.gov) and the US National Institutes of Health (http://ClinicalTrials.gov/) (Table 1). Currently, the highest number of studies and clinical trials is dedicated to *cord blood cells*. As hematopoietic cells, they are traditionally used in the treatment of blood oncology and bone marrow pathologies. Novel studies focus on rehabilitation after chemotherapy, diabetes, and ischemic lesions of the central nervous system and extremities. *MSCs* of placental origin have higher differentiation potential but require more complicated isolation procedures. The number of experimental studies and clinical trials with placental MSCs is generally smaller in comparison to cord blood cells; however, the range of pathological conditions where they can be effectively applied is broader. Among others, this includes autoimmune and endocrine diseases, disorders of the nervous and reproductive systems. Most of the works on MSCs are fundamental and experimental; the number of clinical trials is relatively small. Research dedicated to *amniotic membranes* mainly relates to the coverage of wounds, surgical defects, ulcers, and burns. Fewer studies are dedicated to the application of the amnion in the cell culture. At the same time, the number of publications on clinical application of the amnion is significantly higher in comparison to the basic research. The number of studies on *placental extracts* is relatively low and is mainly associated with degenerative diseases and disorders of the reproductive system. Works on application of *cord blood serum* are mainly performed in corneal pathology. Despite the widespread usage of fetal serum as a rich source of growth factors for the cell culture techniques, the intensity of cord blood serum-associated research is rather low.

12. Biobanking of Placental Components

Active work with biological material requires appropriate biobanks and biobanking technologies. Long-term storage of placental components may be aimed at clinical application and scientific research, as well as development and testing of the novel drugs [3, 120–123].

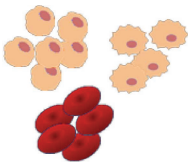
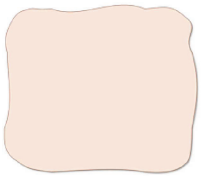
Methods of chemical preservation, high-temperature sterilization, hypothermic storage, low-temperature storage, and cryosublimation have been used for the storage of placental components. Selection of the storage method depends on the type of material and the purpose of its further application [5, 120, 122, 124].

Methods of sterilization by filtration or autoclaving have been used for devitalization of the extracts containing peptides [4]. Devitalization methods allow storing the material at the room temperature without additional equipment; however, the properties of such biological objects are significantly altered. For example, devitalization of amniotic membrane significantly reduces the immunogenicity and highly extends its biodegradation period [3].

Hypothermic and subnormothermic storage of biomaterial ensures preservation for a short period of time, required for delivery of the material to a laboratory or clinics with minor structural and biochemical injuries [122, 125–127].

The most conventional method for storage of biological objects, which provides high levels of preservation for a long period of time, is cryopreservation [124]. Cord blood serum, placental extracts, cell suspensions, chorionic and amniotic membranes, and placental tissue are all suitable for cryopreservation procedures [120, 123]. Possibility to isolate MSCs

Table 1: Worldwide progress in the research, preclinical studies, and clinical application of the placenta-derived material.

Forms of application of placental components	In research (https://www.ncbi.nlm.nih.gov/) 2017	Clinical trials (http://clinicaltrials.gov/) 2017	Pathology
 Cord blood cells	>10,000	351	Aplastic anemia, haematological malignancies, cancer, traumatic brain injury, virus infection, limb ischemia, stroke, and brain ischemia
 MSCs (derived from placental tissue, fetal membranes, Wharton's jelly, and amniotic fluid)	>4000	39	Diabetes mellitus, multiple sclerosis, myocardial infarction, strokes, peripheral neuropathy, trophic ulcers, Crohn's disease, graft-versus-host disease, and pulmonary fibrosis, limb ischemia, cardiomyopathy, knee osteoarthrosis, diabetes mellitus, amyotrophic lateral sclerosis, and erectile dysfunction
 Amniotic membrane	>1900	114	Corneal ulcers, corneal melting, injury, periodontitis, diabetic foot, foot ulcer burns, and adhesions
 Placental extract	65	2	Keratinocytes, wound healing, rheumatoid arthritis, intervertebral disc degeneration, and climacteric symptoms in premenopausal women
 Cord blood serum	7	3	Corneal epithelial wound
 Amniotic fluid	4	—	Preventing fibrosis, adhesion, nerve, and bone regeneration

with a stable genome from cryopreserved placental tissues, similar to the population of cells isolated from the native (fresh) tissues, significantly extends the scopes of cryopreservation of the placental material [128]. The placenta is a unique object for low-temperature biobanking and autobanking [6, 120, 122]. In most countries, the application of placenta does not face ethical issues and women positively evaluate this possibility, otherwise considering the material as a "waste." Donation of the placenta is physiologically indifferent for the donor. At the same time, it provides a large amount of material suitable for direct application and for long-term storage in initial state or after processing, as well as for the preparation of extracts and isolation of cells or individual components [3]. Biobanking technologies might offer a lifelong availability of the autologous placental material or tissue-engineered constructs, readily available for immediate application for a patient [101].

13. Conclusions

Considerable amount of information on the properties, experimental studies, and possibilities of clinical application of placental components is accumulated to date (Table 1). The researchers showed the potential use of placental components in various fields of biology and medicine. However, many findings are not confirmed independently by different research groups and have not been performed on the amount of material sufficient enough for clinically significant statistical conclusions.

Early works were primarily devoted to the study of placental extracts as hormonal agents and biostimulators, as well as amniotic membranes as biocovers. Cord blood cells are the most widely used placental component in modern medicine, being applied in the stem cell transplantation. Amniotic membrane is successfully applied in the ophthalmic practice, surgery, and wound healing. Novel technologies based on the application of placental MSCs and autobanking are considered as the most promising and prospective for the near future in the field of regenerative and reconstructive therapies as well as in bioengineering. The interest of researches in placental extracts and placental blood serum is still low, despite the widespread application of various fetal sera in the cell and tissue culture media.

Independent research groups at different time points received similar data on the properties and effects of application of the various components of the placenta for correction of a wide spectrum of pathologies. In most of their opinion, general therapeutic properties of various placental components are expressed in stimulation of reparative processes and anti-inflammatory and immunomodulatory effects. The mechanism of action of the various components of the placenta on the recipient's organism is associated with a shift from Th1 to Th2 type of immune response, suppression of the synthesis of IL6, TNF, and IFNγ, and enhancement of the synthesis of IL10, VEGF, and trophic factors.

Various research teams have verified the effects from the application of placenta and its derivatives on the nonhealing wounds, ulcers, disorders of the reproductive system (infertility and menopausal syndrome), autoimmune pathologies, and diabetes, as well as neurological disorders. The use of stem cell therapy methods is accepted as an addition rather than an alternative to conventional medical approaches, since it often does not cover all components of the pathogenesis of the targeted diseases.

Considering the clinical potential and high perspectives of the placental material as an object for autobanking, it may be recommended to preserve not only the individual cell populations but also fragments of membranes, tissue, placental extracts, and cord blood serum.

There are no doubts in the conception of placental components as a rich source of biologically active substances and stem cells. At the same time, it should be noted that currently available results on the positive effects of placental components in the treatment of a wide spectrum of diseases have to go through the test of time and statistical observations in order to determine their potential/real therapeutic efficacy. Additionally, adverse effects and contradictions should be carefully and precisely studied and evaluated in the short- and in the long-term periods.

Ethical Approval

Human placental material used in microscopic images for Figure 1 was donated in anonymized manner with a written informed consent of the patients after routine Caesarian section at the Department of Gynaecology and Obstetrics at Hannover Medical School, Germany (approved by the Ethical Commission of Hannover Medical School, ethic votum number 2396-2014) and in Kharkiv municipal maternity hospital number 1, Ukraine (approved by the Bioethics Committee of the Institute for Problems of Cryobiology and Cryomedicine of the National Academy of Sciences of Ukraine, ethic votum number 2-0306-2013). This study did not involve animal experiments.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this article.

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Research Article

Soft Tissue Repair with Easy-Accessible Autologous Newborn Placenta or Umbilical Cord Blood in Severe Malformations: A Primary Evaluation

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Disrupted organogenesis leads to permanent malformations that may require surgical correction. Autologous tissue grafts may be needed in severe lack of orthotopic tissue but include donor site morbidity. The placenta is commonly discarded after birth and has a therapeutic potential. The aim of this study was to determine if the amnion from placenta or plasma rich of growth factors (PRGF) with mononuclear cells (MNC) from umbilical cord blood (UCB), collected noninvasively, could be used as bio-constructs for autologous transplantation as an easy-accessible no cell culture-required method. Human amnion and PRGF gel were isolated and kept in culture for up to 21 days with or without small intestine submucosa (SIS). The cells in the constructs showed a robust phenotype without induced increased proliferation (Ki67) or apoptosis (caspase 3), but the constructs showed decreased integrity of the amnion-epithelial layer at the end of culture. Amnion-residing cells in the SIS constructs expressed CD73 or pan-cytokeratin, and cells in the PRGF-SIS constructs expressed CD45 and CD34. This study shows that amnion and UCB are potential sources for production of autologous grafts in the correction of congenital soft tissue defects. The constructs can be made promptly after birth with minimal handling or cell expansion needed.

1. Introduction

Organogenesis occurs early during embryonic development, and disruption of normal organogenesis results in permanent congenital malformations [1]. These malformations affect up to 3% of all infants [2] and are one of the leading causes of neonatal death [3]. Congenital malformations are also associated with morbidity and are responsible for a significant part of pediatric hospital admissions and costs [4]. Correction of congenital soft tissue defects such as myelomeningocele (MMC), giant omphalocele, gastroschisis, and congenital diaphragmatic hernia (CDH) involves surgical correction short after birth. Affected newborns without spare tissue are dependent of donor tissue. Because of the worldwide perinatal organ donor shortage [1], prosthetic materials are sometimes being used [5, 6], which might be associated with complications such as recurrence, graft dislodgement and/or rejections, erosion of the graft, and infections [7–9]. Biological graft material would be preferable and consists of the extracellular compartment of human or animal tissue. They are also associated with a risk for recurrence but have the advantage of tissue remodeling, neovascularization, and lower rate of infections [10, 11] and also advantages in the situation of high-risk contamination areas, such as congenital high-risk abdominal wall defects [12].

The progress in tissue engineering (TE) paves an alternative route to the traditional use of prosthetic materials [13]. The most common approach involves a combination of biological matrices and cells. Populating the matrix with cells enhances graft integration and restoration of host tissue

and reduces scar formation and bacterial infections [13, 14]. Small intestine submucosa (SIS) consists of cell-free porcine extracellular matrix (ECM) (mostly collagens), an absorbable biomaterial that contains growth factors and supports angiogenesis, frequently used for surgical correction of soft tissue defects [15, 16].

Autologous grafts are considered as the gold standard, but they often include donor site morbidity where healthy tissue is removed from one site of the body to replace injured or missing tissue [17] and have limitations depending on the graft area size. Allogeneic grafts eliminate the donor site morbidity but may induce an immune response in the recipient [18], rejection with subsequent necrosis [19], and allograft removal or replacement.

The placenta is a fetomaternal organ consisting of three layers: outer maternal decidua, fetal chorion membrane and inner fetal amnion membrane surrounding the fetus, and the amniotic fluid. The human placenta has been used in medicine since the 1500s for the treatment of, for example, leg ulcers, wounds, and in skin transplantation [20–24]. The amnion contains multipotent cells, both in the epithelial and the mesenchymal layers, and possesses anti-inflammatory and regenerative properties and also antifibrotic, antimicrobial, immune suppressive, and angiogenic properties [25–29]. Umbilical cord blood (UCB) contains hematopoietic stem cells and has been used for transplantation to cure malignant and other severe diseases for more than 30 years [30]. From UCB, plasma rich of growth factors (PRGF) can be isolated [31]. Both experimental and clinical studies have shown that PRGF is clinically safe and promotes vascularization, tissue regeneration, and wound healing [32, 33].

To overcome the problems with donor site morbidity, allogenic immunological reactions, and long production time, we hypothesized that autologous proangiogenic constructs could be produced from derivatives of the term placenta; amnion and PRGF gel with mononuclear cells (MNC) from UCB. Both are noninvasive sources for autologous tissue engineering that are easily available after birth with no ethical obstacles of their use.

The objective of this study was to analyze amnion and MNC-enriched PRGF gel with respect to tissue and cell integrity, morphology, and viability. In addition, amnion and PRGF gel were combined with SIS in order to improve mechanical strength and to build a durable graft. The tissue and cell integrity of the amnion and PRGF-SIS constructs were analyzed as above, but additionally for changes in cell morphology and protein expression.

2. Material and Methods

2.1. Isolation of the Amnion. Human term placentas ($n = 4$) were obtained after elective caesarian sections from healthy donors after informed oral and written consent according to ethical approval from the Regional Ethical Review Board in Stockholm (Dnr 2012/480-31/1) and in accordance with the Declaration of Helsinki. The placentas were placed in a sterile environment with the cord side up and transported to the laboratory. The amnion was mechanically peeled off from the chorion membrane starting from the cord. Amnion pieces were transferred to petri dishes and washed three times in phosphate buffered saline (PBS) (Life Technologies, Paisley, United Kingdom) before being further processed as described below.

2.2. Comparison of Culture Conditions of Amnion. To evaluate culture conditions, three different culture media were initially compared in regard to amnion-residing cell morphology, viability, and proliferation. The culture media compared were (1) mesenchymal stromal cell culture medium: modified Eagle medium alpha (MEMα) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% inactivated fetal calf serum (FCS) (PAA Laboratories GmbH, Pasching, Austria); (2) epithelial cell culture medium (DMEM-EC): Dulbecco's modified Eagle's medium-high glucose (Invitrogen) supplemented with 10% FCS (PAA Laboratories), 2 mM L-glutamine (Sigma-Aldrich, St. Louis, MO, USA), 1% nonessential amino acid (Sigma-Aldrich), 55 μM 2-mercaptoethanol (Sigma-Aldrich), and 1 mM sodium pyruvate (Sigma-Aldrich); and (3) endothelial cell culture medium (DMEM-F12): Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (Invitrogen) supplemented with 10% FCS (PAA Laboratories) and 1% L-glutamine (Sigma-Aldrich). All culture media were supplemented with 1% antibiotic-antimycotic solution (Anti-Anti, Life Technologies). Approximately, $3 \times 2\,\text{cm}^2$ pieces of amnion were isolated and placed in 12-well cell culture plates (Becton & Dickinson, Franklin Lakes, NJ, USA) equally divided between the different culture conditions. The amnion pieces were maintained at 37°C in a humidified environment containing 5% CO_2, and the media was replaced every 3-4 days. At days 0, 3, 7, 10, 14, and 21, the amnion pieces were washed twice in PBS and fixed in 4% paraformaldehyde solution (PFA) (Solveco AB, Rosersberg, Sweden) for 3 days. Thereafter, the amnion pieces from all time points and culture conditions were paraffin embedded, sectioned, and stained with hematoxylin (HTX) (Meyers HTX, HistoLab Products AB, Gothenburg, Sweden) and eosin for morphology (0.2%, HistoLabs Products AB), Ki67 for proliferation, (1 : 500, Life Technologies), and activated caspase 3 for apoptosis (1 : 200, Invitrogen). To visualize the staining, biotinylated secondary anti-mouse IgG antibodies were used (1 : 500, Vector Laboratories Inc., Burlingame, CA, USA). Avidin-Biotin complex (Vector Laboratories Inc.) was added and subsequently DAB (Dako, Glostrup, Denmark). The sections were counterstained with HTX (HistoLab Products AB).

2.3. Characterization of Migrated Amnion-Derived Cells in the Different Culture Conditions. To characterize amnion-derived cells (ADC) that migrated out from the tissue and adhered to the cell culture surface, the ADC was analyzed with flow cytometry at day 21. The cells were harvested by treatment with 0.05% trypsin and 0.53 mM EDTA (Invitrogen) and resuspended in PBS. The ADC were incubated with conjugated monoclonal antibodies against CD31, CD73 (Becton-Dickinson), and PCK (Dako) for 30 min in the dark at 4°C. The antibodies were conjugated to fluorescein (FITC), phycoerythrin (PE), or cyanine 3 (Cy3). Thereafter, the cells

Table 1: Components of the two studied constructs.

Amnion-SIS constructs	PRGF-SIS constructs
Amnion from term placenta collected from healthy donors undergoing elective caesarean section	Term umbilical cord blood collected from healthy donors was used for isolation of the following: (i) Plasma rich in growth factors (PRGF) (ii) Mononuclear cells (MNC)
Commercially available 4-layer small intestine submucosa (SIS) that consists of cell-free porcine extracellular matrix, mainly collagen	Commercially available 4-layer small intestine submucosa (SIS) that consists of cell-free porcine extracellular matrix, mainly collagen
The components were combined by suturing the amnion onto the SIS.	The components were combined by the following: (1) Mixing MNC with PRGF (2) Casting the MNC-PRGF gel onto SIS

were washed and resuspended in PBS. The cells were analyzed with a flow cytometer with 488 nm excitation and emission filters (FACSCalibur, Becton-Dickinson), and the data was analyzed using the software Flow-Jo (v. 10, Tree Star Inc., Ashland, OR, USA).

2.4. Characterization of Amnion-Residing Cells in Constructs with SIS. To analyze the properties of the amnion-residing cells in the construct with SIS (amnion-SIS), $2 \times 2\,cm^2$ pieces of amnion were placed on dry 4-layer SIS (Biodesign® Tissue Graft, Cook Biotech Incorporated, IN, USA) and let adhered for 5 minutes. To increase the contact, the amnion was sutured onto the SIS matrix (Prolene 6-0, Ethicon Inc., NJ, USA) (Table 1), and the construct was kept in culture for up to 21 days in DMEM-EC medium. At days 0, 3, 7, 10, 14, and 21 amnion-SIS pieces were washed twice and transferred to 4% PFA (Solveco AB) for fixation and thereafter paraffin embedded and sectioned. The sections were stained with HTX/eosin (HistoLab Products AB) for morphology or pan-cytokeratin (PCK, epithelium 1 : 500, Dako), CD31 (endothel, 1 : 500, Dako), and CD73 (common stromal marker, 1 : 500, Abcam, Cambridge, United Kingdom) for surface epitope expression, and Ki67 (1 : 500, Life Technologies) and activated caspase 3 (1 : 200, Invitrogen) as above. Stained sections of amnion without SIS cultured in DMEM-EC served as a control.

2.5. Preparation of Cell-Enriched Gel from Plasma Rich in Growth Factors. Term UCB was collected after birth, before delivery of the placenta, by puncturing the umbilical vein using a sterile collection system (Macopharma MSC1206DU cord blood collection kit, Tourcoing, France) containing 21 mL anticoagulant (citrate phosphate dextrose solution). The UCB was collected by gravity until flow ceased. Fresh unbanked (because of too low cell counts) UCB units ($n = 3$, mean total volume 109 ± 15 mL) were obtained from the Swedish National Cord Blood bank, Karolinska University Hospital, Sweden, after informed oral and written consent according to ethical approval from the Regional Ethical Review Board in Stockholm (Dnr 2012/480–31/1) and in accordance with the Declaration of Helsinki.

To produce a gel from PRGF, 45 mL of the UCB was centrifuged at 500*g* for 10 min at room temperature. The UCB separated into red blood cells at the bottom of the tube, PRGF in the middle layer, and plasma poor in growth factors (PPGF) in the top layer. 10 mL of the PPGF layer was discarded, and the remaining PRGF, including 3-4 mm of the RBC fraction to maximize the platelet recovery [34], was transferred to a new tube. To enrich the PRGF with a high concentration of cells, the remaining UCB was diluted four times with PBS and the MNC were isolated by density gradient separation (Lymphoprep, density 1.077 g/mL, Axis-Shield PoC AS, Oslo, Norway). The MNC were counted in Türk's solution (Merck, KGaA, Darmstadt, Germany) and resuspended to 60×10^6 cells/mL in PRGF. 42 μL of 10% $CaCl_2$ in distilled water per mL PRGF was added for gel formation.

2.6. Characterization of PRGF-Residing Cells in Constructs with SIS. To evaluate the durability of the produced gel and to analyze the protein expression of the MNC in the PRGF, pieces of SIS ($0.9\,cm^2$) were placed in 8-chamber slides (Merck Millipore, Cork, Ireland) and 350 μL PRGF was added on top and incubated for 30–45 min in room temperature until gel was formed (Table 1). PRGF (1 mL) without SIS was produced as a control. The gel pieces and the PRGF-SIS constructs were transferred to 12-well plates (Becton-Dickinson) and 350 μL RPMI 1640 medium (Life Technologies) supplemented with 10% pooled inactivated human AB serum, 100 U/mL penicillin, and 100 mg/mL streptomycin (HyClone Laboratories, South Logan, UT, USA), and 20 mM L-glutamine (Invitrogen) was added per well. At days 0, 3, 7, 10, and 14, the PRGF gel and PRGF-SIS constructs were collected, washed in PBS, and transferred to 4% PFA for 3 days and thereafter paraffin embedded and sectioned. The sections were stained as above with HTX/eosin for morphology, CD34 and CD45 (both hematopoietic surface markers, 1 : 100, Dako) for surface epitope expression, Ki67 (1 : 500, Life Technologies), and for activated caspase 3 (1 : 250, Invitrogen).

2.7. Statistical Analysis. Data from the flow cytometry analysis were analyzed using one factor analysis of variance (ANOVA) and paired Student *t*-test, assuming unequal variances between samples. Results were considered statistically significant when p values were <0.05.

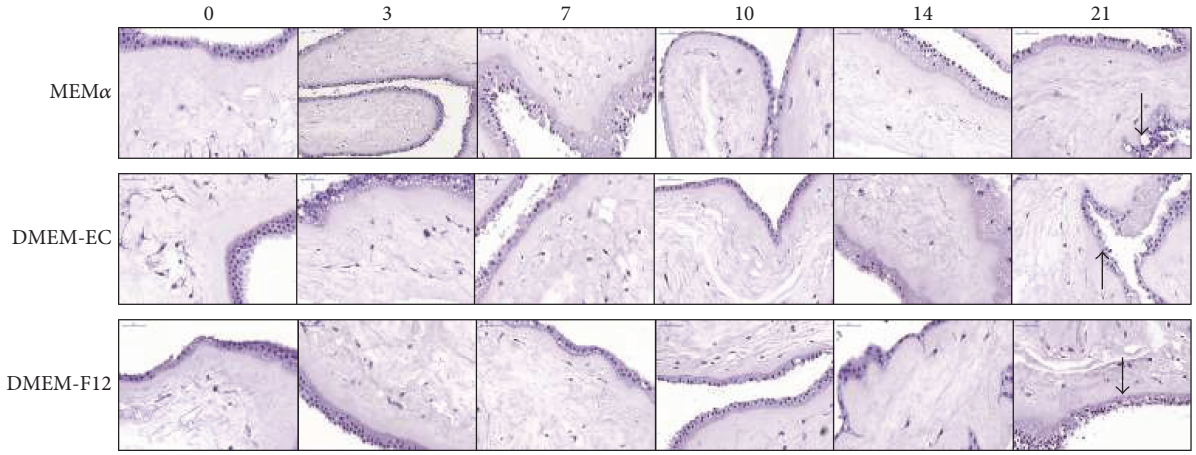

Figure 1: Morphology of amnion-residing cells. Pieces of amnion were kept in culture for up to 21 days in three different culture media and thereafter stained with HTX/eosin. The different culture conditions did not induce morphological changes, but there was a decreased integrity of the epithelial layer integrity over time (indicated by the arrows). Magnification 20x.

3. Results

3.1. Isolation and Culture of the Amnion. The amnion could easily be isolated from the placentas and kept in culture to the endpoint of 21 days. To evaluate different culture media, amnion pieces were cultured in MEMα, DMEM-EC, and DMEM-F12. The amnion pieces were evaluated morphologically with HTX/eosin, which showed that the mesenchymal cells had maintained morphology throughout the time in culture, but there was a decreasing integrity of the epithelial layers at the end of culture in all conditions (Figure 1). There were no differences in proliferation and viability of the amnion-residing cells when testing the different culture media. A uniform low expression of Ki67 was detected at all time points up to day 21 when no expression was detected (Figure 2). Expression of caspase 3 was low, but slightly increased over time (Figure 2). The ADC in MEMα and DMEM-EC showed fibroblast-like morphology whereas the cells in DMEM-F12 had neither typical epithelial-like morphology nor fibroblast-like morphology (Figure 3(a)). The ADC were further investigated by surface epitope expression by flow cytometry, and the majority of cells from the different culture conditions expressed CD73 with a significantly higher expression in DMEM-EC compared to the other cell culture media (DMEM-F12 66.7%, MEMα 87.8%, and DMEM-EC 96.6%, $p = 0.03$ and 0.008, resp.) (Figure 3(b)). There was no expression of CD31 or PCK of the ADC in any of the culture conditions (data not shown). Based on these results, DMEM-EC was selected for the remaining experiments.

3.2. Properties of Amnion-SIS Constructs. To investigate whether the amnion-residing cells would migrate and integrate into SIS and to analyze protein expression, amnion pieces were combined with SIS and kept in culture for 0–21 days and thereafter stained for protein expression. Amnion without SIS served as a control. The HTX/eosin staining showed no morphological differences compared to amnion pieces without SIS, and no cells were detected in the SIS. Cells in both the epithelial and mesenchymal layers expressed CD73, and the epithelial cell layer expressed PCK. There was a low expression of Ki67; less than 3% of the cells at all time points. At day 0, a few cells within the inner mesenchymal layer expressed Ki67, and at later time points, no Ki67-positive mesenchymal cells were detected. At day 0, the epithelial layer contained no Ki67-positive cells, but the Ki67 expression increased with a peak at day 7. At day 21, there were no Ki67-positive cells detected in any samples. Overall, there was a low expression of caspase 3 in both mesenchymal and epithelial cells, but it slightly increased in the epithelial cells over time. There was no CD31 expression at any time points (data not shown). There were no differences in protein expression between amnion-SIS and amnion without SIS (Figure 4 and Supplementary Figure 1).

3.3. Properties of PRGF-SIS Constructs. The PRGF gel with or without SIS could be kept in culture for up to 14 days with sustained morphology, but with decreasing number of cells and with an increasing degradation of the gel. At day 14, the gel was completely dissolved and only SIS remained. Therefore, day 14 was excluded from further analysis. There were no differences in protein expression if the PRGF gel was combined with SIS or not. The cells expressed CD45, and a few CD34-positive cells could be detected at all time points. Cells were positive for Ki67, but with decreasing number over time. The caspase 3 expression was low but increased over time (Figure 5 and Supplementary Figure 2).

4. Discussion

Conventional harvesting of autologous biological starting material for TE involves at least one invasive donor site, leading to at least two sites of surgical interventions. To circumvent the donor site morbidity, a noninvasive technique for collection is desirable. In this study, we investigated two highly proangiogenic types of autologous constructs made from the noninvasive source term placenta: (1) amnion and (2) MNC-enriched PRGF gel from UCB.

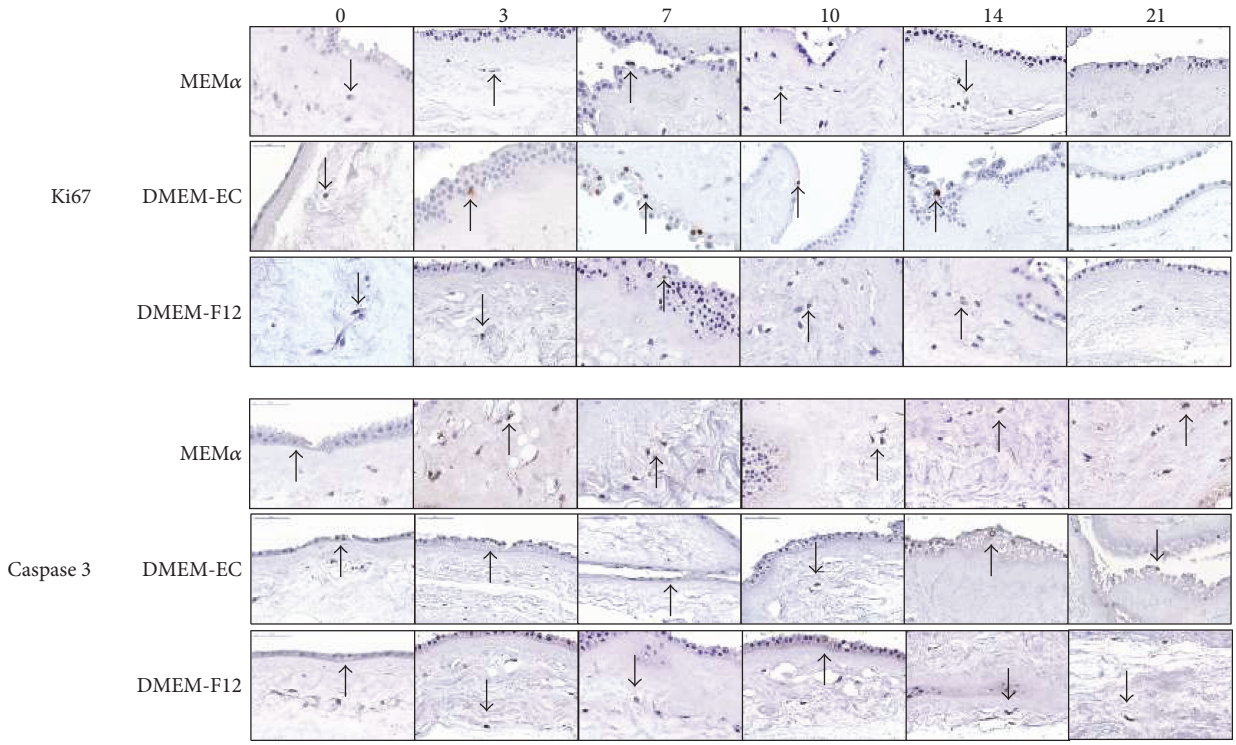

Figure 2: Proliferation and viability of amnion-residing cells *in vitro*. Pieces of amnion were kept in culture for up to 21 days in three different culture media and thereafter stained for Ki67 (proliferation) and caspase 3 (apoptosis) at days 0 to 21. There was no significant difference between the culture conditions. A low expression of Ki67 and caspase 3 was detected up to day 14, and on day 21, Ki67 was not detected and caspase 3 showed a slight increased expression. Single positive cells stain brown as indicated by the arrows. Magnification 60x.

This is, to our knowledge, the first study on fresh amnion and PRGF combined with a biological graft. Our results demonstrate that the placenta and UCB can easily be collected directly after birth and the amnion can be separated from the chorion and is ready for use in less than one hour after birth. The PRGF can be produced within 30 min after retrieving the UCB, isolation of MNC took up to one hour with minimal addition of compliant products (Lymphoprep and $CaCl_2$), and gel formation occurred within 45 min. In total, a complete MNC-enriched PRGF construct can be prepared within 2-3 hours after birth. At this point, the constructs can be used for surgical correction of the defect.

We wanted to investigate if the properties of the amnion were maintained after being kept in culture. Cell culture conditions such as culture medium is per se selective for different subpopulations of cells, and therefore, we compared three different culture media for amnion in regard to morphology (visualized with HTX/eosin) and viability of the cells (Ki67 for proliferation and caspase 3 for apoptosis). The HTX/eosin staining showed no morphological differences in the amnion-residing cells despite different culture media, and there were no significant dissimilarities in cell viability at any time points. When the amnion was in direct contact with the culture surface, cells (ADC) migrated from the amnion and multiplied. When cultured in DMEM-F12, the ADC had a nonfibroblast and nonepithelial morphologies whereas when cultured in MEMα and DMEM-EC, the ADC had a fibroblast-like phenotype. We next investigated the ADC in a flow cytometric assay for surface marker expression. We chose established markers for epithelial (PCK), mesenchymal (CD73), and endothelial (CD31) cells. All ADC expressed CD73, but no PCK or CD31 expression was detected, indicating that mesenchymal and not epithelial or endothelial cells had migrated from the amnion. ADC cultured in DMEM-EC had the highest percentage of CD73-expressing cells, fibroblastic morphology and with no differences in protein expression of the amnion-residing cells between the culture medium, and DMEM-EC was used for the forthcoming analyses.

Amnion and MNC-enriched PRGF gel were combined with a matrix and thereafter analyzed. Conventional commercially available biological grafts usually consist of decellularized ECM proteins, mainly collagens, from various tissue sources such as skin (human, bovine, and porcine) and pericardium (equine and bovine) [35]. One of the most commonly used grafts is SIS (porcine) [36], and since it is currently used in the clinic, is well studied, non-cross-linked, and has a good mechanical strength, we decided to use 4-layer SIS in this study. We wanted to investigate if the properties of the amnion-residing cells would change over time when combined with SIS, and therefore, we kept the constructs in culture for up to 21 days before analysis. We stained the constructs for PCK, CD73, CD31, Ki67, and caspase 3. After 21 days of culture, the amnion remained highly viable with a low apoptotic environment as indicated by few caspase 3-positive cells, and little activation of cell

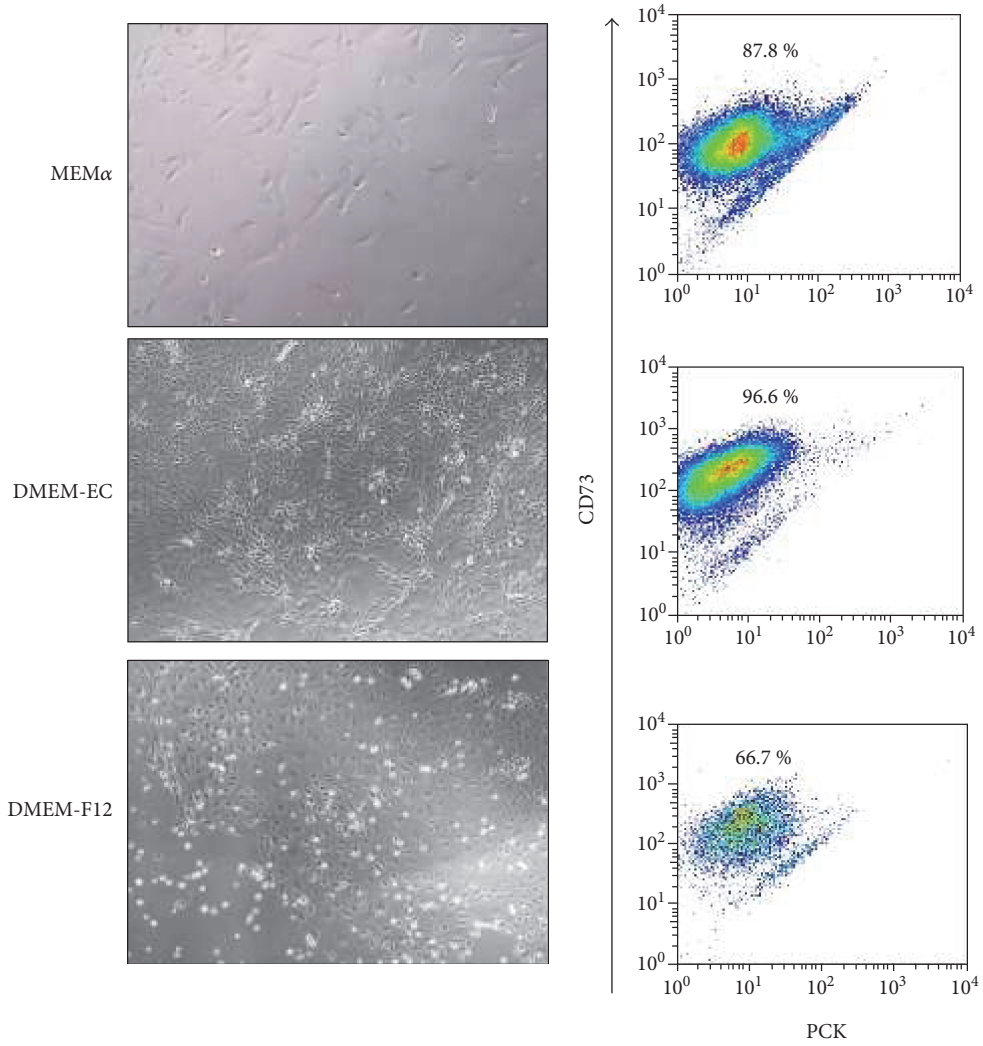

Figure 3: Properties of amnion-derived cells (ADC). ADC migrated from cultured amnion and adhered onto the plastic culture surface. (a) Typical images of the morphology of the ADC at day 14 in the different culture conditions. Magnification 10x. (b) Cell surface expression of CD73 and PCK by ADC was analyzed by flow cytometry. Typical plots are shown with the mean CD73 expression in percent.

proliferation, as indicated by Ki67-positive cells. Also, the cells present in the amnion when combined with SIS did not alter their phenotype, and both the epithelial and stromal cells had a stable protein expression throughout the analysis period. However, the morphological investigations showed a reduction of the epithelial cell layer integrity over time, which means that prolonged storage is not possible or require further optimization.

In the amnion-SIS constructs, the amnion-residing cells did not migrate or integrate into the SIS matrix. One explanation to this could be the manufacturing process of SIS where the tissue is harshly decellularized and sterilized, which may impact on the collagens and thereby change the protein properties and influence the biocompatibility and host integration. The processing is required for the graft to persist rapid degradation by endogenous collagenases [37, 38]. Spelzini et al. could show integration of rat mesenchymal stromal cells into SIS, but they were isolated single cells in suspension and not cells migrating from a tissue [39]. In our construct, the SIS only acts as support and will gradually degrade over time as endogenous extracellular tissue forms and replaces the SIS. By these means, cell migration into the SIS is not of major significance. In summary, these results demonstrate that in cases of delayed correction of the defect, the amnion-SIS constructs could be kept in culture for 21 days, indicating a potential for short-term storage of the construct.

The PRGF-SIS constructs were characterized, which showed that the MNC in the PRGF gel, with or without SIS, maintained a robust protein expression of both CD45 and CD34 over time. No increased proliferation (Ki67) was detected but a small increase in caspase 3 expression was observed. The increased number of apoptotic cells might be explained by a loss of PRGF-derived growth factors and cytokines at medium changes through the degradation process. The gel in the PRGF-SIS constructs slowly degraded and was completely dissolved at day 14. PRGF releases growth

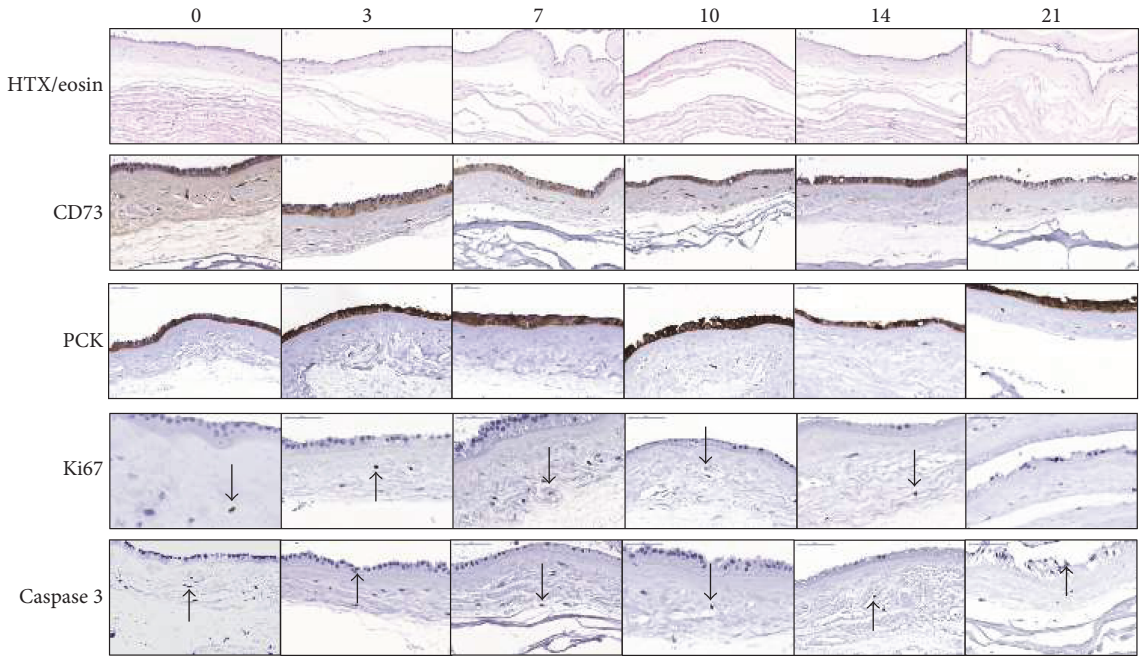

Figure 4: Morphology and protein expression in amnion-SIS constructs. Amnion sutured to SIS was analyzed for protein expression at days 0 to 21. All amnion-residing cells expressed CD73, and epithelial cells expressed PCK at all time points (brown staining). A low expression of Ki67 and caspase 3 was detected up to day 14. On day 21, Ki67 was not detected and caspase 3 showed a slight increased expression. Single positive cells stain brown as indicated by the arrows. Magnification 40x (CD73 and PCK) and 60x (Ki67 and caspase 3).

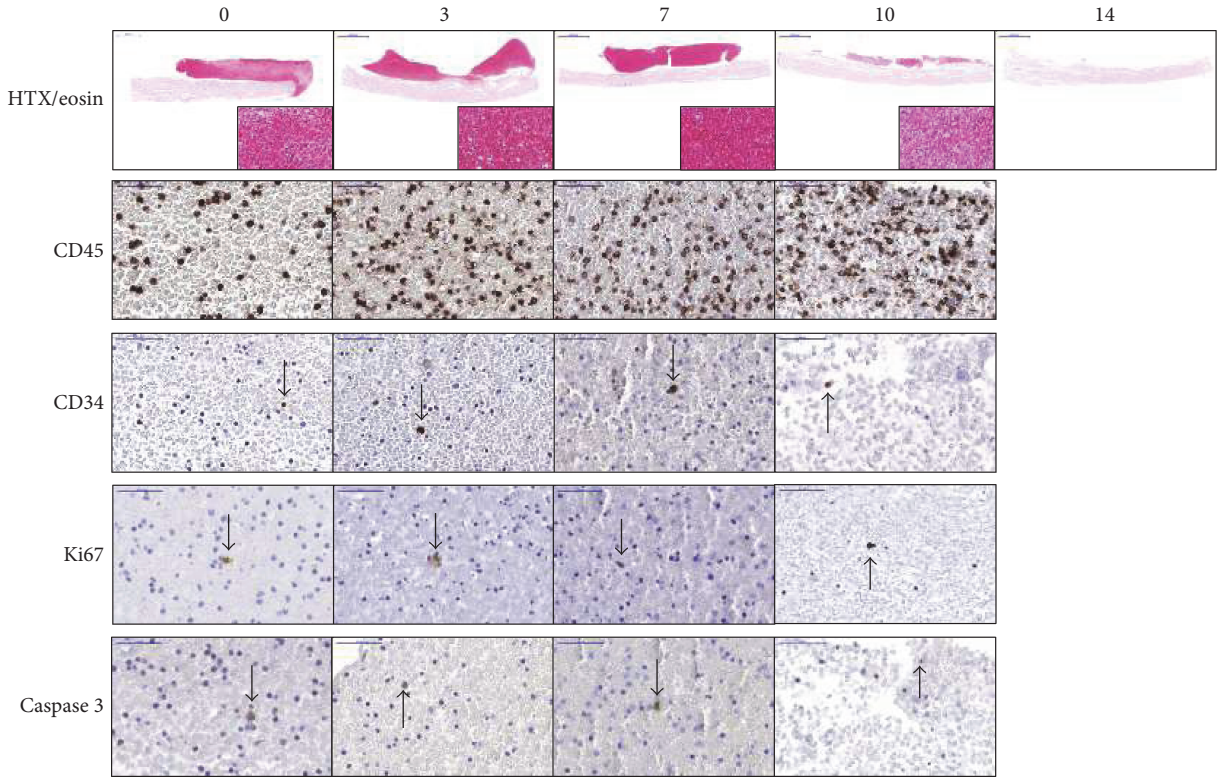

Figure 5: Morphology and protein expression in PRGF-SIS constructs. The PRGF-SIS constructs were kept in culture for up to 14 days and thereafter analyzed. The HTX/eosin staining showed decreasing number of cells and a complete degradation of the gel at day 14. The cells expressed CD45 (brown staining), and few CD34-positive cells were detected throughout the time points. Ki67-positive cells were detected, but with decreasing number over time. The caspase 3 expression was low but increased over time. Single positive cells stain brown as indicated by the arrows. Magnification 2x and 60x.

factors and cytokines; promotes cell recruitment, tissue regeneration, angiogenesis, and reperfusion of damaged tissue; reduces apoptosis [33, 40–42], properties that promote tissue formation, and wound healing; and reduces the risk for bacterial colonialization [43]. For these reasons, degradation of the gel within 14 days may not be a concern in a clinical setting.

When preparing the PRGF gel, which is induced clotting, we allowed a small fraction of RBC to be included, following an established protocol [33]. Even though RBC may be toxic to the cells, other studies have shown that the maximum recovery of platelets and leucocytes is when a small portion of the RBC fraction is included in platelet-enriched blood products [34].

Platelet-enriched derivatives from blood have previously been characterized in regard to platelet concentration and growth factor and cytokine content [43, 44]. In our study, the PRGF was also enriched with MNC, and similarly, the cellular content of UCB is well known [45]. Therefore, we judged it unnecessary to include such characterizations.

5. Conclusions

This study demonstrates that amnion and MNC-enriched PRGF gel, after minimal handling, are promising candidates as noninvasive sources for autologous grafts in correction of congenital soft tissue defects. Further studies are needed to evaluate the full potential of these constructs.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

Acknowledgments

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Supplementary Materials

Supplementary figure 1. Protein expression of amnion-residing cells. Amnion cultured in DMEM-EC was analyzed for protein expression at day 0 to 21. All cells expressed CD73 and the epithelial cells expressed PCK at all time points. There was a low expression of Ki67 and caspase 3 up to day 14, and at day 21 no Ki67 expression was detected but a slightly increased caspase 3 expression. Positive cells stain brown. Magnification 20x (HTX/eosin), 40x (CD73 and PCK) and 60x (Ki67 and caspase 3). Supplementary figure 2. Morphology and protein expression of MNC in PRGF gel. MNC-enriched PRGF gel was kept in culture for up to 14 days and thereafter analyzed. The HTX/eosin staining showed decreasing number of cells and also a degradation of the gel to a total absence at day 14. The cells expressed CD45 and few CD34 positive cells were detected throughout the time points. Cells positive for Ki67 were detected, but with decreasing number over time. The caspase 3 expression was also low but increased over time. Positive cells stain brown. Magnification 60x. (*Supplementary materials*)

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Research Article

Maternal Adaptive Immune Cells in Decidua Parietalis Display a More Activated and Coinhibitory Phenotype Compared to Decidua Basalis

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The maternal part of the placenta, the decidua, consists of maternal immune cells, decidual stromal cells, and extravillous fetal trophoblasts. In a successful pregnancy, these cell compartments interact to provide an intricate balance between fetal tolerance and antimicrobial defense. These processes are still poorly characterized in the two anatomically different decidual tissues, basalis and parietalis. We examined immune cells from decidua basalis and parietalis from term placentas ($n = 15$) with flow cytometry. By using multivariate discriminant analysis, we found a clear separation between the two decidual compartments based on the 81 investigated parameters. Decidua parietalis lymphocytes displayed a more activated phenotype with a higher expression of coinhibitory markers than those isolated from basalis and contained higher frequencies of T regulatory cells. Decidua basalis contained higher proportions of monocytes, B cells, and mucosal-associated invariant T (MAIT) cells. The basalis B cells were more immature, and parietalis MAIT cells showed a more activated phenotype. Conventional T cells, NK cells, and MAIT cells from both compartments potently responded with the production of interferon-γ and/or cytotoxic molecules in response to stimulation. To conclude, leukocytes in decidua basalis and parietalis displayed remarkable phenotypic disparities, indicating that the corresponding stromal microenvironments provide different immunoregulatory signals.

1. Introduction

During pregnancy, the fetus receives nutrients, gas exchange, and immunological protection against infections from the mother via the placenta. At the same time, the maternal immune system must be kept from attacking the allogeneic fetus. The fetus, umbilical cord (UC), and placenta are encased by the decidua, a maternal membrane originating from differentiated endometrial cells in early pregnancy [1]. The decidua can be divided into two anatomically different parts; the decidua basalis covers the basal plate of the placenta, while the decidua parietalis lines the fetal membranes. Decidual stromal cells (DSCs) make up the foundation of the connective structures of both decidua basalis and parietalis and have been shown to utilize a specific epigenetic program of gene silencing in order to minimize the attraction of maternal effector T cells in mice [2]. *In vitro*, DSCs have been shown to induce T regulatory cells as well as suppress T cell

proliferation by utilizing IDO and PGE_2 and mediate an induced impaired ability of T cells to respond to IL-2 [3–5]. Trophoblast cells make up the outer barrier of the fetal side of the placenta, and over the course of pregnancy, trophoblasts invade the decidual layer surrounding the placenta. These extravillous trophoblasts lack classical polymorphic HLA class I molecules but express HLA-C, HLA-G, and HLA-E [6]. This provides some aspects of how the fetus escapes immunological recognition and response.

Previous studies on different subsets of maternal immune cells infiltrating decidual tissues rarely discern between decidua parietalis and basalis. In early pregnancy, the majority of decidual leukocytes are noncytotoxic NK cells, which have been shown to be crucial in the forming and remodeling of the blood supply to placental tissues. Decidual macrophages have an important role in maintaining a tolerogenic environment in healthy pregnancies, but can also respond with proinflammatory cytokines upon bacterial stimulation [7]. The decidual macrophage population also contains a subclass with an M2 phenotype, which produces high levels of IL-10 [8]. The number of T cells increases over the course of pregnancy and constitutes between 30 and 80% of the total lymphocytes in the decidua at term [9]. The number of T regulatory cells is elevated in both decidual compartments and has the ability to suppress immune responses against the fetus [10]. The expression of PD-1 and TIM-3 is greatly increased on decidual T cells, and *in vivo* blockade of these molecules in mice results in increased miscarriage rates [11].

As placental tissues have emerged as a promising source of stem cells for clinical trials [12], it is of importance to characterize the physiological state of the surrounding immune cell populations in this compartment. The factors influencing immune cell composition and activation status in the decidua basalis and parietalis are still poorly characterized, but it is likely that these two sites are differentially influenced by the stromal microenvironment.

The aim of this study was to examine the immune cell composition of these two decidual tissues. Using flow cytometry, we have made an in-depth characterization of lymphocyte populations in the different decidual compartments from term placentas donated after uncomplicated pregnancies. This provides new basic knowledge of the immunological landscape in these tissues, as well as potential insights into how the stromal environment in different decidual sites can mediate immune regulation.

2. Material and Methods

2.1. Placental Donors. Following uncomplicated term pregnancies (median gestation week 39, range 38–42), healthy individuals ($n = 15$, median age 32, range 21–40) donated their placentas following elective caesarian sections. Written informed consent was obtained from the donors, and the regional review board of ethics in research of Karolinska Institutet approved the donation of peripheral blood and placentas (entry numbers 2009/418-31/4, 2010/2061-32, and 2015/1848-31/2). Data on some immune parameters in decidua parietalis from 11 out of 15 donors have partly been included in another publication [13], but no data on the decidua basalis immune cells has previously been published.

2.2. Cell Isolation. Placentas were transported straight to our laboratory from the operating room in the adjacent building, and the cell isolation started in less than 30 minutes following the placental delivery. Paired samples of tissue-resident lymphocytes were collected from decidua basalis and parietalis using a method similar to that used by others [14]. The fetal membranes (including the decidua parietalis) were cut ≥1 cm from the edge of the placenta and placed in a sterile petri dish and washed extensively with PBS. The parietalis was dissected from the chorion, which was then discarded together with the amnion. The tissue was cut into smaller pieces and placed in PBS. Thereafter, the placenta was placed with the umbilical cord facing down and washed extensively with PBS. The basalis is strongly attached to the placental tissue, and hence we used a scalpel to carefully scrape off the thin grey basalis membrane which was placed in PBS. The two types of tissue were washed in PBS by centrifugations at $600g$ for 1 minute. The supernatant was discarded, and the process was repeated five times or more until the supernatant was clear. Lymphocytes were released from the tissue by nonenzymatic mechanical disaggregation using the gentleMACS Dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany). The tissue was then consecutively filtered through a 100 μm metal mesh and then through 70 μm and 40 μm cell strainers (VWR, Radnor, PA). Following centrifugation, the cell pellets were resuspended in lysis buffer (IOTest 3, Beckman Coulter, Fullerton, CA, USA), washed once, and stained for extracellular flow cytometric analysis. The remaining cells were frozen in liquid nitrogen in RPMI (HyClone, GE Health Sciences, South Logan, UT) medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (complete medium) containing 10% DMSO.

2.3. Flow Cytometry. The majority of the extracellular flow cytometry experiments were performed directly on fresh cells, but for six donors, the T cell markers CD69, CD25, CXCR3, CCR6, CD38, HLA-DR, CD127, and PD-1 were analyzed on paired frozen samples. Staining was carried out in 96-well plates with $\leq 1 \times 10^6$ cells/well in 50 μl CliniMACS PBS/EDTA buffer (Miltenyi Biotech, Bergish Gladbach, Germany) supplemented with 0.1% bovine serum albumin. The cells were incubated with mAbs for 30 min at 4°C. Intracellular staining was performed after extracellular staining using the BD Cytofix/Cytoperm™ kit (BD Biosciences, Franklin Lakes, NJ) according to the manufacturer's instructions. 7AAD staining was used to sort live and dead cells if no intracellular staining was performed. The antibodies used in this study are listed in Supplementary Table 1 available online at https://doi.org/10.1155/2017/8010961. Data was collected using a BD FACSCanto flow cytometer and analyzed with FlowJo software (Tree Star, Ashland, OR, USA). Results from subgating were only included if the parent population consisted of ≥80 cells.

2.4. Bacterial Stimulation Assay. Mononuclear cells were isolated by density gradient centrifugation (Lymphoprep,

Axix-Shield, Dundee, Scotland) and resuspended in complete medium. Fresh cells were then cultured at 37°C and 5% CO_2, at a concentration of 3×10^6 cells/ml, in 96-well plates alone or together with UV-irradiated *Escherichia coli* (*E. coli*) at a multiplicity of infection of 30 together with 1.25 μg/ml anti-CD28mAb (CD28.2, BioLegend, San Diego, CA). After 12 hours of culture, 10 μg/ml Brefeldin-A (BFA, Sigma-Aldrich, St. Louis, MO) was added, followed by an additional 4 hours of culture. After the total 16 hours of culture, cells were harvested and stained for flow cytometry.

2.5. PMA/Ionomycin Stimulation Assay. Frozen cells were thawed and mononuclear cells were isolated by density gradient centrifugation (Lymphoprep, Axix-Shield). Peripheral blood mononuclear cells (PBMCs) from healthy blood donors (healthy controls) were used as controls. Cells were resuspended in complete medium and cultured at 37°C and 5% CO_2, at a concentration of 2×10^6 cells/ml in 96-well plates. 10 μg/ml BFA (Sigma-Aldrich) was added to all wells, and half of the samples were stimulated with 25 ng/ml PMA (Sigma-Aldrich) and 1 μg/ml ionomycin (Sigma-Aldrich). After 5 hours of culture, cells were harvested and stained for flow cytometry.

2.6. Statistics. Multivariate orthogonal projection to latent structures by means of partial least squares discriminant analysis (OPLS-DA) was used to obtain a maximum separation of *X*-variables, that is, immune cell variables, based on class information, that is, basalis and parietalis in Figures 1(b), 2(a), and 3(b) (SIMCA software, Sartorius Stedim Biotech, Umeå, Sweden). The contribution of each *X*-variable, VIP values, to the OPLS-DA model in Figure 1(a) was calculated (Supplementary Figure S1). *X*-variables with a VIP value below 0.98 were excluded, and a new model was generated based on remaining variables (Figure 1(b)). The scale presented on the *y*-axis of the OPLS plot is a dimensionless scale; the loading vector is normalized to unit length. The quality of OPLS analyses is based on R2, which indicates how well the variation of the variables is explained by the model, and Q2, an estimate of the model's predictive ability. Utilizing the OPLS-DA analysis as screening for differences between the groups, the factors contributing most to the separation were further analyzed using a two-tailed Wilcoxon matched-pairs signed rank test (GraphPad Software, La Jolla, CA). An alpha value of <0.05 was considered significant.

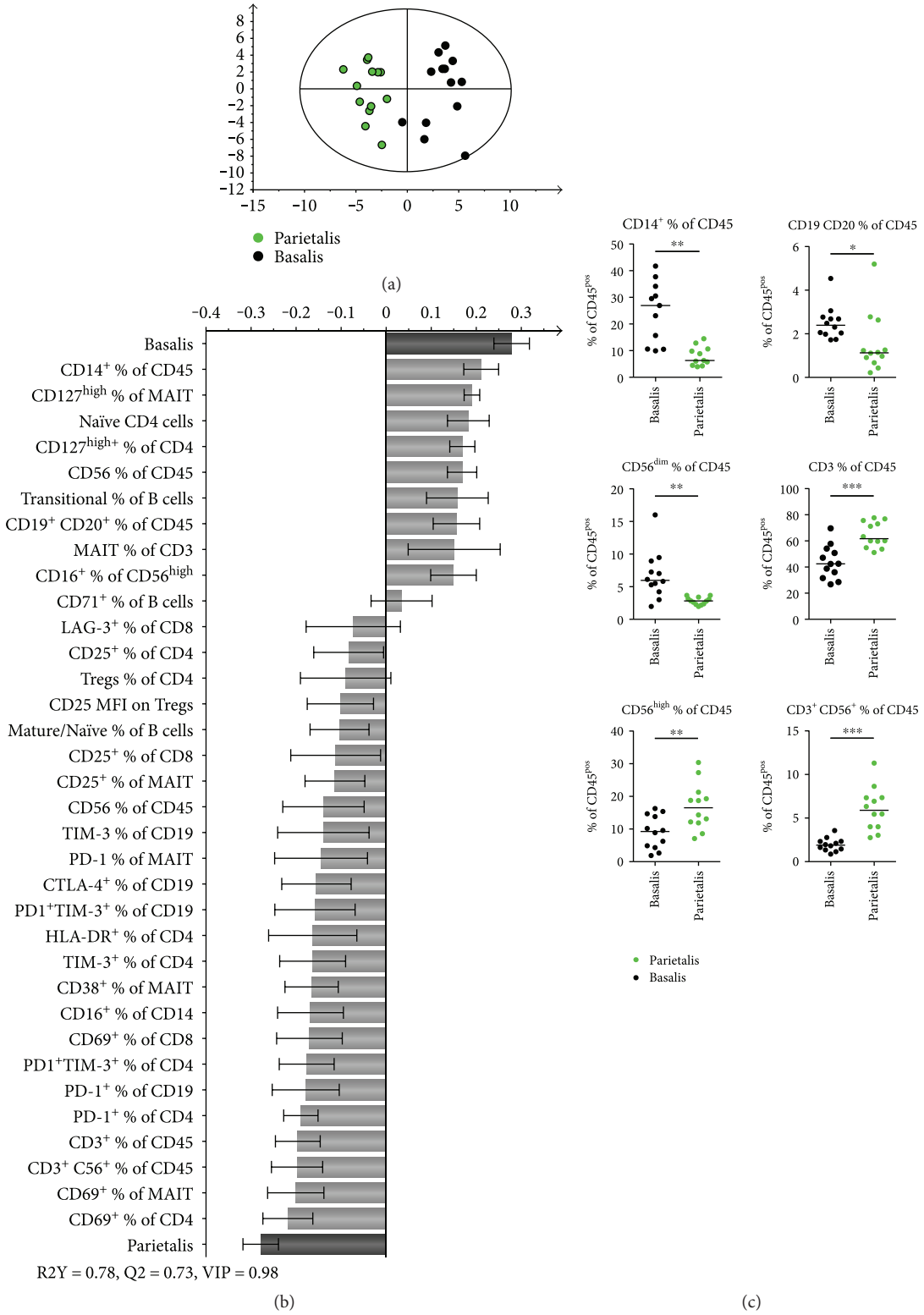
3. Results

3.1. The Proportions of Several Immune Cell Populations Differ in Decidua Basalis and Parietalis. By multivariate OPLS-DA, we examined if the 81 immunological immune cell variables assessed differed between decidua basalis and parietalis. As depicted in the observational plot in Figure 1(a), a clear separation between decidua basalis and parietalis was found. The *X*-variables that showed the strongest association with basalis or parietalis are shown in the OPLS-DA loading plot in Figure 1(b), a model based on *X*-variables with VIP values ≥ 0.98. A VIP column plot for all parameters is shown in Supplementary Figure S1. Further investigating the two groups using univariate statistical analysis, differences were seen in all main immune cell populations investigated. The proportions of monocytes, B cells, and $CD56^{dim}$ NK cells were higher in basalis compared to parietalis, whereas total $CD3^+$ T cells, $CD56^{bright}$ NK cells, and NKT-like cells were found in higher frequencies in parietalis relative to basalis (Figure 1(c), gating strategies in Figure 1(d)).

3.2. Decidua Parietalis T Cells Display a More Activated Phenotype Compared to Decidua Basalis. As demonstrated by the OPLS-DA in Figure 1(b), phenotypic differences in T cells, B cells, and NK cells were revealed between decidua basalis and parietalis. Based on CCR7 and CD45RA expression, we found that $CD4^+$ and $CD8^+$ T cells from both compartments were dominated by an effector memory T cell phenotype at the expense of naïve cells (Figure 4(a)). This contrasts to the general composition in peripheral T cells in pregnant and nonpregnant women [13, 15]. Decidua parietalis contained a higher proportion of both effector memory and central memory $CD4^+$ T cells compared to decidua basalis (Figure 4(a)). Gating strategies are shown in Figure 4(b). Markers of T cell activation and homing also had different expression pattern between the two compartments (Figures 4(c), 4(d), 4(e), 4(f), 4(g), 4(h), and 4(i)). T cells from parietalis had a higher expression of the early activation and/or tissue residency marker CD69 (Figure 4(d)), as well as an increased expression of the α-chain of the IL-2 receptor, CD25 (Figure 4(e)). No significant difference was observed for the expression of the late activation marker HLA-DR, although there was a trend towards an increased expression on T cells from parietalis (Figure 4(f)). The expression of the α-chain of the IL-7 receptor, CD127, was significantly decreased in parietalis, further indicating a more activated phenotype compared to the T cells from basalis (Figure 4(g)). The expression of the chemokine receptors CXCR3 and CCR6 was both increased in parietalis compared to basalis (Figures 4(h) and 4(i)).

Upon stimulation with PMA/ionomycin, both $CD4^+$ and $CD8^+$ T cells responded with the production of interferon-γ (IFN-γ) in similar levels to that of PBMCs from healthy donors (Figure 4(j)). Decidual $CD8^+$ T cells expressed granzyme B (GrzB) in a resting state, but the median expression was lower compared to peripheral T cells from healthy donors (Figure 4(j)). As expected, the expression of GrzB was low in $CD4^+$ T cells.

3.3. Differences in B Cell and NK Cell Subsets and T Regulatory Cells in Decidua Parietalis and Basalis. B cells can be divided into immature/transitional, mature/naïve, and memory cells based on CD24 and CD38 expression (Figure 5(a)). The frequency of transitional B cells ($CD24^{high}CD38^{high}$) among $CD19^+CD20^+$ B cells was higher in basalis compared to parietalis, whereas the mature/naïve compartment ($CD24^{int}CD38^{int}$) was higher in parietalis (Figure 5(a)). There was no significant difference in the B cell memory compartment ($CD24^{high}CD38^{low/neg}$).



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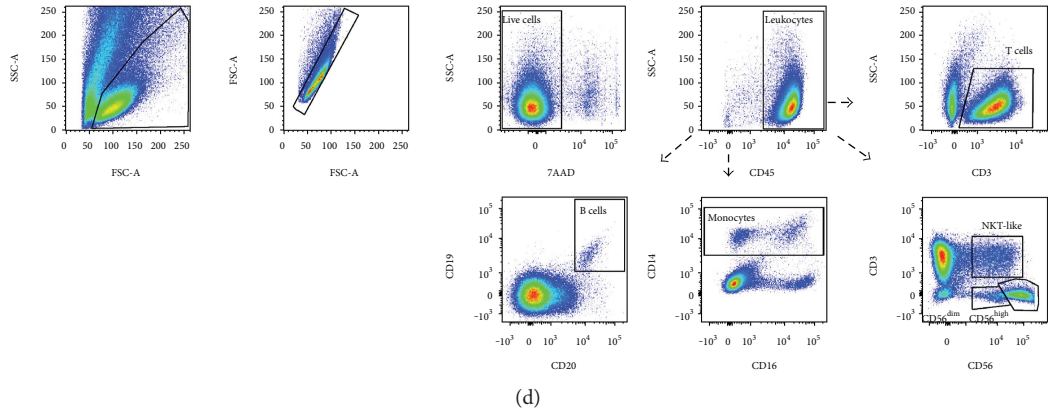


(d)

Figure 1: Different leukocyte populations in decidua parietalis and basalis. (a) OPLS-DA observation plot displaying a separation between leukocytes from decidua basalis and parietalis. (b) OPLS plot following a variable influence on projection (VIP) of 0.98, showing associations between decidual compartment and phenotypic leukocyte markers ($n = 8$–13 for (a, b)). (c) Distribution of major leukocyte subsets in paired samples of decidua basalis and parietalis compared with the nonparametric Wilcoxon test. Line in graphs depicts the median among values ($n = 11$-12). (d) Representative flow cytometry plots showing the initial gating strategy used throughout the paper, as well as the gating of the subsets in (c). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

It has previously been shown that $CD4^+CD25^+CD127^{low}$ T cells in decidual tissues largely overlap with $Foxp3^+$ T regulatory cells (Tregs) and that they are increased in the first trimester compared to peripheral blood [16]. We found that parietalis contained a significantly higher proportion of $CD4^+CD25^+CD127^{low}$ Tregs among the $CD4^+$ T cells compared to basalis and that the median intensity of CD25 expression in Tregs was higher in parietalis (Figure 5(b)).

The expression of the Fc receptor CD16 was investigated on the two NK cell populations residing in decidual tissue. As expected, the classical $CD3^-CD56^{dim}$ NK cells had a high CD16 expression (Figure 5(c)), whereas the expression on the decidual $CD3^-CD56^{bright}$ NK cells was low (Figure 5(d)). The CD16 expression was significantly lower on both NK cell populations in parietalis compared to basalis (Figures 5(c) and 5(d)). On the other hand, there was a trend towards a lower expression of CD16 on monocytes in parietalis compared to basalis (Supplementary Figure S2a).

After stimulation with PMA/ionomycin, we observed that IFN-γ was expressed in the $CD56^{dim}$, as well as the $CD56^{bright}$ NK cells from both basalis and parietalis (Figure 5(e)). The proportion of NK cells from decidua expressing IFN-γ was as least as high as peripheral NK cells from healthy donors. We further noted that $CD56^{dim}$ NK cells showed a pattern of background expression of IFN-γ, which was not observed in $CD56^{bright}$ cells. Although too few experiments were performed to do statistical analysis, the proportion of $CD56^{dim}$ NK cells expressing GrzB appeared to be lower in decidua compared to peripheral blood (Figure 5(e)). However, there was no such difference in GrzB expression in $CD56^{bright}$ NK cells.

3.4. Decidua Parietalis Immune Cells Express Higher Levels of Coinhibitory Markers Compared to Basalis. The surface expression of the coinhibitory molecules PD-1, TIM-3, LAG-3, and CTLA-4 was assayed on $CD4^+$ and $CD8^+$ T cells, $CD19^+$ B cells, and $CD56^+$ NK cells. Based on these 20 parameters from the flow cytometry results, the OPLS-DA model demonstrated that there were higher proportions of cells expressing coinhibitory molecules, mainly B cells and $CD4^+$ T cells, in parietalis compared to basalis (Figure 2(a)). Further analysis with univariate statistics in large confirmed this model. Expression of PD-1, LAG-3, and TIM-3 and coexpression of both PD-1 and TIM-3 were higher in parietalis compared to basalis $CD4^+$ T cells (Figure 2(b)). The same results were seen regarding LAG-3 expression in $CD8^+$ T cells, but only trends were apparent for the other markers (Figure 2(b)). The expression of CTLA-4 on T cells was not statistically different between basalis and parietalis (Supplementary Figure S2b). An increased frequency of B cells from parietalis expressed PD-1, TIM-3, and CTLA-4, as well as the dual expression of PD-1 and TIM-3 (Figures 2(c) and 2(e)). Regarding the NK cells, the expression of LAG-3 was higher in samples from parietalis compared to basalis (Figures 2(d) and 2(e)). Although not significantly different, we also noted a trend towards a higher median expression of TIM-3 on NK cells in parietalis (67%) compared to basalis (51%, $p = 0.0537$, Supplementary Figure S2c).

3.5. Mucosal-Associated Invariant T (MAIT) Cell Proportion and Phenotype Differs in Decidual Basalis and Parietalis. MAIT cells can rapidly respond with secretion of proinflammatory cytokines and cytotoxic molecules when stimulated by microbial-derived vitamin B metabolites in an MHC-related molecule 1- (MR1-) dependent manner [17], but little is known about their importance in pregnancy. MAIT cells were identified as $V\alpha7.2^+$ and $CD161^{high}$ T cells (Figure 3(a)). OPLS-DA demonstrated a separation between decidua basalis and parietalis based on MAIT cell proportion and phenotype (Figure 3(b)). The frequency of MAIT cells among $CD3^+$ T cells was higher in basalis compared to parietalis (Figure 3(c)). The distribution of $CD8^+$, double negative (DN), and $CD4^+$ MAIT cells was similar as previously reported in peripheral blood [17], but the proportion of $CD4^+$ MAIT cells was higher in parietalis compared to basalis at the expense of the $CD8^+$ MAIT cells (Figure 3(d)).

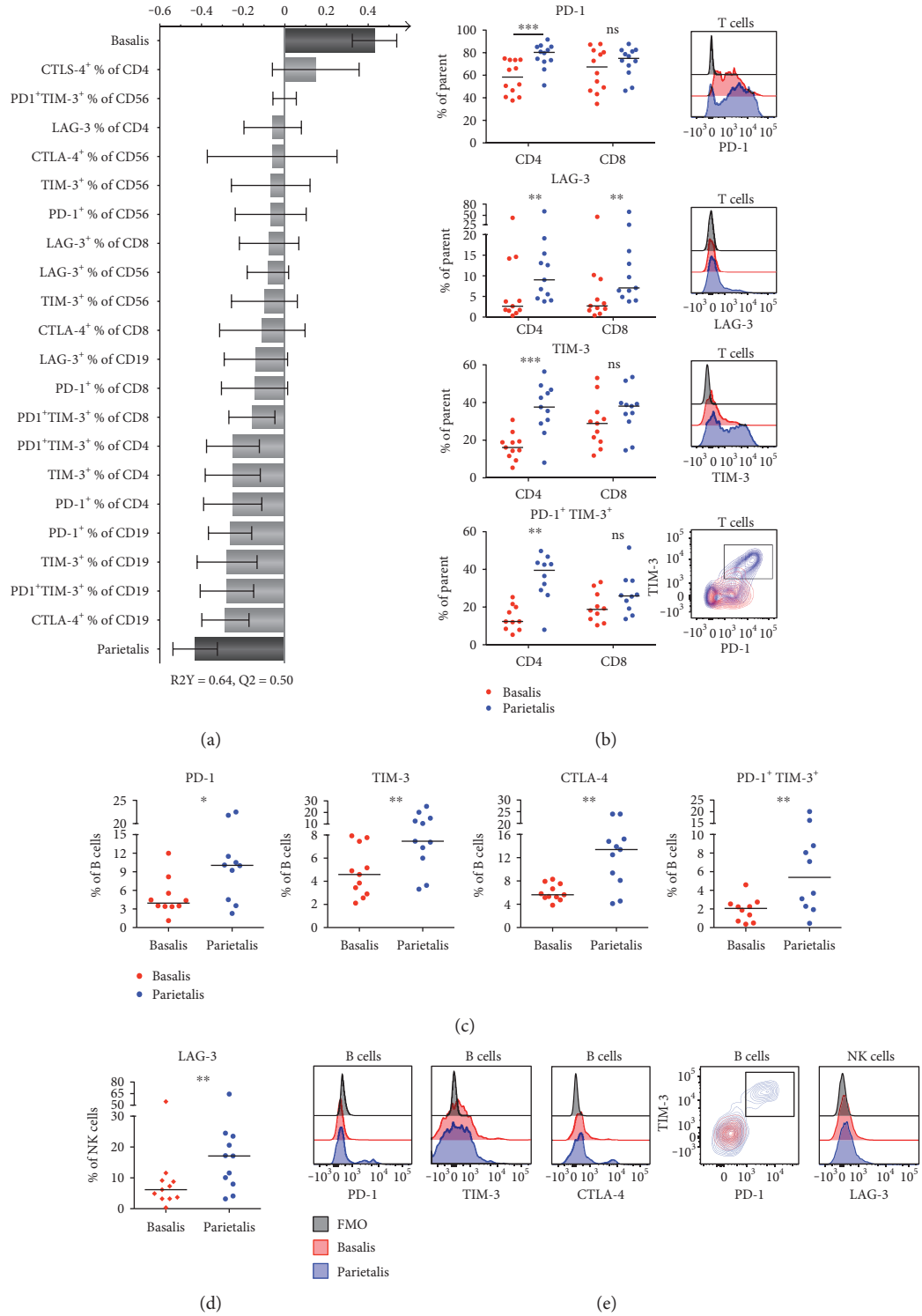
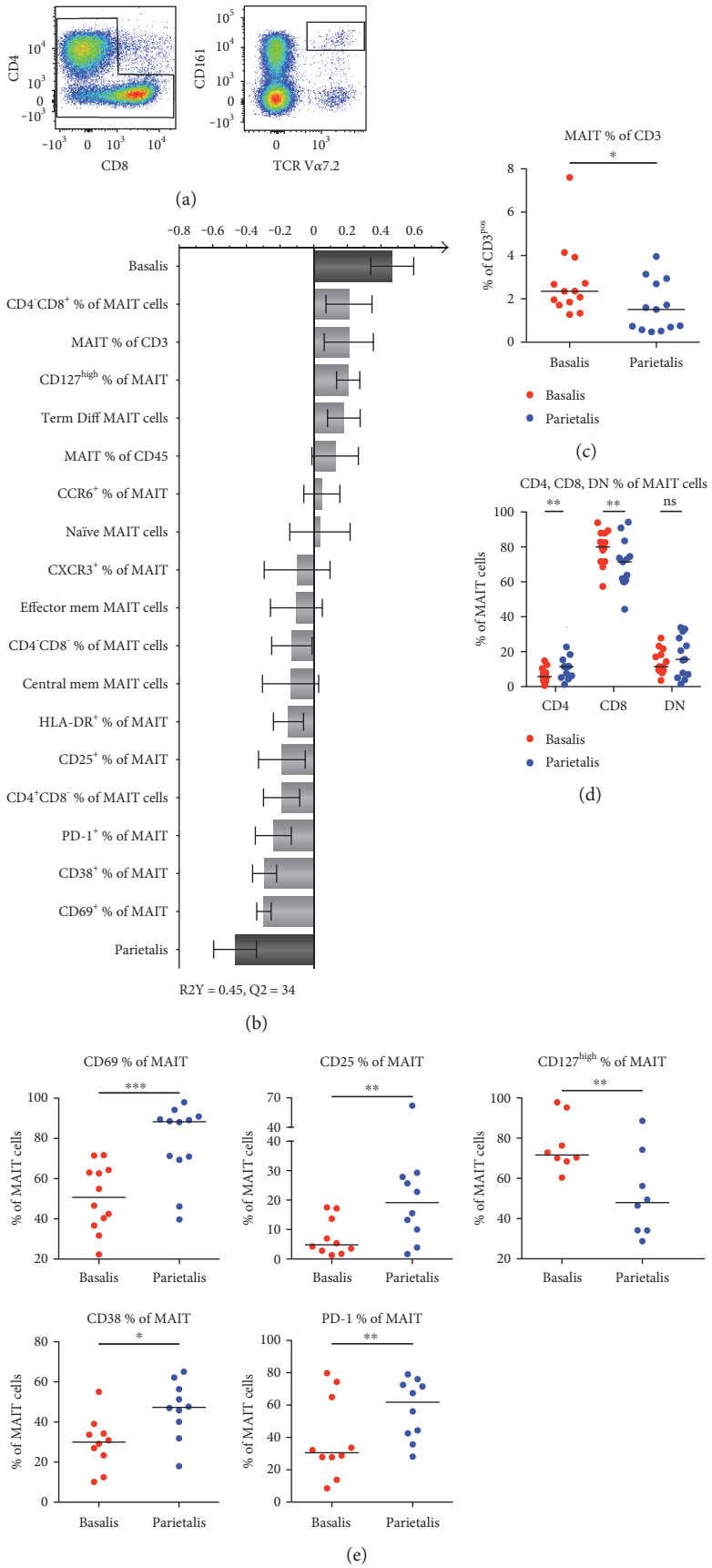


Figure 2: Lymphocytes in decidua parietalis express more coinhibitory markers compared to basalis. (a) OPLS plot showing associations between decidual compartment and phenotypic coinhibitory markers ($n = 10$–12). (b) Surface expression and representative histograms and contour plots showing the expression of the indicated extracellular markers on decidua basalis and parietalis T cells ($CD3^+$) compared to the fluorescent minus one (FMO) control of PD-1 ($n = 12$), LAG-3 ($n = 11$), TIM-3 ($n = 11$), and $PD\text{-}1^+TIM\text{-}3^+$ ($n = 10$). (c) B cell ($CD19^+$) surface expression of PD-1 ($n = 10$), TIM-3 ($n = 11$), CTLA-4 ($n = 11$), and $PD\text{-}1^+TIM\text{-}3^+$ ($n = 10$). (d) NK cell ($CD56^+CD3^-$) surface expression of LAG-3 ($n = 11$). (e) Representative histograms and contour plots showing the expression of the indicated extracellular markers on decidua basalis and parietalis cells from (c) to (d) compared to the fluorescent minus one (FMO) control. Line in graphs depicts the median. Comparisons between the paired samples were made using the nonparametric Wilcoxon test. ns = not significant; $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.



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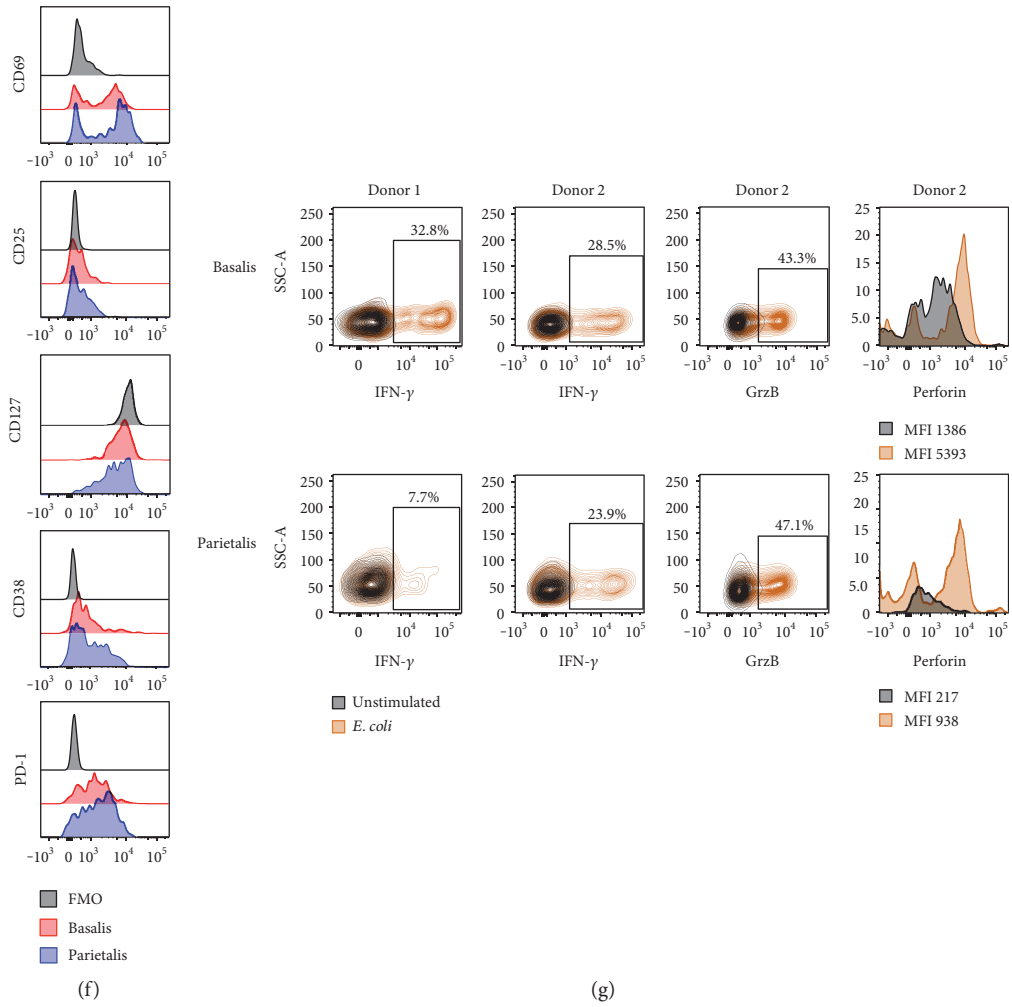


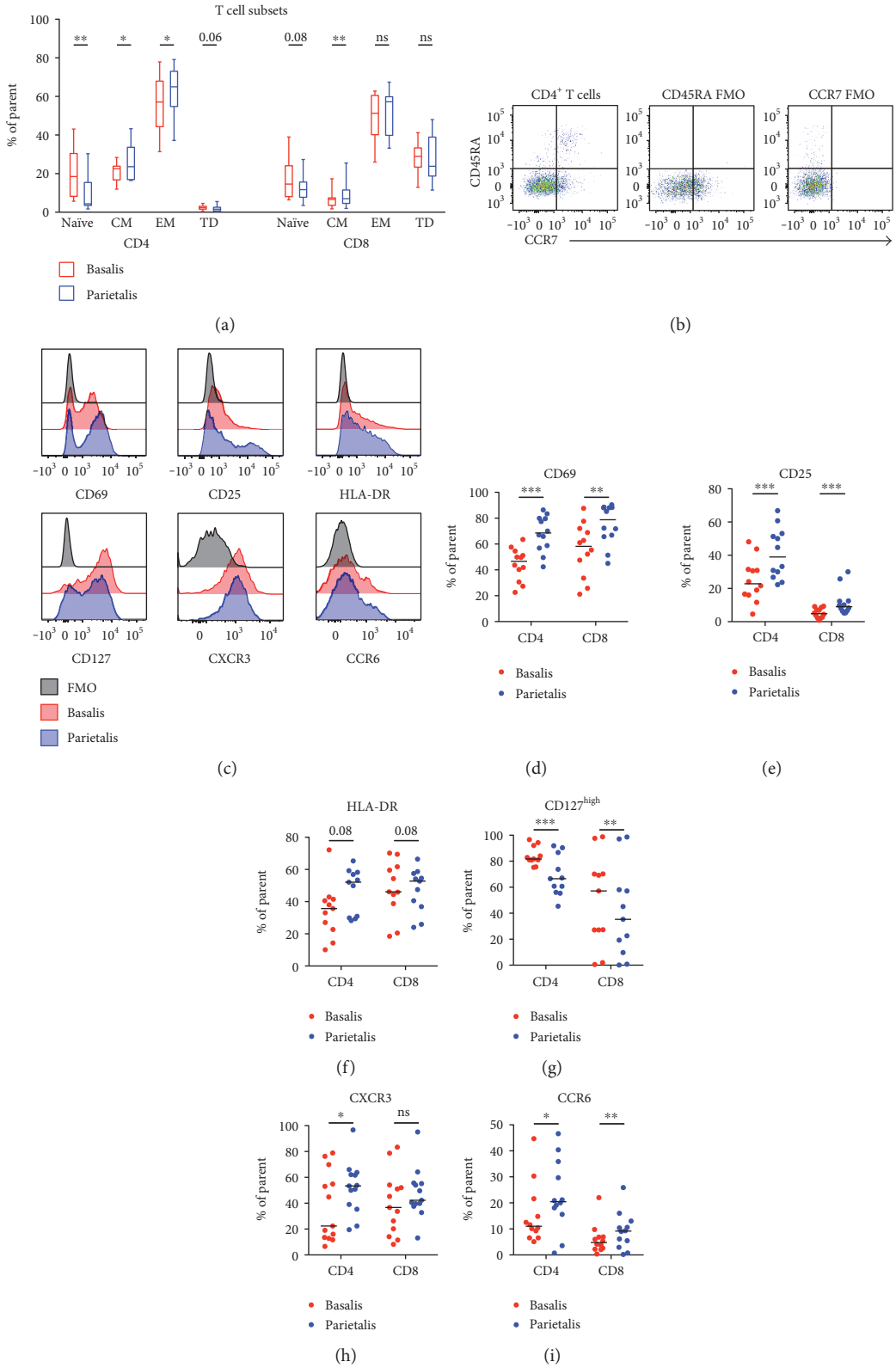
Figure 3: MAIT cells in decidua basalis are more numerous, but parietalis MAIT cells display a more activated phenotype. (a) Representative flow cytometry plots showing the gating strategy for MAIT cells based on $CD3^+$ T cells (left to right). (b) OPLS plot showing associations between decidual compartment and phenotypic MAIT cell markers ($n = 8$–13). (c) Number of MAIT cells expressed as percentage of $CD3^+$ T cells in paired samples of decidua basalis and parietalis ($n = 13$). (d) MAIT cell surface expression of CD4 and CD8 in paired samples of decidua basalis and parietalis ($n = 13$). (e) MAIT cell surface expression of CD69 ($n = 12$), CD25 ($n = 10$), $CD127^{high}$ ($n = 8$), CD38 ($n = 10$), and PD-1 ($n = 10$). (f) Representative histograms showing the expression of the indicated extracellular markers on MAIT cells from decidua basalis and parietalis compared to the fluorescent minus one (FMO) control. (g) Contour plots and histograms showing the expression of the indicated intracellular molecules on decidua basalis (top) and parietalis (bottom) in unstimulated (black) compared to the *E. coli* stimulated conditions (orange) for interferon-γ (IFN-γ, $n = 2$), granzyme B (GrzB, $n = 1$), and perforin ($n = 1$). Line in graphs depicts the median. Comparisons between the paired samples were made using the nonparametric Wilcoxon test. ns = not significant; $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

Similar to what we observed for the conventional T cells, the MAIT cells from parietalis had a higher expression of CD69 and CD25 than basalis and a lower expression of CD127 (Figures 3(e) and 3(f)). CD38, another marker for MAIT cell activation [18] and the coinhibitory marker PD-1, was also increased on parietalis MAIT cells as compared to basalis (Figures 3(e) and 3(f)). Thus, decidua parietalis MAIT cells showed a more activated phenotype compared to basalis MAIT cells.

To examine the functionality of MAIT cells in decidual tissue, mononuclear cells were stimulated with UV-irradiated *E. coli* for 16 hours. The response to the bacteria was measured by intracellular expression of IFN-γ (two donors) and GrzB and perforin (one donor). A substantial proportion of the decidual MAIT cells from both donors responded with IFN-γ expression (Figure 3(g)). Bacterial stimulation also induced expression of the cytotoxic molecules GrzB and perforin in decidual MAIT cells (Figure 3(g)).

4. Discussion

This study provides the most comprehensive mapping of immune cell subsets in decidua parietalis and basalis to date. By analyzing such a large number of factors using paired samples, we can present a multifaceted overview of how the immune system is polarized in the different decidual compartments at term pregnancies. Using OPLS-DA to create a model on which further univariate testing is based provides



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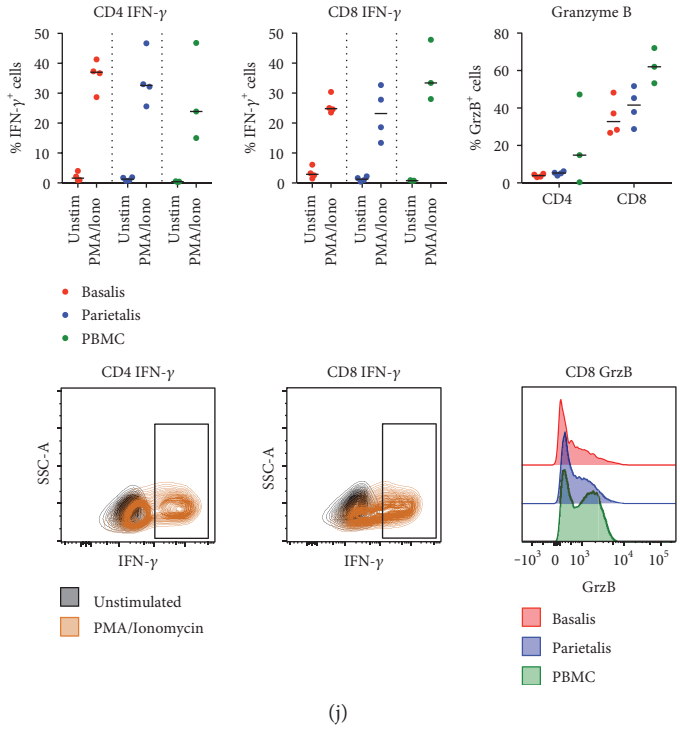

Figure 4: Decidua parietalis contains more activated T cells than basalis. (a) $CD4^+$ and $CD8^+$ T cells were gated according to the expression of CD45RA and CCR7 (gating strategy in (b)) and divided into naïve, central memory (CM), effector memory (EM), or terminally differentiated (TD) subsets ($n = 13$). (c) Representative histograms showing the expression of the indicated extracellular markers on decidua basalis and parietalis compared to the fluorescent minus one (FMO) control. Comparisons between decidua basalis and parietalis $CD4^+$ and $CD8^+$ T cells regarding the expression of CD69 (d), CD25 (e), HLA-DR (f), $CD127^{high}$ (g), CXCR3 (h), and CCR6 (i) ((d, e) and (i) $n = 12$, (f, g) $n = 11$, (h) $n = 13$). (j) Paired mononuclear cells from basalis ($n = 4$) and parietalis ($n = 4$) were stimulated for 5 hours with PMA/ionomycin or left unstimulated. Data on cells from healthy controls (PBMC) were included as controls ($n = 3$). Intracellular expression of interferon-γ (IFN-γ) and granzyme B (GrzB) was determined by flow cytometry. T cells were divided into $CD4^+$ (upper left) and $CD8^+$ (upper middle), representative contour plots below the respective cell subset. GrzB was measured in unstimulated samples only (upper right), representative histograms shown in the bottom right corner. Line in graphs depicts the median among values. Comparisons between the paired samples were made using the nonparametric Wilcoxon test. ns = not significant; $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

a more objective basis of statistical analysis as all parameters are used and weighed depending on their relative addition to the model. The discriminate analysis revealed large differences in the immune cell composition between decidua parietalis and basalis.

Among the general leukocyte populations, decidua basalis contained more B cells, monocytes, and $CD56^{dim}$ NK cells, whereas parietalis was comprised of more T cells, NKT-like cells, and $CD56^{bright}$ NK cells. This pattern is in general in line with previous publications examining decidual immune cell subsets [19–22].

Decidual NK cells constitute >70% of decidual leukocytes during the first trimester and are important for directing the invasion of trophoblasts into the decidua by cytokine and chemokine gradients, as well as in the growth and remodeling of spiral arteries. Over the course of pregnancy, these numbers decline to reach similar levels at term as the endometrium of nonpregnant women [23]. Bartmann et al. found that CD16 expression on decidual NK cells in basalis increased over the course of pregnancy [21]. We observed that CD16 expression was significantly higher in basalis compared to parietalis on both $CD56^{dim}$ and $CD56^{bright}$ NK cells. It is not known if the decidual NK cells upregulate the expression of CD16 expression during pregnancy or if there is an infiltration of $CD16^+$ NK cells as pregnancy proceeds. Both NK cell subsets produced IFN-γ when stimulated with PMA/ionomycin, but the $CD56^{dim}$ cells had a background expression, whereas the $CD56^{bright}$ cells did not. It would be of interest to further study decidual NK cells and their responsiveness to physiological stimulus.

The decidual T cell population was polarized towards an effector memory phenotype at the expense of naïve T cells, a finding most prominent in parietalis. This is in line with the findings of Sindram-Trujillo et al., as well as the consecutive work of Tilburgs et al., who showed an enrichment of effector memory T cells in decidual tissues compared to peripheral blood, also with the most prominent changes seen in parietalis [20, 24]. These effector cells contained large amounts of mRNA coding for cytotoxic molecules, but the translation into functional proteins was impaired. In line with these findings, we observed a trend towards T cells from both basalis and parietalis having lower amounts of GrzB than T cells from peripheral T cells from healthy controls. An elevated activation status of T cells in decidual tissues has also been demonstrated previously [20]. Our data confirm these findings, and the observation that CD127 expression is decreased

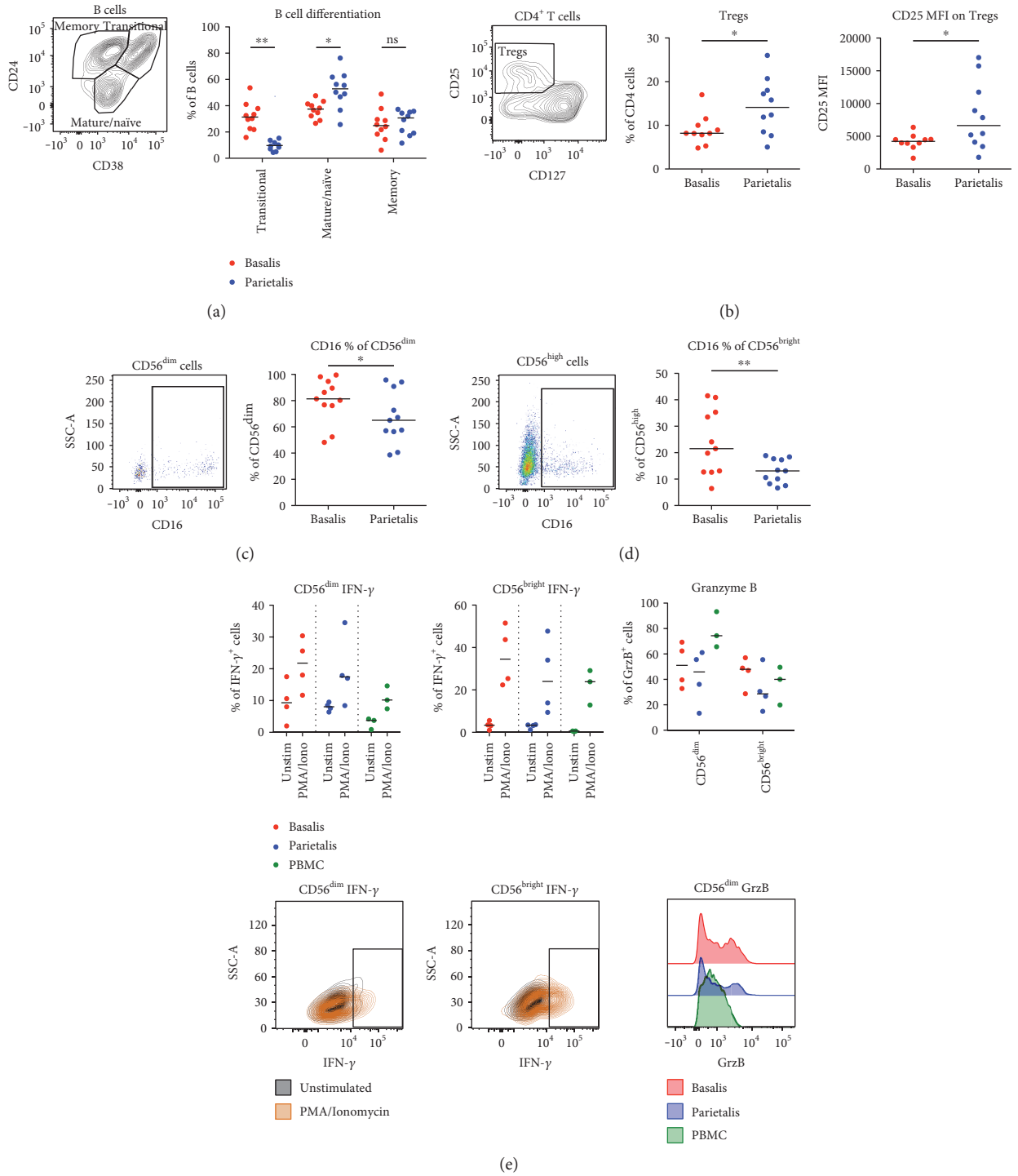

Figure 5: Maturational state of B cells and frequencies of T regulatory cell differ between decidual compartment. (a) Gating strategy (left) and graphical representation (right) of differentiation status of B cells from paired samples of decidua basalis and parietalis ($n = 10$). (b) Gating strategy and proportions of T regulatory cells (Tregs) as percentage of $CD4^+$ T cells (middle) and the median fluorescence intensity (MFI) of CD25 of the gated Tregs (right) ($n = 10$). (c) Gating strategy (left) and graphical representation (right) of CD16 expression on $CD56^{dim}$ cells from paired samples of decidua basalis and parietalis ($n = 11$). (d) Same as in (c) but for $CD56^{high}$ cells ($n = 11$). (e) Paired mononuclear cells from basalis ($n = 4$) and parietalis ($n = 4$) were stimulated for 5 hours with PMA/ionomycin or left unstimulated. Data on cells from healthy controls (PBMC) were included as controls ($n = 3$). Intracellular expression of interferon-γ (IFN-γ) and granzyme B (GrzB) was determined by flow cytometry. T cells were divided into $CD56^{dim}$ (upper left) and $CD56^{bright}$ (upper middle), representative contour plots below the respective cell subset. GrzB was measured in unstimulated samples only (upper right), representative histograms shown in the bottom right corner. Line in graphs depicts the median. Comparisons between the paired samples were made using the nonparametric Wilcoxon test. ns = not significant; $^*p < 0.05$; $^{**}p < 0.01$.

in decidual T cells further supports an effector memory status and activated phenotype [25].

The expression of the chemokine receptors CXCR3 and CCR6 was higher on T cells from decidua parietalis compared to basalis. This probably reflects the findings of a dominant effector memory phenotype but could also imply a higher proportion of Th1 cells and Th17 cells [26, 27]. DSCs have been shown to have an impaired production of the CXCR3 ligands CXCL9 and CXCL10 [2], but extravillous trophoblasts infiltrating decidual tissue constitutively produce CXCL10 [28]. Svensson-Arvelund et al. further showed a high production of the CCR6 ligand CCL20 by cells from placental tissue explants [28]. $CCR6^+$ T cells in the 1st trimester decidua were shown to be fewer compared to matched peripheral blood, indicative of a decreased Th17 polarization during early pregnancy [16]. On the other hand, Feyaerts et al. showed that decidua parietalis contained higher proportions of IFN-γ and IL-17 producing T cells compared to peripheral and endometrial T cells [15]. This, together with our findings, indicates that the relative frequency of Th1 cells and Th17 cells increases in parietalis over the course of pregnancy, further promoting the activated T cell profile in this compartment.

Although B cells have generally been ignored in the context of decidual immunology, their presence has been demonstrated in previous publications both by flow cytometry [20, 22, 29, 30] and by immunohistochemistry [31]. We have previously shown that DSCs from both decidua basalis and parietalis readily produce B cell-activating factor of the tumor necrosis factor family (BAFF) after stimulation with interferons [22], suggesting a role for B cell maturation in the decidua. We found that the frequency of B cells was higher in basalis compared to parietalis, but that parietalis contained a higher proportion of mature/naïve B cells at the expanse of transitional B cells. This may indicate that microenvironmental factors in parietalis, including BAFF, induce decidual B cell maturation. Interestingly, it was recently shown that an altered B cell composition in decidua and higher BAFF expression were associated with preterm labor [31].

Tregs have been proposed to play an important role in fetal-maternal tolerance and have been investigated in decidual tissues by several others [14, 30, 32–34]. Tilburgs et al. demonstrated a correlation between HLA-C mismatch between mother and fetus and proportions of both activated $CD4^+CD25^{dim}$ T cells and $CD4^+CD25^{high}$ putative Tregs in decidua parietalis [35]. This indicates that fetal alloantigens in the decidua promote maternal T cell activation and also induction of Tregs. In line with the increased activation status of T cells in parietalis, we found that the proportion of $CD4^+CD25^+CD127^{low}$ Tregs was higher in parietalis than in basalis. The intensity of CD25 expression on Tregs was also higher in parietalis, further indicating a more activated Treg phenotype. We have previously shown that DSCs from parietalis promoted elevated expression of CD25 on alloantigen-stimulated T cells, which partly was explained by an induction of Tregs [4] and also by elevated IL-2 levels in the supernatant due to a diminished capacity of the T cells to signal through the IL-2 receptor complex [5]. Thus, DSCs could potentially have a role in shaping the T cell phenotype in the decidual tissues.

Although coinhibitory marker expression in decidua has been investigated to some degree previously, our study provides a more complete analysis of these markers on T, B, and NK cell subsets, as well as the comparison between decidual compartments. The OPLS-DA model based on the expression of the coinhibitory markers PD-1, TIM-3, LAG-3, and CTLA-4 gave a clear separation between parietalis and basalis. Parietalis was associated with an increased expression of the vast majority of the investigated markers on several lymphocyte subsets. A large proportion of the T cells in both decidual compartments expressed PD-1 and TIM-3, which is in line with other studies showing that decidual T cells express higher levels of these markers compared to peripheral blood [11, 36]. PD-1, TIM-3, and LAG-3 expression on T cells was most pronounced in parietalis, which also contained a substantial $CD4^+$ T cell population expressing both PD-1 and TIM-3. The ligands for PD-1, PD-L1 and PD-L2, have been shown to be expressed on decidual macrophages and stromal cells and to be functional in suppressing T cell proliferation and cytokine production [4, 36, 37]. Pregnant mice that were treated with antibodies blocking PD-1 and TIM-3 had a decreased number of live fetuses compared to the control animals, thus demonstrating the importance of these markers for a successful pregnancy outcome in mice [11]. LAG-3 has not been extensively studied in pregnancy, but it has been showed that decidual Tregs express LAG-3 [38]. When we stimulated mononuclear cells with PMA/ionomycin, we found that both $CD4^+$ and $CD8^+$ T cells had an IFN-γ response in line with that observed in PBMCs from healthy controls. It would be of interest to explore decidual T cell responsiveness to fetal and viral antigens to further investigate the role of coinhibitory markers in suppressing the decidual T cell response.

A relatively large proportion of the decidual parietalis B cells expressed PD-1, TIM-3, and CTLA-4, and the expression was consistently higher in parietalis compared to basalis. PD-1 expression has been shown to increase in B cells as a response to activation [39] and to be expressed by a subpopulation of regulatory B cells involved in hepatoma progression [40]. Although CTLA-4 has mainly been described to be expressed by T cells, it has been reported that it can also be expressed by murine B cells after T cell-induced activation [41]. CTLA-4 expression in decidual B cells has not been described previously, but we observed a distinct $CTLA\text{-}4^+$ B cell population in parietalis. Direct effects of these markers on B cells are however still unclear, and the general role of B cells in fetal-maternal tolerance warrants further investigation.

MAIT cells are activated by vitamin B metabolites derived from the metabolism of certain strains of bacteria [17]. Little is known about MAIT cells in pregnancy, but we have previously shown that MAIT cells are present in decidua parietalis and have a similar capacity to respond to stimulation as peripheral MAIT cells [13]. Interestingly, we could separate basalis and parietalis in an OPLS-DA model based on data collected only on MAIT cells, demonstrating major phenotypic differences on MAIT cells between these

two compartments. Basalis contained higher frequencies of MAIT cells among the $CD3^+$ cells, but a larger proportion of MAIT cells in parietalis were $CD4^+$ at the expense of $CD8^+$ MAIT cells. Similar to the conventional T cells, the MAIT cells in parietalis displayed a more activated phenotype compared to basalis, but parietalis MAIT cells also had a significantly higher expression of the activation marker CD38. In addition, parietalis MAIT cells had higher expression of the exhaustion marker PD-1, further demonstrating their chronically activated phenotype in decidual tissues. Although the number of donors for functional analysis was few, we found that MAIT cells from both basalis and parietalis rapidly produced IFN-γ, GrzB, and perforin in response to *E. coli* stimulation. Our previous work on MAIT cells in decidua parietalis also showed that decidual MAIT cells produced these factors at similar proportions as peripheral blood MAIT cells [13]. PD-1 ligation has been described to hamper MAIT cell function [42], but despite the high PD-1 expression on decidual MAIT cells, their functional capacity was still intact. Our study provides the first evidence of the presence, phenotype, and functionality of MAIT cells in basalis, as well as comparison with MAIT cells from parietalis.

The physiological reasons for the differences in lymphocyte phenotypes in parietalis and basalis are not known. One potential explanation is that term basalis is more vascularized compared to parietalis, which could allow a higher influx of peripheral immune cells with a less activated phenotype, whereas the parietalis could be more static in terms of immunological composition. This could also mean that basalis is more exposed to blood-borne infectious agents, whereas parietalis mainly provides protection against mucosal pathogens as well as commensal microbiota. Another hypothesis is that labor is induced by an activation or recruitment of inflammatory immune cells to the membranes [43] and that parietalis is the site for membrane rupture. Marcellin et al. recently showed that the area of parietalis overlying the cervix displayed an altered immunological phenotype shift at term compared to the other parts of the parietalis membrane [44]. The expression of polymorphic HLA molecules was upregulated, the NK cell population shifted towards the classical cytotoxic phenotype, and immunotolerant M2 macrophages declined. We isolated lymphocytes from the entire decidua parietalis membrane and found the general leukocyte population to be dominated by the more activated phenotype. It would be interesting to investigate the T cell phenotype in parietalis tissue overlying the cervix in term decidua to examine if the effects observed by Marcellin et al. are also apparent for T cells.

With our isolation method, we aimed at procuring tissue resident immune cells as representative as possible of physiological conditions. Although the tissue was extensively washed before processing, we cannot be sure that our samples were completely devoid of contaminating peripheral blood. Since the decidua basalis is a more vascularized tissue compared to parietalis, it is also possible that the contamination was larger in the basalis samples. Also, although we did not use enzymatic digestion of the tissue, the mechanical disaggregation of the tissue could possibly have changed the expression of certain surface molecules. Another limiting factor is that our sample size was not large enough to investigate whether factors such as time of placental delivery to analysis, age of the donor, or the gestation week did influence either the isolation of immune cells or results of the analysis.

Placenta-derived stromal cells are being explored as potential candidates for cell therapy, since they are involved in regulating inflammatory immune responses [12, 45, 46]. Stromal cells and fibroblasts from different anatomical sites of the body have been shown to have distinct transcriptional patterns even after *in vitro* expansion, indicating a positional memory of stromal cells [47]. It may therefore be of importance to consider the immunological phenotype of the tissue from which the stromal cells are isolated. Differences between stromal cells isolated from decidua parietalis and basalis have not been thoroughly explored, but as evidenced by our immunophenotyping, it is possible that they have different capacities to affect immune responses.

5. Conclusion

We have performed a comprehensive characterization of leukocyte populations in paired samples from decidua parietalis and basalis. We found that immune cell populations differ strongly between the two investigated compartments. Decidua parietalis lymphocytes as well as MAIT cells displayed a more activated phenotype with a higher expression of coinhibitory markers than those isolated from basalis. Since decidua parietalis is the site for membrane rupture, it is interesting that the parietalis generally contained T cells of a more activated and differentiated phenotype compared to basalis. Also, these two different anatomical sites could be influenced by different factors and may play different roles during pregnancy. The results from this study may also give clues on how the stromal microenvironments at the different decidual sites may affect immune cell regulation, which potentially should be considered when selecting the source of tissue for stromal cell isolation for cell therapy.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this article.

Authors' Contributions

Martin Solders designed, performed, and analyzed the experiments, interpreted the results, and wrote the paper. Laia Gorchs designed, performed, and analyzed the experiments and interpreted the results. Anna-Carin Lundell conceived and analyzed the experiments, interpreted the results, and performed statistical analysis. Eleonor Tiblad and Sebastian Gidlöf interpreted the results. Helen Kaipe conceived, designed, and analyzed the experiments, interpreted the results, and wrote the paper. All authors participated in the final approval of the manuscript.

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Review Article

Lymphocytes in Placental Tissues: Immune Regulation and Translational Possibilities for Immunotherapy

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Immune modulation at the fetomaternal interface is crucial to ensure that the fetal allograft is not rejected. In the present review, the focus is to describe basic functions of lymphocyte populations and how they may contribute to fetomaternal immune regulation, as well as determining what proportions and effector functions of these cells are reported to be present in placental tissues in humans. Also explored is the possibility that unique cell populations at the fetomaternal interface may be targets for adoptive cell therapy. Increasing the understanding of immune modulation during pregnancy can give valuable insight into other established fields such as allogeneic hematopoietic stem cell transplantation and solid organ transplantation. In these settings, lymphocytes are key components that contribute to inflammation and rejection of either patient or donor tissues following transplantation. In contrast, an allogeneic fetus eludes rejection by the maternal immune system.

1. Introduction

Immunological tolerance and defensive mechanisms of foreign tissue were first discussed by Murphy [1] and Owen [2]. However, the concept of acquired immune tolerance was introduced definitively by Billingham and Medawar in 1953 [3].

The sites at which the fetal and maternal tissues are in contact can be referred to as the fetomaternal interface and can be divided into two compartments. The first of which is between the maternal decidua and the fetal chorionic plate and chorionic membrane. Depending on whether the decidua is in contact with the site of implantation or with the fetal membranes is referred to as the decidua basalis or decidua parietalis, respectively. The second interface is where the maternal blood is in contact with the placental body and interacts with fetal trophoblasts. Thus, fetal and maternal tissues are not completely separated and immune cells have access to fetal tissues, driving complex tolerogenic immunological mechanisms to prevent rejection of the fetal allograft. The objective of this review is to discuss some of these mechanisms in the light of the current literature, with particular emphasis on lymphocyte function at the fetomaternal interface and how these cells may contribute to immune modulation during pregnancy.

2. T Cell Priming and Fetal Antigen Presentation

The placenta can be regarded as a haploidentical transplant. However, transplantation of a solid organ or hematopoietic stem cells leads to rejection or graft-versus-host disease (GVHD) without proper immunosuppressive interventions, while pregnancy is tolerated. Thus, there must be fundamental differences in these two entities in the priming and effector responses of the immune system to nonself. Acute graft rejection is driven by direct and indirect allorecognition [4].

Donor or recipient tissue-resident antigen presenting cells (APCs) collect graft antigens and migrates to adjacent lymphoid organs. Presentation of a foreign peptide to a T cell by a foreign APC elicits a stronger response in a larger quantity of T cell clones than if a foreign peptide is presented by self APCs [5]. Interestingly, studies have indicated that indirect allorecognition—and not direct allorecognition—is the major pathway for the maternal immune system to recognize fetal antigens [6, 7]. Using an Act-mOVA system [8], predominant maternal APC presentation of fetal antigens is suggested, as OVA-specific T cells respond to Act-OVA transgenic fetuses but not to fetuses deriving from control males [7, 9].

Moreover, trophoblasts have shown to have no expression of major histocompatibility complex (MHC) class II molecules, which limits the priming of $CD4^+$ T cells by fetal cells in the placenta [10]. Trophoblasts also have expression of human leukocyte antigen- (HLA-) C, HLA-G, and HLA-E, while expression of the more polymorphic HLA-A and HLA-B is limited, resulting in a reduced recognition of alloantigens. HLA-C is the only classical HLA molecule expressed by fetal trophoblasts. Interestingly, a study with HLA-C mismatch between mother and father showed an increase in frequencies of $CD4^+CD25^{dim}$ T cells in decidual tissue [11]. Additionally, the placental tissues also contained $CD4^+CD25^{high}$ cells, supposedly regulatory T cells (Tregs). This was not seen in pregnant women when the mismatch was for HLA-DR or HLA-DQ.

Besides the restriction of indirect allorecognition for activation of T cells, studies have indicated that the dendritic cells resident in the decidua are constrained in their ability to leave the tissue and migrate to adjacent lymph nodes where they can activate circulating T cells [12]. Collins et al. have suggested that the dendritic cells (DCs) in the decidua are immobile despite being responsive to the chemokine CCL21, one of the ligands for CCR7 that enable homing to lymphatics. The DCs stay immobile even after being activated through exposure to lipopolysaccharides.

Lymphangiogenic molecules are produced by the first or second trimester *ex vivo* cultured invasive cytotrophoblasts. These cells have shown to stimulate lymphatic remodeling and growth of lymphatics when transplanted into an *in vivo* model [13]. Thus, the process by which cytotrophoblasts enable lymphatic remodeling may be important in implantation and vascularization [14]. In contrast, a later study showed that the lymphatics disappeared in human endometrium following decidualization [15], limiting the possibility of primed DCs to migrate to lymphoid organs. Interestingly, extravillous trophoblasts may enter decidual veins as early as week 5 of gestation [16, 17]. The implication of this for fetomaternal tolerance is not known. This does not rule out the possibility of fetal-derived peptides reaching the lymph nodes and being taken up by APCs. Exosomes containing fetal peptides are readily produced by placental tissues throughout pregnancy and may contribute to modeling immune responses and contribute to transport fetal peptides for antigen presentation [18]. To our knowledge, there are no studies investigating if these exosomes can contribute to partial central tolerance towards the fetus during pregnancy.

In addition to reduced DC migration for efficient T cell priming, reports are suggesting that gene silencing in the decidua prevents migration of effector T cells to the fetomaternal interface [19]. Specifically, upon activation by tumor necrosis factor- (TNF-) α, the myometrium upregulates the levels of transcription for RNAs encoding *Ccl5*, *Cxcl9*, and *Cxcl11* whereas stromal cells from the decidua do not. These genes encode chemokines that are ligands for CXCR3, which is present on Th1 cells and increase their homing towards tissues expressing these chemokines. Altogether, the results of these studies indicate that one way of maintaining fetomaternal tolerance is to limit allorecognition, inhibit APC migration to lymph nodes, and reduce infiltration of effector T cells.

3. Lymphocyte Cell Composition at the Fetomaternal Interface and Their Role in Tolerance

3.1. NK Cells. A major difference between a transplanted allograft and the fetomaternal interface is their different immune cell composition. The majority of immune cells present in these tissues are natural killer (NK) cells [20]. One of the known roles of these cells is to increase and modulate the vascularization between the placenta and the uterus to enable sufficient blood flow at the fetomaternal interface. Deficiencies in this process inhibits the growth of the fetus with adjacent tissues and may lead to preeclampsia [10, 21]. NK cell is also immunologically relevant in maintaining tolerance during pregnancy.

The NK cells found in the decidua during pregnancy are functionally and phenotypically different from conventional NK cells found in adults and children [20, 21] and are referred to as decidual NK (dNK) cells. Phenotypically, these cells have a very high expression of CD56. During the first trimester, dNK cells may comprise up to two thirds of the total lymphocyte repertoire [22]. NK cells in peripheral blood range between 5–30 percent and approximately 0–7 percent of the NK cells are activated [23]. Compared to conventional NK cells, dNK cells have an increased expression of natural killer group 2 (NKG2) receptors [24–26]. These receptors recognize antigens presented by HLA-C and HLA-E. Moreover, dNK cells have a high expression of killer cell lectin-like receptor subfamily D (KLRD1) [27], CD9 [28], and CD49a [29] and an increased frequency of expression of CD69 [22]. This shows that many dNK cells display a more activated phenotype. Indeed, the cytokine production in dNK cells is high [30] but despite having granules for cytotoxic ability, these granules fail to work efficiently and dNK cell cytotoxicity is therefore low [26]. There are a couple of different studies suggesting mechanisms for how the cytotoxic ability of dNK cells is impaired. Decidual macrophages can inhibit dNK cells cytolytic ability in a transforming growth factor- (TGF-) β-dependent manner [31]. The known roles of decidual macrophages in fetomaternal tolerance have been thoroughly covered in a review by Svensson-Arvelund and Ernerudh [32]. Furthermore, the cytotoxic ability of dNK cells is reduced by recognition of

HLA-E expressed on trophoblasts [26, 33]. Additionally, dNK cells may interact with HLA-G$^+$ extravillous trophoblasts (EVT). During the interaction of dNK cells and EVT, HLA-G can be acquired by the dNK cells, while during internalization and degradation, possible signaling of HLA-G leads to reduced cytotoxicity in dNK cells. However, dNK cell cytotoxicity can be increased in an inflammatory milieu [34, 35]. One study showed that those dNK cells in contact with cytomegalovirus-infected cells became cytotoxic following activation through NKG2C/D/E receptors [36]. Another study demonstrated dNK cells expressing the killer cell Ig-like receptor 2DS1 had cytotoxic function towards human cytomegalovirus presented by HLA-C2 on decidual stromal cells *in vitro* [37]. The secretory functions of dNK cells are distinctive from conventional NK cells *in vitro*. When stimulated with interleukin- (IL-) 15, dNK cells can produce interleukin- (IL-) 8, interferon-inducible protein- (IP-) 10, vascular endothelial growth factor (VEGF), placental growth factor [30], interferon- (IFN-) γ, and TNF-α [38]. These cytokines and growth factors are crucial for angiogenesis and arterial remodeling in early pregnancy.

IFN-γ is associated with Th1-like responses, including activation of macrophages, upregulation of MHC class I on APCs and epithelia. dNK cells may also contribute to modulating Tregs. IFN-γ induces production of indoleamine-2,3-dioxygenase (IDO) [39]. IFN-γ production by dNK cells can induce IDO expression in CD14$^+$ cells in the decidua. When dNK cells were cocultured with CD14$^+$ cells from the decidua and T cells, the frequency of Tregs was increased [40]. In the same setting, using conventional NK cells from the peripheral blood or only decidual CD14$^+$ cells did not affect Treg frequencies. The frequency of Tregs in this system was also decreased when an anti-TGF-β antibody was added. Furthermore, decreased expression of killer immunoglobulin receptors (KIR) on NK cells in peripheral blood of women is associated with recurrent spontaneous abortions or unsuccessful implantation [41]. However, it is difficult to assess whether the shift in expression has implications in the implantation or in maintaining tolerance against paternal antigens. NK cell receptor activation leads to IFN-γ secretion through distinct pathways [42–44], and IFN-γ seems to be important for both implantation and to maintain tolerance during pregnancy. Investigating this axis would be interesting, especially with regard to expression of 2B4 and other coreceptors in dNK cells. This may give additional insight on the role of dNK cells in fetomaternal tolerance.

One study that used different toll-like receptor (TLR) stimulation on dNK could show differences in IFN-γ and IL-6 and TNF-α concentrations in supernatants when cultured *in vitro* [45]. The highest amount of these cytokines were produced when the cells were stimulated with TLR3 or TLR9. TNF-α production was favored when the cells were stimulated with TLR2, TLR3, and TLR9. dNK cells also showed to produce chemokines that may attract both granulocytes and T cells, including chemokine ligand (CCL) 5, IL-8, CCL3, and CCL4. NK cells in peripheral blood can produce trace amounts of IL-10 and TGF-β [46], but the translation of this regulatory NK cell subset in dNK cells or the presence of IL-10 producing NK cells in placental tissues has not been investigated to our knowledge.

A possible mechanism of immune regulation during pregnancy is through the downregulation of NKG2 on PBMCs [47]. This inhibits NKG2-dependent cytotoxic responses at the fetomaternal interface and is caused by production of MHC class I chain-related proteins A and B by syncytiotrophoblasts in the placenta.

3.2. B Cells. Certain maternal antibodies are known to be able to cross the placental barrier to provide a passive defense to the fetus [48, 49].

As early as the 1990s, a potential role for B cell frequencies in blood was identified. Maternal serum was shown to stimulate B cells to reduce production of IgM and increase production of IgA and IgE, while IgG production remained stable [50].

B cell activating factor (BAFF) is important for maturation of B cells [51]. A study by Lundell and colleagues can show that BAFF is produced by decidual stromal cells and BAFF levels are high at birth [52]. BAFF is induced in DSCs by interferon-γ/interferon-α, which can be produced by dNK cells. Increased BAFF levels in children are associated with a higher frequency of CD27$^+$ memory B cells [53]. The same study also showed that infants with allergies at 18 months were associated with lower cord blood BAFF levels compared to infants with no allergic symptoms.

Changes in B cell subsets during pregnancy have also been linked to disease outcome in children postpartum. For instance, atopic asthmatic mothers were shown to have increased frequencies of transitional B cells (IgM^{hi}CD38^{hi}) as opposed to healthy pregnant mothers. Moreover, the atopic asthmatic mothers with the highest frequencies of transitional B cells were shown to have an increased risk of progeny with allergies [54]. Additionally, CD5$^+$ B cells were shown to be reduced during pregnancy in the blood of healthy women, only to return to normal levels within 2 months' postdelivery. No difference was seen for other B cellular subsets [55].

B cells in the fetomaternal interface may protect against fetal rejection by asymmetric IgG production. This is thought to be influenced by Th2 cytokines produced from switching from a Th1 to Th2 phenotype in blood during pregnancy [56, 57].

Regulatory B (Bregs) cells are currently a focal point of research in the fetomaternal interface. In a recent review by Guzman-Genuino and Diener, the role of Bregs in transplantation, cancer, autoimmunity, and pregnancy are discussed in detail. IL-10 producing Bregs are of special interest in the pregnancy context, as IL-10 can be detected at heightened levels in the fetomaternal interface and is thought to counterbalance the proinflammatory response associated to fetal rejection [58]. The role for IL-10 in pregnancy can however be debated, as *in vivo* models have shown that successful pregnancy can be IL-10 independent [59].

3.3. $\gamma\delta$ T Cells. Another cell population that has been inadequately discussed regarding a role during gestation and fetomaternal tolerance is $\gamma\delta$ T cells. These cells constitute 5–10%

of circulating T cells in adult peripheral blood and represent a bridge between innate and adaptive immunity [60]. The frequency of these cells is similar in decidua [61]. In umbilical cord blood, $\gamma\delta$ T cells are less frequent than in peripheral blood, representing less than 1% of the lymphocytes [62]. Additionally, $\gamma\delta$ T cells in umbilical cord blood are more naive and show a polyclonal repertoire with predominance of the Vδ1 subtype in contrast to peripheral blood where Vδ2 is in abundance [63]. $\gamma\delta$ T cell function and their specific phenotype in the human placenta and at the fetomaternal interface have been described to some extent. Earlier studies suggest that $\gamma\delta$ T cells in the decidua are skewing the immune response towards Th2 at the maternal-fetal interface [64, 65]. This is mediated by the production of IL-10 and TGF-beta [65, 66]. The importance of decidual $\gamma\delta$ T cells for Th2 skewing via cytokines was later confirmed by Fan et al. [67] which also showed that the cells enhance trophoblast growth and invasion. This implies their dual role in both modulating the immune response in favor of fetomaternal tolerance as well as placental growth. What complicates the interpretation of the role of $\gamma\delta$ T cells is that their phenotype differs greatly between the parietalis and basalis. In the former, more than 50% express Vδ1, while the latter more resembles peripheral blood with more than 90% expressing Vδ2 [68]. Studies on peripheral blood comparing healthy pregnant women and women at risk of premature pregnancy termination suggest that an increase in the Vδ2/Vδ1 ratio is associated with an increased risk of termination via a shift in Th1/Th2 balance [69].

3.4. Invariant Natural Killer (iNK) T Cells. iNKT cells are identified by expression of the complementarity-determining region 3 conserved invariant T cell receptor chain Vα24-Jα18 and the more diverse Vβ11 [70, 71] receptor chain. They are activated through interaction with glycolipids presented by the nonclassical MHC complex CD1d [72]. These cells are potent effector cells and can produce cytokines such as IFN-γ, IL-4, IL-13, TNF-α [73, 74], and IL-10 [75]. They also have cytotoxic ability [72], primarily through the Fas/FasL pathway [76].

The iNKT : T cell ratio is increased early following allogeneic hematopoietic stem cell transplantation in patients receiving conditioning with total lymphoid irradiation (TLI) combined with antithymocyte globulin (TLI) [77, 78]. These patients had a reduced incidence of GVHD. Moreover, a high number of iNKT cells in donor grafts are associated with of GVHD-free survival [79]. In a phase IIa trial, one third of the patients that received an iNKT ligand (RGI-2001) combined with sirolimus had increased frequencies of regulatory T cells [80]. However, it is difficult to assess the role of iNKT in this setting as SRL alone can promote Tregs [81, 82]. Murine studies further demonstrate the importance of iNKT cells for reduced incidence of GVHD and expansion of Tregs [83]. Thus, high numbers of iNKT cells in the allogeneic setting seem to be favorable for maintaining tolerance.

Comparatively, iNKT cells are highly enriched in the decidua compared to peripheral blood [84]. Upon stimulation with αGalCer or anti-CD3, decidual iNKT cells produced more IFN-γ and granulocyte macrophage-colony stimulating factor than IL-4 compared to iNKT cells from peripheral blood. Thus, it can be speculated that immunomodulation by iNKTs at the fetomaternal interface is directed towards functions associated with IFN-γ.

There are several murine studies linking iNKT function to pregnancy loss following administration of αGalCer [85–89]. αGalCer-stimulated pregnancy loss was associated with perforin when given early. In contrast, when given at later stages of pregnancy, it was associated with IL-2 and TNF-α [89]. This emphasizes the importance of different functions at different time points during gestation. Li et al. showed that administration of αGalCer reduces the number of decidual Tregs, as well as their ability to produce IL-10 and TGF-β [88]. The same study also shows that administration of both αGalCer and an anti-IFN-γ antibody restored Treg function *in vitro*. Although the majority of studies regarding iNKT cells during pregnancy indicates that iNKT cells may contribute to loss of tolerance and labor induction, there is a need for more studies exploring mechanisms for iNKT enrichment and function at the fetomaternal interface, especially in the human setting.

3.5. Innate Lymphoid Cells. Innate lymphoid cells (ILCs) can be divided into three groups: ILC1, ILC2, and ILC3. The ILC1 and ILC3 groups also have transcriptional, phenotypical, and functional heterogeneity [90–92]. Vacca et al. described the presence of ILCs in the decidua, showing that human first trimester decidua is host to two types of ILC3s [93]. One type was capable of producing IL-22 and IL-8, and the other one could secrete TNF-α and IL-17A. ILC1 cells were also present and produced IFN-γ. The same group also showed that ILC3s were present in the decidua in the proximity of neutrophils, suggesting that ILC3s contribute to the recruitment of neutrophils during the first trimester [94]. Moreover, coculture of ILC3s and decidual neutrophils increased survival of the neutrophils, whereas dNK cells could not. ILC3s were able to produce GM-CSF and CXCL8, which promote survival and migration in neutrophils. Thus, present data suggests that ILCs at the fetomaternal interface may contribute to microbial defense. Their role in fetomaternal tolerance is yet unexplored. A summary of findings in humans of lymphocytes discussed so far is presented in Table 1.

4. $CD8^+$ T Cells

Compared to the NK and macrophage compartment, the number of T cells at the fetomaternal interface is low [95]. Compared to peripheral blood, where the T cell compartment contains more $CD4^+$ cells than $CD8^+$ cells, the fetomaternal interface contains more $CD8^+$ T cells [61, 96, 97]. A recent study has shown that there is an accumulation of virus-specific effector memory (T_{EM}) $CD8^+$ T cells in the decidua during uncomplicated pregnancy, which may suggest that the skewing of the $CD8^+$ T cell compartment may be to manage infections rather than allogeneic responses against fetal tissues [98]. $CD8^+$ dT cells have been shown to be less cytotoxic than their counterpart in peripheral blood [99, 100]. These cells also appear to almost exclusively exert

Table 1: Proportions of lymphocytes in human placental tissue and findings ex vivo relating to outcome or lymphocyte function.

Cell	Compartment	Approximate abundance	Comment	Reference
NK cells	T1 Dec	50–70% of Dec cells	CD3-CD56$^{+(+)}$	[22, 219, 220]
	T1 Dec basalis		Low cytotoxic function	[26]
	T1 Dec		↑ Tregs by IFN-γ → IDO	[40]
	T1 Dec basalis		TLR chemokine patterns	[45]
B cells	Term CB		↓ BAFF → allergy and decreased B cell maturation	[53]
$\gamma\delta$ T cells	T1 Dec	5% of CD3$^+$	Naive. Vδ2 skewing	[61]
	Term UCB	1% of lymph		[62]
iNKT cells	T1 Dec	0.5% of CD3$^+$ versus 0.01% in PB	Th1 cytokine bias	[84]
ILCs	T1 Dec		IL-17A and IL-22 ICS	[93]

BAFF: B cell activating factor; CD: cluster of differentiation; Dec: decidua; CB: cord blood; ICS: intracellular staining; IDO: indoleamine-2,3-dieoxygenase; ILC: innate lymphoid cells; lymph: lymphocytes; NK: natural killer; PB: peripheral blood; T1–3: trimester 1–3; UCB: umbilical cord blood.

an effector memory phenotype [100]. Interestingly, despite having a lower expression of perforin and granzyme (GZM) B, the mRNA expression of these proteins are high [100]. Two studies from the Rao lab have presented data indicating that persistent IL-2 receptor α (IL-2Rα) signaling can induce endoribonuclease dicer to produce microRNA to control perforin of CD8$^+$ T cells during inflammation [101, 102]. The same studies also suggest that perforin transcription is regulated by signal transducer and activator of transcription 5 (STAT5) and eomesodermin [102]. We performed a study where we investigated how decidual stromal cells affected IL-2 production and IL-2R expression and signaling. We could observe that DSCs increased IL-2 production in an allogeneic setting *in vitro* [103]. This was followed by a high expression of the IL-2Rα in CD8$^+$ T cells. Furthermore, we showed that even though IL-2Rα expression was high, the expression of the high-affinity IL-2R$\alpha\beta\gamma_c$ complex was reduced. This was associated with a significantly reduced IL-2 internalization and STAT5 signaling in CD8$^+$ T cells. Thus, we suggest a hypothesis where DSCs induce a persistent high IL-2 production that through several pathways limits the effector function of CD8$^+$ T cells. The increased production of IL-2 by DSCs may also contribute to the abundance of CD8$^+$ dT cells with an effector memory phenotype, although the role of IL-2 in CD8$^+$ T cell differentiation is difficult to determine [102, 104]. One recent study suggested that these cells have an increased expression of programmed death-1 (PD-1), indicating exhaustion as a possible mechanism with reduced effector function as a consequence [105]. We did not observe this when allostimulated CD8$^+$ T cells were cultured with DSCs, but in the absence of DSCs and addition of equivalent concentrations of IL-2 as provided through DSCs, the cells expressed increased levels of PD-1 [103]. This indicates that DSCs contribute to reduce exhaustion in T cells, despite high concentrations of IL-2. How DSCs induces IL-2 is not known. A recent report by Fragiadakis et al. compared paired samples from maternal peripheral blood and cord blood at term and compared the evoked immune features upon stimulation with a cocktail of IL-2, IL-6, IFN-α2A, and granulocyte macrophage-colony stimulating factor (GM-CSF) [106]. Although the aim of the study is more directed towards peripheral immunity, they were able to show that T cells in maternal peripheral blood were hyporesponsive compared to T cells from cord blood. The study did not include immune cells isolated from the fetomaternal interface.

5. CD4$^+$ T Cells

5.1. Th1 T Cells. A relatively high frequency of the T helper cell subset Th1 (identified by the transcription factor Tbet$^+$ [107]) is present in maternal blood compared to umbilical cord blood at term [106]. These cells are characterized by their ability to produce IL-2, IFN-γ, and TNF-β, and can be differentiated following exposure of IL-12 [108]. Th1 cells (identified as CXCR3$^+$CCR4$^-$CCR6$^-$ [109]) have been shown to be more abundant in the first trimester decidua compared to peripheral blood, while Th2 and Th17 ratios are lower compared to peripheral blood [110]. The study does not specify if the decidual lymphocyte cells were isolated from decidua basalis or decidua parietalis. This is in line with the high number of CD8$^+$ T cells, where Th1 cells may be important for enhancement of the probability of activation of CD8$^+$ cells [111], while Tregs support tolerance of the fetus. There has also shown to be a correlation between NK cells and Th1 priming [112]. The ability of dNK cells to prime Th1 cells at the fetomaternal interface is not known. Interestingly, this observation is in contrast to the study by Karjalainen et al., which showed in mice that decidual stromal cells (DSCs) may be able to use chemokine gene silencing to prevent CXCR3$^+$ Th1 cells from reaching the placenta. There is also additional work in murine models suggesting that CXCR3 is involved in spontaneous preterm birth [113]. A study by Saito et al. showed that there was no significant increase of IFN-γ secreting cells in human peripheral blood during any trimester in pregnancy [114], whereas Matthiesen et al. showed increased numbers of IFN-γ^+ and IL-4$^+$ cells in all trimesters [115]. The disparity in results is probably due to differences in methods to detect these cells and that many cells can produce IFN-γ. However, the high ratio of Th1 cells in decidua is interesting as they secrete IFN-γ, although dNK is likely the largest producer of IFN-γ in this setting. IFN-γ activation leads to a broad transcription profile and diverse immunological functions [116, 117],

including priming adaptive immune responses, inhibiting cell proliferation and inducing apoptosis [118]. IFN-γ is also important for production of IDO [119], which can be important for tolerance and induction of Tregs [120–123]. During uncomplicated pregnancy, concentrations of IFN-γ are low in the periphery and are significantly lower in the third trimester compared to healthy controls [124], but the concentration is nonsignificantly increased in patients with preeclampsia. The frequency of Th1 cells has shown to be increased in preeclampsia [114]. In women with multiple sclerosis (MS), symptoms are reduced during pregnancy and are associated with a decrease of Th1 cells [125, 126]. Interestingly, relapse of MS postpartum is associated with a continued decline of Th1 cells, while patients that remain in remission have restored Th1 values. Murine studies have shown that Th1 cells inhibit Treg induction during pregnancy and induce fetal loss [127]. The broad spectrum of function initiated by IFN-γ points towards an understanding that the regulation of IFN-γ is important during pregnancy and that the balance of IFN-γ may be important for maintaining fetomaternal tolerance. Inhibiting IFN-γ increases proliferation of allostimulated PBMCs that were otherwise suppressed by DSCs [120]. In contrast, DSCs suppress the secretion of IFN-γ in the same system [128]. Furthermore, pretreatment of DSCs with IFN-γ reduced their ability to inhibit alloantigen-induced proliferation [120].

5.2. Th2 T Cells. In contrast to Th1 cells, a Th2 phenotype is somewhat increased in peripheral blood during pregnancy and a Th2 predominant immunity has been thought to be associated with uncomplicated pregnancy [129]. Indeed, early studies have indicated that the cytokine profile at the fetomaternal interface was skewed towards Th2 [130]. However, as also stated by Saito et al. [129], the importance of Th2 cells in maintaining fetomaternal tolerance should probably be revised. There are several studies questioning the importance of Th2 cells for the maintenance of fetomaternal tolerance during pregnancy, and their role appears to be of less importance compared to other T helper effector cells. It is, however, important to also note that the proportions of these T cells can vary over time during pregnancy, and many studies focus in a set time point, mostly early pregnancy. Th2 cells are characterized by expression of the transcription factor GATA3 [131, 132] and are induced by IL-4 [133, 134]. The frequencies of Th2 ($CCR4^+CXCR3^-CCR6^-$) cells have in one study been shown not to differ significantly in blood compared to the first trimester decidua [110]. Additionally, another murine study demonstrated that knockout of the Th2 effector cytokines IL-4, IL-5, IL-9, and IL-13 did not necessarily lead to fetal loss [135]. In a recent study investigating the transcriptional profile of human decidual lymphocytes, it was shown that the T cells in the decidua had a predominant Th1, Th17, and Treg profile [136]. In contrast, Kostlin et al. isolated granulocytic myeloid-derived suppressor cells (MDSCs) from human placenta. Where placenta-derived MDSCs were cocultured with PBMCs, they saw a shift towards a Th2 phenotype in an *in vitro* system [137]. Thus, the Th1/Th2 paradigm and its importance in fetomaternal tolerance still require exploration.

5.3. Th17 T Cells. Although a proportion of cells in the decidua have been shown to be associated with secretion of IL-17 [136], the frequency of Th17 cells is lower in the decidua compared to peripheral blood in the first trimester [110]. A similar study suggested that there is an increased frequency of IL-17 producing cells among lymphocytes in the decidua compared to peripheral blood [138]. The master regulator of Th17 cells is RORC [139], and the cells can be induced by IL-6, IL-1β [140], IL-21, and TGF-β [141–143]. Th17 cells have been associated with a wide range of inflammatory complications where mechanisms of central/peripheral tolerance are insufficient, including autoimmunity [144–146] and graft-versus-host disease [147, 148]. Elevated levels of IL-6 could be detected at the onset of spontaneous abortions [149]. Indeed, patients with recurrent miscarriage also had increased frequencies of $CD4^+$ T cells expressing CCR6 and IL-17 [150]. A similar result was shown by Santner-Nanan et al., where the ratio of Tregs/Th17 was increased in healthy pregnancies compared to pregnancies ending in preeclampsia [151]. IL-17 produced by T cells has also been linked to contribute to inflammation in human amniotic mesenchymal cells by enhancing the production of IL-8 synergistically with TNF-α [152].

It is difficult to assess whether it is the Th17 cells that promotes inflammation in these settings, or if the results are a consequence of a reduction in Tregs. Tregs, or more specifically the transcription factor forkhead box P3 (FOXP3), suppress differentiation of Th17 cells [153] which in part can result in a Treg-dependent balance between Tregs and Th17 cells. Interestingly, IFN-γ has also been shown to suppress differentiation of Th17 cells from naive T cells [154], adding more complexity to the regulation of Th17 cells. In contrast to Th1 and Tregs, the mechanisms of Th17 function and immunity at the fetomaternal interface are yet relatively unexplored, including the role of Th17 cells for production of antimicrobial peptides [155] and recruitment of neutrophils [156].

5.4. Regulatory T Cells. The concept of cellular immunity was formed in the seventies and eighties when patients that received blood transfusions showed weaker responses to mitogenic stimuli *in vitro* [157, 158]. However, it would be more than a decade before Tregs were identified [159–162]. Treg function is dependent on expression of FOXP3 [160, 162]. Tregs have a high expression of CD25, cytotoxic T lymphocyte antigen- (CTLA-) 4 [163], and a low expression of IL7-Rα [164–166]. Tregs can be divided based on origin and effector function [167]. They can either be generated in the thymus or be induced in the periphery. Tregs can be divided into central Tregs and effector Tregs. Effector Tregs have low expression CCR7 and CD62L, while nonactivated Tregs have higher expression of CCR7 and CD62L [167, 168]. Upon TCR, CD28, and IL-2 stimulation, central Tregs will develop an effector phenotype and can have suppressive functions. Tregs are critical for maintaining peripheral tolerance and reduced levels are associated with poor outcomes in pregnancy [169, 170]. Tregs have a central role for fetomaternal tolerance and are detected in increased frequencies at the fetomaternal interface. In comparison, the

frequencies of Tregs in peripheral blood between pregnant and nonpregnant women are similar [110, 123, 171, 172]. Effector $CD45RA^{-}$ Tregs from term decidua parietalis are increased compared to peripheral blood and the endometrium [173, 174]. This is not seen in the peripheral blood of second trimester women [175, 176].

Increased levels of Tregs have been shown in patients with miscarriages [177], and Mjosberg et al. observed that Treg frequencies were reduced in the second trimester as a possible result of hormonal changes [178]. Peripheral Tregs in the second trimester of women with preeclampsia had an increased expression of CTLA-4 and CCR4 [175].

Continuous TCR, CD28, and IL-2 stimulation is important for Treg expansion and survival [179–181]. *FOXP3* represses production of IL-2 by Tregs themselves [182] and thus Tregs are dependent on other cells to provide IL-2. In contrast to conventional T cells, which seem to accumulate a reduced sensitivity to IL-2 signaling following alloactivation, Tregs maintain a high expression of pSTAT5 following exposure to IL-2 [103]. A recent study by Chinen and colleagues showed that IL-2 induced STAT5 signaling facilitated suppressive Treg function independently of TCR activation [183]. An earlier observation by Szymczak-Workman et al. also showed that TCR signaling is not necessary for suppressive Treg function [184]. Interestingly, we demonstrated that DSCs altered T cell responsiveness by inducing a high production of IL-2. One may speculate that this mechanism may favor Tregs at the fetomaternal interface, as they are less sensitive to IL-2R depletion and can have suppressive function in presence of IL-2.

Tregs can produce IL-10 to suppress immune responses [185, 186] and are important in maintaining tolerance [187]. CTLA-4 is crucial for Treg function [188–190]. CTLA-4 binds to CD80/CD86 on APCs and through this, interaction induces indoleamine-2,3-dioxygenase (IDO) in the APCs [191, 192]. CTLA-4 has been shown to reduce the expression of costimulatory molecules on APCs [188, 193] by transendocytosis [194, 195]. The intrinsic effects of CTLA-4 on Tregs are not known and under investigation [196, 197]. Tregs may also use lymphocyte activation protein- (LAG-) 3 [198], CD39/CD73 [199, 200], inducing IL-10 and TGF-β production in APCs [201] and production of TGF-β [202] or IL-35 [203] by the Tregs for suppression.

While conventional T cells that respond to self-antigens are terminated during thymic selection, part of the naturally occurring Treg repertoire responds to self-antigens [204]. These Tregs are dependent on continuous TCR activation to maintain functionality [205]. Mapping the TCR repertoire at the fetomaternal interface and linking its reactivity to maternal and/or paternal antigens using similar assays [206, 207] could elucidate Treg and conventional/invariant T cell evolution and specificity at the fetomaternal interface. Kahn et al. showed in murine models that Tregs recognize paternal antigens and that the suppressive function of these Tregs is antigen specific [208]. In the same study, ablation of Tregs in pregnant mice using a fusion protein of IL-2 and diphtheria toxin [209] led to significantly reduced number of births and reduced fetal birth weight. Moreover, adoptive transfer of Tregs to a T cell-depleted murine model may reverse an otherwise high rate of fetal loss [210]. In a review by Erlebacher, the data regarding the antigen specificity of the Tregs at the fetomaternal interface is debated, as so far they only are able to show that fetal absorption rates increase in T cell-/Treg-depleted allogeneic *in vivo* models, whereas this is not the case in syngeneic models [6]. Thus, identifying patterns of repertoire and antigen disparity in successful and complicated pregnancies could lead to further progress in the understanding of maintaining a tolerogenic environment during pregnancy.

Induction of Tregs at the fetomaternal interface has also been associated to activity through the IDO and PD-L1 pathways [6, 211]. *In vitro*, inhibition of IDO activity reduces the frequency of Tregs in cultures with DSCs. This could not be seen when PD-L1 was neutralized [120]. One experimental study has shown that IL-10 is not necessarily needed for successful pregnancy [59] and neutralization of IL-10 does not influence stromal-induced inhibition *in vitro* [120], despite stromal cells from the decidua and umbilical cord are able to promote production of IL-10 [128]. In the same setting, neutralizing PD-L1, but not PD-L2, inhibits alloinduced proliferation [120]. These experiments suggest that many immunosuppressive functions overlap in order to maintain fetomaternal tolerance. The role of IL-10 in this setting seems to be of lesser importance if ablated alone. The immunomodulatory properties of cells originating from placental tissues still need further investigation. This is also discussed by PrabhuDas and colleagues [212]. Direct effector functions of Tregs in pregnancy and their importance for tolerance require further investigation. A summary of findings in humans regarding conventional T cells in pregnancy is shown in Table 2.

6. Lymphocytes from Placental Tissues for Adoptive Cell Therapy

The present review has discussed the heterogeneity of lymphocytes in placental tissues and their potential role in maintaining homeostasis during pregnancy. The main role of these cells in this context is summarized in Figure 1. As there has to be a completely different immune paradigm during pregnancy to maintain a low alloreactivity towards the fetus while ensuring sufficient protection towards infections, mechanisms for fetomaternal tolerance could be investigated for translational purposes in several areas. This includes improving tolerance and reducing inflammation following transplantation, prolonging remission or prevention of autoimmunity, or enhancing immunological responses towards infections. In all of these areas, lymphocytes are key components for determining outcome in patients. As the placenta is normally discarded following delivery, these tissues may provide a source for large amounts of primary effector cells that may be investigated in *ex vivo* studies.

The complexity of the to date identified mechanisms for fetomaternal tolerance highlights the difficulties of identifying effective diagnostic and treatment protocols to promote successful uncomplicated pregnancies. As we have discussed how lymphocytes exert effector functions at the fetomaternal interface, we can also identify unique

Table 2: Proportions of T cells in human placental tissues or peripheral blood and findings ex vivo relating to T cell function or outcome during pregnancy.

Cell	Compartment	Approximate abundance	Comment	Reference
$CD8^+$	T1 Dec		Reversed CD4/8 versus PB	[61, 96]
	Term Dec basalis/parietalis		Virus-specific viral control	[98]
	Term Dec basalis/parietalis	70% of $CD3^+$	↓ perforin versus PB	[100]
	Term PB		Hyporesponsiveness	[106]
	Term DSC		Hyporesponsiveness	[103]
$CD4^+$	T1 Dec	20% of $CD3^+$		[61]
Th1	T1 Dec	15% of $CD4^+$	$CCR4^-CXCR3^+CCR6^-$	[110]
	T1, T2, T3, PP PB	112, 110, 156, 53/100000 lymph	IFN-γ secreting cells	[115]
	T1 → T3 PB	20 → 17% of $CD4^+$	IFN-γ^+ ICS	[114]
	T1 → T3 PB	1% lower $CD4^+CD45RA^+$	IFN-γ^+ ICS, Rel free versus Rel MS PP	[125]
Th2	T1 Dec	5% of $CD4^+$	$CCR4^+CXCR3^-CCR6^-$	[110]
	T1, T2, T3, PP PB	84, 87, 119, 54/100000 lymph	IL-4 secreting cells	[115]
	T1→ T3 PB	2.3 → 3% of $CD4^+$	IL-4 ICS	[114]
	T3 PB	3% versus 1.5% of $CD4^+$	No PE versus PE	[114]
Th17	T1 Dec	1-2% of $CD4^+$	$CCR4^+CXCR3^+CCR6^+$, $IL17^+$ ICS	[110] [138]
	T1 → T3 PB	1-2% of $CD4^+$	$IL\text{-}17^+$ ICS	[138]
	Preterm amniotic fluid, decidua		$Il\text{-}17^+$ ICS Th17 Inflammation in hAMSC	[152]
	T1 PB		↑ IL-17/$CCR6^+$ in spontaneous abortion	[150]
Treg	T1 Dec	2% of $CD4^+$	$CD25^{high}FOXP3^{high}$	[110]
	Term Dec, PB		Dec ↑ CTLA-4^+, HLA-DR^+, $CD69^+$ versus PB	[123]
	T1, T2, T3 PB	6.7, 10.9, 8.9% of $CD4^+$	$CD4^+CD25^+$	[171]
	T2 PB	6 versus 7.5% of $CD4^+$	$FOXP3^+$ pregnant versus nonpregnant. Reduced by hormones	[178]
	Term DSC		↑ Tregs, partly by IDO	[120]

CD: cluster of differentiation; Dec: decidua; DSC: decidual stromal cells; hAMSC: human amniotic mesenchymal stromal cells; ICS: intracellular staining; IDO: indoleamine-2,3-dieoxygenase; lymph: lymphocytes; PB: peripheral blood; PE: preeclampsia; PP: postpartum; MS: multiple sclerosis; Rel: relapse; Treg: regulatory T cells; T1–3: trimester 1–3; TLR: toll-like receptor; UC: umbilical cord.

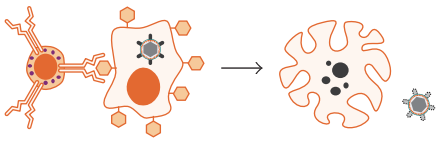
cell populations that may be of particular interest for immunotherapy applications.

The extent of placental lymphocytes for cell therapy is limited to possible autologous applications. The reduction of MS symptoms during the third trimester identifies a potential patient group that may benefit by further investigations with the approach to decrease inflammation-induced neurodegeneration using cell therapy. One cell type that critically contributes to peripheral tolerance during pregnancy is Tregs. Tregs at the fetomaternal interface have shown in murine models to have response specifically towards paternal antigens [208]. An intriguing future study would be to investigate the extent of exosome-derived peripheral fetal antigen presentation during pregnancy [213]. This can later be used to determine if the reduction of MS symptoms in the third trimester is due to altered immune homeostasis in general or perhaps a local antigen-specific response by Tregs.

Moreover, the lymphocyte population in placental tissue that differs compared to their peripheral counterpart are mainly dNK cells. However, unpredictable plasticity of placental tissue-resident lymphocytes outside the unique placental compartment is not thoroughly explored and NK cell has a high plasticity [214]. NK cells expressing a single type of killer cell immunoglobulin-like receptor are under investigation as cell therapy towards cancer.

iNKT cells are also enriched in placental tissues. Just like NK cells, iNKT cells can be activated by different receptors present on the cells. Thus, specificity of these cells can be difficult to control. iNKT cells in humans also have diverse effector functions within subpopulations, indicating a high plasticity or a higher heterogeneity than presently described [73]. Nevertheless, iNKT cells are potent cytokine-secreting effector cells and the literature is associating small quantities of these cells with large biological impact [79].

Allogeneic donor T cell therapy is utilized in transplantation to prevent leukemic relapse. Okas et al. show that T cells from umbilical cord grafts can be expanded and used as donor lymphocyte infusions (DLI) [215]. A clinical study by the same group also showed the results of treatment with these cells. The small patient material limits evaluation of clinical benefit and safety. One of the patients developed GVHD, but it is difficult to assess if this was a direct consequence of the DLI [216]. The highly immunosuppressive

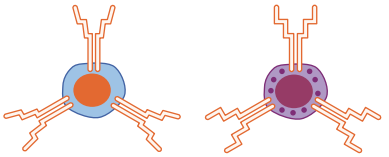


Virus-specific CTLs
+ Boosts very specific part of immune response
+ Low chance of autoimmunity
+ Increases immune response to infections
– Increases inflammation
– May reduce tolerance

dNK cells
+ Increases vascularisation
+ Increases Treg frequency
+ Increases tolerance
± Low cytotoxicity
– Extensive cytokine production, may have unclear effects on other cell types

$CD8^+$ T cells
+ Boosts response to infections
+ Less cytotoxic than blood counter parts
± Exhausted phenotype

(a)

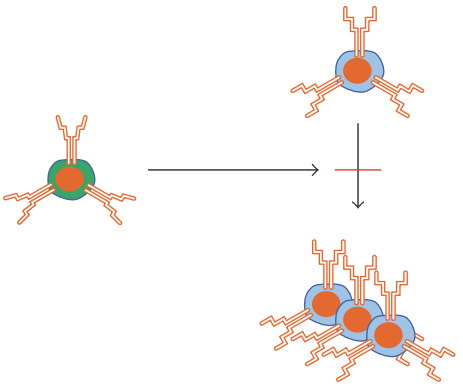


$\gamma\delta$ T cells
+ Forms a bridge between innate and adaptive system
+ Enhances trophoblast formation and invasion
± Skews towards Th2 response

iNKT cells
+ Increases Treg frequency
± Depending on situation may increase and decrease tolerance
– Extensive cytokine production, may have unclear effects on other cell types

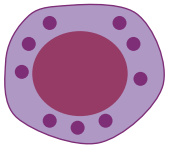
$CD4^+$ T cells
+ Specific Th1, Th2, or Th17 responses may vary in time
+ Th1 may increase tolerance via IFNγ production
– Th1 linked to preeclampsia
– Th17 promotes inflammation and autoimmunity

(b)

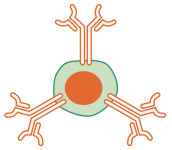


Tregs
+ Suppresses immune response
+ Improves tolerance
+ Reduces inflammation
+ Prevents autoimmunity
+ Supresses differentiation into Th17
– May decrease immune response to infections

(c)



ILCs
+ Promotes neutrophil survival and migration
+ Aids microbial defense
– Large phenotypical and functional heterogeneity



B cells/Bregs
+ Suppress immune response
+ Passive defense
– Increases chance of autoimmunity via autoantibodies

(d)

Figure 1: Summary of placental lymphocyte populations and features that may be of interest in the context of adoptive cell therapy.

state in transplantation patients is a prerequisite for the use of allogeneic T cell therapy. There are clinical trials showing that third party Epstein-Barr virus-specific T cells can be used as adoptive cell therapy [217]. T cells can be expanded a thousandfold and still maintain effector functions *in vitro* [218]. However, the benefit of isolating placental T cells compared from peripheral blood can be discussed, as autologous T cells of a high clonal diversity are present in peripheral blood and specific clones can be expanded *in vitro*.

7. Conclusion

The placenta is an interesting site of immune modulation which ensures survival of the fetus. We have discussed some of the mechanisms which may be of importance for immune tolerance during pregnancy.

As the placenta is normally discarded following delivery, it can provide an accessible source of large numbers of unique tissue-resident effector cells that can be investigated for cellular therapies; both for immune modulation and improving microbial defenses. Lymphocytes from cord blood are established for several cell therapy applications, primarily within HSCT. Although lymphocyte therapies are utilized successfully, future applications of placenta-derived lymphocytes other than from cord blood appear to be restricted to small patient groups and conditions. Cells with unique functions at the fetomaternal interface need more investigation for these purposes and possible applications still remain to be identified.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

Allogeneic Umbilical Cord-Derived Mesenchymal Stem Cells as a Potential Source for Cartilage and Bone Regeneration: An *In Vitro* Study

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Umbilical cord (UC) may represent an attractive cell source for allogeneic mesenchymal stem cell (MSC) therapy. The aim of this *in vitro* study is to investigate the chondrogenic and osteogenic potential of UC-MSCs grown onto tridimensional scaffolds, to identify a possible clinical relevance for an allogeneic use in cartilage and bone reconstructive surgery. Chondrogenic differentiation on scaffolds was confirmed at 4 weeks by the expression of sox-9 and type II collagen; low oxygen tension improved the expression of these chondrogenic markers. A similar trend was observed in pellet culture in terms of matrix (proteoglycan) production. Osteogenic differentiation on bone-graft-substitute was also confirmed after 30 days of culture by the expression of osteocalcin and RunX-2. Cells grown in the hypertrophic medium showed at 5 weeks safranin o-positive stain and an increased CbFa1 expression, confirming the ability of these cells to undergo hypertrophy. These results suggest that the UC-MSCs isolated from minced umbilical cords may represent a valuable allogeneic cell population, which might have a potential for orthopaedic tissue engineering such as the on-demand cell delivery using chondrogenic, osteogenic, and endochondral scaffold. This study may have a clinical relevance as a future hypothetical option for allogeneic single-stage cartilage repair and bone regeneration.

1. Introduction

Cartilage and bone lesions represent a common problem in the orthopaedic practice, and tissue engineering is constantly proposing innovative approaches to improve their repair. Current treatments for cartilage defects are bone marrow stimulation (microfractures), autologous osteochondral transplantation, and autologous chondrocyte implantation. However, these options have specific limitations and disadvantages: the poor quality of the repair tissue, the donor-site morbidity, and the limited availability of tissue [1]. For bone repair, the available bone substitutes are acellular and do not possess any osteogenic potential, representing simple "gap-filling scaffold" to be populated by resident cells.

To overcome these issues, the use of autologous mesenchymal stem cells (MSCs) has gained popularity due to the ability of these cells to differentiate toward chondrogenic or osteogenic pathways. Generally, MSCs are derived from bone marrow aspirations or from lipoaspirates, which contain an undifferentiated population of precursors, both $CD34^+$ and $CD34^-$ along with a great number of blood mononuclear cells: These cell concentrates are currently used for one-stage treatment of cartilage or bone defects [2–7]. The main disadvantage of this approach is the limited number of MSCs in the final product [8]. Thus, the use of selected and precultured MSCs is under investigation [9]. In this perspective, allogeneic cells would eliminate the morbidity of harvesting procedures and the costs linked to these procedures. Indeed,

a cell factory may host a great number of selected allogeneic stem cell lines from different donors, readily available for clinical use.

Besides the well-known sources as the bone marrow and the fat, new allogeneic cell sources are emerging, such as the umbilical cord stroma (UC) [8, 10–12]. The application of cells derived from UC structure has some nonnegligible advantages compared to other sources; these cells are indeed isolated from a formerly discarded material that has a virtual unlimited availability [12]. Moreover, UC contains two umbilical arteries and one umbilical vein and a mucous proteoglycan-rich connective tissue, named Wharton's jelly, covered by amniotic epithelium: Stem cells may be isolated from each of these structures with a promising efficiency [10, 13]. These cells have unique properties compared to other stem cell types as they lie between embryonic stem cells (ESCs) and adult mesenchymal stem cells (MSCs) on the development map, they share stemness markers with ESCs and MSCs, they do not induce tumorigenesis, and they are hypoimmunogenic [14]. When taken together, the different UC-MSC subtypes constitute a "mixed" heterogeneous MSC population, which is able to differentiate toward the osteogenic, adipogenic, or chondrogenic lineage [15].

Thus, UC-MSCs may represent an appealing cell source with a potential for clinical allogeneic use to treat chondral, osteochondral lesions, and bone defects, being a possible candidate for a "universal off-the-shelf" stem cell product in the field of orthopaedic tissue engineering [13].

The goal of this *in vitro* study was to evaluate the capability of allogeneic UC-MSCs to differentiate toward chondrogenic or osteogenic pathway in a tridimensional environment and to test the possibility to address these cells toward a hypertrophic stage, as a first step to recapitulate the endochondral ossification.

2. Materials and Methods

Approvals were obtained both from the Ethical Committee of MBC (Molecular Biotechnology Center), University of Turin, and from the Ethical Committee of Mauriziano Hospital, Turin (Italy); protocol number is CS792 approved on January 11, 2016.

2.1. UC Collection and Processing. After obtaining patient's informed consent, 15 fresh UC samples were retrieved during caesarean deliveries from the Department of Obstetrics and Gynecology of Mauriziano Hospital (Turin, Italy). The UC samples were collected in a phosphate-buffered saline (PBS) transfer medium containing 200 mg/100 ml ciprofloxacin, 500 IU heparin, and they were immediately processed. After transferring samples under a sterile laminar flow cell culture hood, the cord length and weight were estimated and the UC was washed in PBS to remove traces of contaminant red blood cells. The UC was first cut into 3 cm long segments, which were subsequently cut longitudinally and split open to expose the inner surface.

The UC segments were then manually minced into small cuboidal fragments (4–7 mm length). The fragments were seeded in 60 cm^2 Petri dishes with the same expansion medium in which they have been minced. This mesenchymal stem cell expansion medium contained Dulbecco's Modified Eagle Medium/F-12 (DMEM) enriched with 5% human platelet lysate obtained from healthy donors, 10% fetal bovine serum (FBS), 1X penicillin/streptomycin, 1X sodium pyruvate, 1X nonessential amino acids, 500 IU heparin (Pharmatex). The small UC fragments were distributed into 6–7 different 60 cm^2 Petri dishes (approximately 40–45 fragments/Petri dish) and incubated in the MSC expansion medium at 37°C in a humidified atmosphere with 5% CO_2 (day 0). Fragments of UC were left undisturbed in culture and monitored for up to 2 weeks to allow identification of MSC in the dishes. Cell isolation was successful for 11 samples out of 15.

2.2. UC-MSC Culture. After 2 weeks (day 14), the UC debris were removed and adherent cells were expanded for 2 additional weeks; cell expansion was reached for 11 samples out of 15. Forty percent of the medium was changed every 3–4 days. After 2 weeks, the adherent cells (P0) were trypsinized, centrifuged at 1200 rpm for 10 min, resuspended in the MSC expansion medium, and replated for one consecutive expansion step at a density of 100–200 cells/cm^2, until full confluence was reached (P1). Cell confluence at P1 was reached after approximately 14 days (day 42). At the end of P1 passage (day 42), living cells were counted by trypan blue dye exclusion.

2.3. Telomere Length Analysis. Telomere length was evaluated on UC-MSCs at P1 from 4 UCs, and results were compared to telomere length of the same cell line at sequential passages from P2 to P5, following a previously reported method [15].

2.4. UC-MSC Immunophenotypic Characterization. Immunophenotyping of the expanded UC-MSCs was done by flow cytometry analysis at P1. $1{,}5 \times 10^6$ UC-MSCs were used for flow cytometry. The following antibodies were used: CD90-peridinin chlorophyll protein- (PerCP-) cyanine dye Cy5.5 (Biolegend, San Diego, CA), CD105-fluorescein isothiocyanate (FITC) (Biolegend, San Diego, CA), CD73-allophycocyanin (APC) (BD Biosciences, San Jose, CA), CD34-phycoerythrin (PE) (BD Biosciences, San Jose, CA), HLA-DR-FITC (BD Biosciences, San Jose, CA), HLA-PerCP (BD Biosciences, San Jose, CA), HLA-ABC-PE, CD29-APC (BD Biosciences, San Jose, CA), CD44-Alexa Fluor (Cell Signaling Technology, Danvers, MA), PE-conjugated antimouse immunoglobulin G (IgG) (Southern Biotechnology Associates, Birmingham, Alabama, USA), isotype-matched IgG-FITC (Biolegend, San Diego, CA), IgG-PE (Biolegend, San Diego, CA), and IgG-PE-Cy5 (Biolegend, San Diego, CA) control antibodies. Analysis was performed on a FACScan (Becton Dickinson (BD), Buccinasco, Italy) for at least 10.000 events and using CellQuest software (BD, Buccinasco, Italy).

2.5. Section 1: UC-MSC Differentiation on Chondrogenic Scaffold. UC-MSCs at P1 were assessed for chondrogenic differentiation on scaffolds. UC-MSCs were loaded onto two different scaffolds: Hyaff-11 (FIDIA Advanced Biopolymers,

Italy) or Chondro-gide (Geistlich Biomaterials, Italy S.r.l.) membranes. Hyaff-11 is a nonwoven esterified HA derivative membrane. Chondro-gide is a double-layer matrix of pig collagen type I and type III, with a smooth compact side and a porous side, where cells are seeded. After the first passage of cell culture, 2×10^6 UC-MSCs were resuspended in 50 μl of the chondrogenic differentiation medium and seeded onto a scaffold surface of 1 cm^2 (either Hyaff-11 or Chondro-gide). The scaffolds were left at 37°C in a humidified atmosphere with 5% CO_2 for 3–4 hours to allow UC-MSC adhesion on the scaffolds. Then, two drops of commercial fibrin glue (Tissucol-Tisseel, Baxter) were added as a surface sealing, and the final constructs were incubated at 37°C, in a humidified atmosphere for 1 month in the presence of the chondrogenic differentiation medium (EUROMED Chondrogenic Differentiation Kit, EuroClone, Pavia, Italy). Cultures were performed both in normoxic conditions (21% O_2) or at low oxygen tension (8% O_2).

After 1 month, constructs were fixed in formalin, included in paraffin, and sectioned. Sections were stained for haematoxylin/eosin and examined under light microscopy. For cell counting, three different areas in two different sections per construct were examined under light microscopy at 20x magnification by two independent observers. Cell number for each single area was defined by the arithmetical mean of the cell counts from both observers. Mean numbers of migrating cells from every area were statistically compared and graphed with GraphPad Prism®. Safranin o staining was also performed on sections.

Expression of chondrocyte markers, sox-9 (AB5535, Merck Millipore, Milano, Italy), and collagen type II (clone 6B3, MAB8887, Merck Millipore, Milano, Italy) was assessed using immunofluorescence techniques. The primary monoclonal antibodies were diluted in PBS-BSA1% and incubated with the sections for 2 h at room temperature. The secondary dye light 488 antibody (KPL, Kirkegaard & Perry Laboratories, Maryland, USA), diluted 1 : 100, was incubated for 1 h at room temperature. The stained sections were visualized with an Apotome fluorescence microscope. We collected digital images using a ×20 dry lens within 0–5 days after labelling.

The same culture conditions were used for UC-MSC pellet culture. Proteoglycan (PG) : DNA ratio was calculated as the best approximation of ECM production per cell following a previously reported method [16].

2.6. Section 2: UC-MSC Differentiation in Osteogenic Scaffold. For osteogenic differentiation on scaffold, we used the Orthoss® bone graft (Geistlich Biomaterials, Italy S.r.l.), a bovine-derived natural commercial bone substitute. Its inorganic bone matrix has a macro- and microporous structure similar to human cancellous bone. Scaffolds were cut into cubes of approximately 1 cm^3. The cubes were then coated with fibronectin by soaking in a solution containing 50 mg/ml fibronectin for 4 h at room temperature. The cubes were air dried overnight in a sterile bio safety cabinet. The cubes were seeded with UC-MSCs resuspended in fibrin glue (6×10^6 cells/scaffold). Then the osteogenic differentiation medium was added (EUROMED Osteogenic Differentiation Kit, EuroClone, Pavia, Italy), and constructs were incubated at 37°C in a humidified atmosphere with 5% CO_2 for 10, 20, and 30 days.

At the end of culture, constructs were initially decalcified and then fixed in formalin, included in paraffin, and sectioned. Sections were stained for haematoxylin/eosin and Alizarin red and examined under light microscopy. Expression of osteocalcin ([Tyr28-, Phe42-, and Phe46-] bone Gla protein, Phoenix Pharmaceuticals Inc., Burlingame, CA, USA) and core-binding factor subunit alpha-1/runt-related transcription factor 2 Cbfa1/RunX-2 (Ab 114,133, Abcam, Cambridge, MA, USA) markers was assessed using immunofluorescence techniques described above. The stained sections were visualized with an Apotome fluorescence microscope. We collected digital images using a × 20 dry lens within 0–5 days after labelling.

2.7. Section 3: UC-MSC Hypertrophic Differentiation. To verify the UC-MSC potential for endochondral differentiation, cells at P2 were seeded onto Orthoss 3 g granules of approximately 4 mm^3 of volume (concentration: 25×10^4 cells/granule) and incubated at 37°C in a humidified atmosphere with 5% CO_2, in three different conditions:

(i) Hypertrophic culture (3 weeks + 2 weeks)

  (1) Chondrogenic medium (Chondrogenic Differentiation Kit, EuroClone), for 3 weeks

  (2) Hypertrophic medium (DMEM, 10 mM Hepes Buffer, 1 mM Na pyruvate, 1% penicillin/streptomycin/glutamine, 1% ITS-A, 4.7 μg/ml linoleic acid, 1.25 mg/ml human serum albumin, 0.1 mM ascorbic acid 2-phosphate, 10^{-8} M dexamethasone, 10 mM β-glycerophosphate, 0.05 μM L-thyroxin) for the following 2 weeks

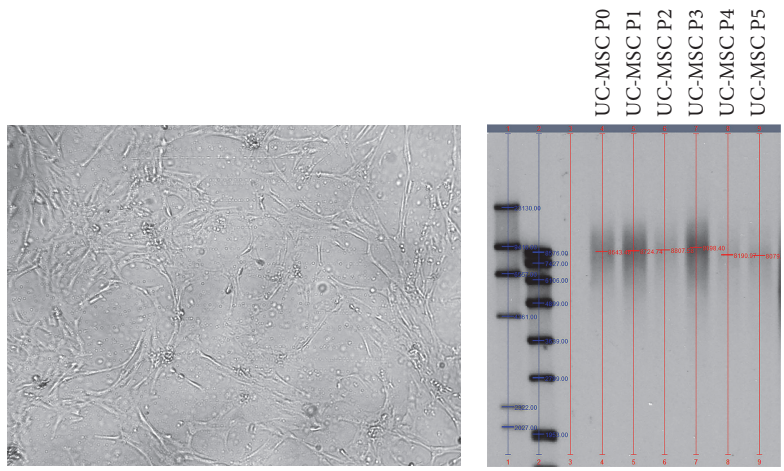
  This peculiar condition was compared with two other culture conditions:

(ii) Osteogenic culture (αMEM, 10% fetal calf serum, 0.1 mM ascorbic acid 2-phosphate, 10^{-8} M dexamethasone, 10 mM β-glycerophosphate) for 3 and 5 weeks

(iii) Basal (control) culture: DMEM +10% fetal calf serum, 50 U/ml penicillin/streptomycin for 3 and 5 weeks

At the end of culture (3 and 5 weeks), H&E and safranin o staining were performed, following the manufacturer's protocols (Bio-Optica Milano SpA, Milan, Italy). Furthermore, real-time PCR analysis was completed to analyze gene expression of the major chondrogenic and osteogenic markers (sox-9, Cbfa1).

2.7.1. RT-PCR Analyses. Total RNA was extracted from constructs using TRIzol® (Life Technologies), and cDNA was generated as previously described [17]. The PCR master mix was based on AmpliTaq Gold DNA polymerase (Perkin Elmer/Applied Biosystems). TaqMan® gene expression or on-



(a) (b)

Figure 1: UC-MSC morphological characterization. (a) UC-MSCs at P1. Cells show a fibroblast-like morphology. Magnification 10x. (b) UC-MSC telomere length analysis at different passages (between P1 and P5); no difference is detected; n of samples = 4.

demand assays (Life Technologies) were used on a ABI 7900 fat real-time PCR system (Life Technologies) for 40 cycles to measure gene expression of sox-9 (Hs00165814_m1) and Cbfa1 (Hs00231692_m1) using GAPDH (Hs99999905_m1) as the housekeeping gene.

2.8. Evaluation of Fluorescence Intensity. The difference of fluorescence intensity between the constructs was evaluated using ImageJ program. This software generated numerical semiquantitative evaluations corresponding to the mean fluorescence intensity of each image examined. Ten cellular fields were randomly chosen among the different areas of migrated chondrocytes in each slide. Briefly, a point tool enables the marking of locations on an image. With each "click," the coordinates of the mark (*xx*, *yy*) and brightness values (0–255) are recorded in a data window. ImageJ brightness units are in a scale where 0 = pure black and 255 = pure white. Brightness values for each image were calculated as the arithmetical mean of all values in all fields recorded for that image. For each construct, mean fluorescence intensity of each marker was calculated and plotted as a graph. The difference in intensity allowed for evaluating the change in marker expression between the different culture conditions.

2.9. Statistical Analysis. All data in text and figures are provided as medians. Statistical analysis was carried out with the statistical software package GraphPad Prism 5.0. The results are shown as box plots, where the transverse line represents the median value, and the width of the box is given by the minimum and the maximum value of the data. If only two conditions are compared, we used Mann–Whitney test and we did not assume Gaussian distribution; if more than two conditions are compared, we used one-way ANOVA and Bonferroni adjustment.

3. Results

3.1. UC-MSC Morphologic and Immunophenotypic Characterization. In primary cultures, typical spindle-shaped adherent cells were observed migrating from the UC tissue fragments and initiating colony formation approximately at day 14 after seeding. After removing the UC fragments at day 14 postseeding, cells took approximately 10 days to gain 60% confluence (Figure 1(a)), while full confluence was observed approximately at day 28 postseeding. The UC-MSC clones (defined as passage P0) were thus collected at day 28 postseeding and replated for further expansion (defined as passage P1). Confluence at P1 was observed approximately after 14 days of culture (day 42 postseeding).

At day 42 (confluence at P1 passage), we obtained a mean of 23.05×10^6 (SD 1.48) cells from each umbilical cord. From the initial seeding (day 0), we obtained at the end of the P1 (day 42) 0.80×10^6 (SD 0.28) cells/g of UC seeded (mean weight of the UC 30.65 g − mean length 40.9 cm) (Supplemental Figure 1 available online at https://doi.org/10.1155/2017/1732094). UC cells' phenotype was analyzed by flow cytometry. The majority of collected UC cells showed a positive expression of the main MSC markers CD73, CD90, and CD105, as well as of CD44 and CD29. Furthermore, they stained negative for the typical hematopoietic marker CD34 (Supplemental Figure 2). The data also demonstrated the presence of HLA-ABC proteins and the absence of HLA-DR. Additionally, we visualized a notable presence (40%) of negative double cells for both HLA-ABC and HLA-DR proteins.

Telomere length analysis performed on UC-MSCs at different culture passages (from P1 to P5) did not show any significant difference (Figure 1(b)).

3.2. Section 1: Chondrogenic Differentiation in Scaffold. During chondrogenic differentiation onto Chondro-gide and Hyaff-11 scaffolds, cells showed chondrogenic commitment both in normoxic conditions and in low oxygen tension, albeit UC-MSCs cultured at lower oxygen tension showed more positive safranin o staining, consistent with increased sulfated glycosaminoglycan (s-GAG) production (Figure 2(a), C and D). Furthermore, histological analysis

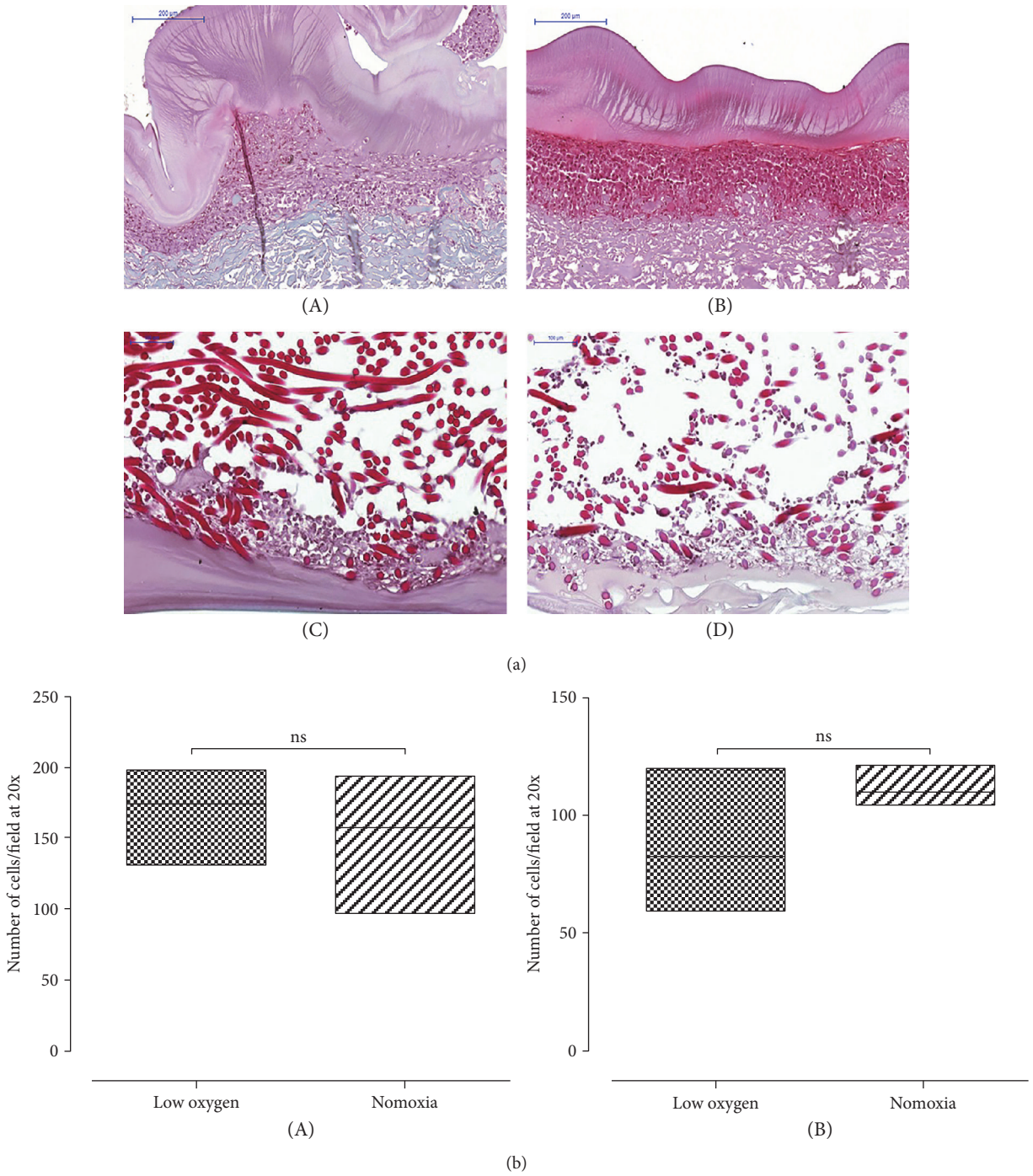


Figure 2: Chondrogenic differentiation of UC-MSCs seeded on a scaffold. (a) A representative image of safranin o staining of UC-MSCs seeded onto Chondro-gide (A and B) or Hyaff (C and D) membranes and cultured in normoxic (A and C) or low oxygen tension conditions (B and D). UC-MSCs differentiate inside the three-dimensional structure of the HYAFF-11, while they are confined to the surface of the Chondro-gide scaffold. A stronger safranin o staining is observed in the low oxygen tension constructs than in normoxic culture. Magnification 20x. Scale bar: 200 μm in (A and B); 100 μm in (C and D). (b) No significant difference in cell number per field is observed comparing normoxic and in low oxygen tension conditions in Chondro-gide (A) (median value and standard deviation: 174 ± 32.07; 157.5 ± 40.32 for low oxygen tension and normoxia, resp.) and Hyaff (B) (median value and standard deviation: 82.50 ± 24.96; 110 ± 6952 for low oxygen tension and normoxia, resp.) scaffolds. p value > 0.05; n of samples $= 3$.

showed that MSCs migrated inside the tridimensional structure of the Hyaff-11 scaffold, while they remained almost complete at the porous surface on the Chondro-gide membrane (Figure 2(a), A and B), probably due to the different composition of the scaffolds. No significant difference in the number of cells per field was observed between normoxic cultures and low oxygen tension conditions for each specific scaffold (Figure 2(b)).

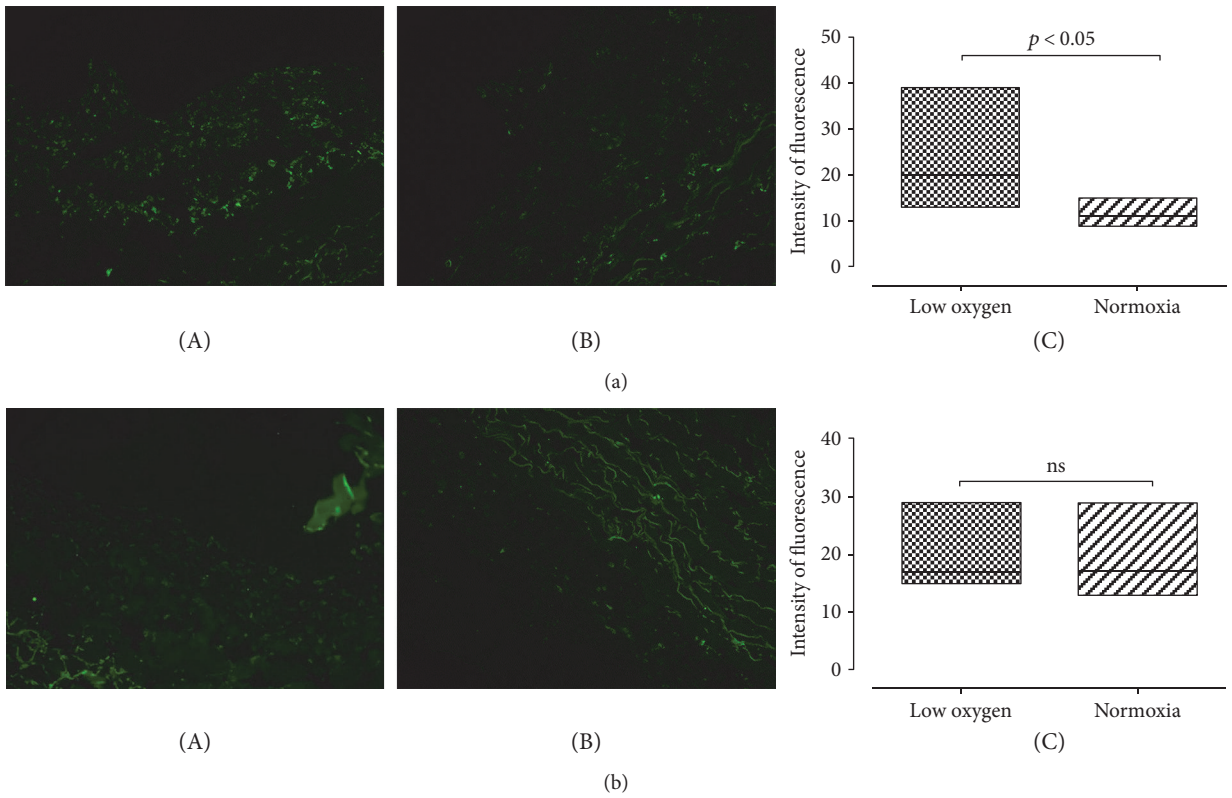


Figure 3: Chondrogenic differentiation of UC-MSCs seeded on Chondro-gide. (a) A representative image of immunofluorescence staining for sox-9 in Chondro-gide scaffold at low oxygen tension (A) and normoxic (B) conditions. Quantification of the signal is shown in (C): Fluorescence intensity is significantly higher in constructs grown at low oxygen tension (median value and standard deviation: 20 ± 6122; 11 ± 2102 for low oxygen tension and normoxia, resp.). p value < 0.05. Magnification 20x; n of samples $= 3$. (b) A representative image of immunofluorescence staining for collagen type II in Chondro-gide scaffold at low oxygen tension (A) and normoxic (B) conditions. Signal quantification is shown in (C); no significant difference in fluorescence intensity is observed (median value and standard deviation: 17 ± 3279; 17 ± 4025 for low oxygen tension and normoxia, resp.). p value $= 0.6926$. Magnification 20x; n of samples $= 3$.

In Chondro-gide scaffolds, positive immunostaining for sox-9 was present both in normoxic culture and at low oxygen tension conditions (Figure 3(a), A and B). A significantly higher fluorescence intensity was observed in constructs cultured art low oxygen tension (p value < 0.05) (Figure 3(a), C). Collagen type II expression in Chondro-gide scaffold was present both in low oxygen tension and under normoxic conditions (Figure 3(b), A and B); however, fluorescence intensity was not significantly different (p value $= 0.6926$) (Figure 3(b), C). Negative controls are shown in Supplemental Figure 3.

In Hyaff-11 scaffolds, we noticed a similar trend: Positive immunostaining for sox-9 was noticed both at low oxygen tension and in normoxic cell cultures (Figure 4(a), A and B). The difference in fluorescence intensity resulted statistically significant (p value < 0.05) (Figure 3(a), C). Collagen type II expression in Hyaff-11 scaffolds was significantly greater at low oxygen tension (p value < 0.05) (Figure 4(b), C). Negative controls are shown in Supplemental Figure 4.

A similar trend was observed in UC-MSC pellet cultures when exposed to low oxygen tension during culture period. We observed a stronger safranin o staining (Figures 5(a) and 5(b)) and higher PG/DNA ratio (Figure 5(c)) in pellet culture grown at low oxygen tension compared to those exposed to normoxic conditions (p value < 0.05).

3.3. Section 2: Osteogenic Differentiation in Bone Substitutes. Scaffolds showed a considerable increasing cellularity in constructs at different time points (10, 20, and 30 days) (Figure 6(a)). Alizarin red stain showed calcium deposits gradually increasing from 10, 20, to 30 days (Figure 6(b)). Semiquantitative analysis of osteocalcin immunostaining (Figure 7(a), A–C) showed a significant increased intensity of osteocalcin expression between 20 days and 30 days; for the other time points, there was an increased expression, though it did not reach a significant difference (Figure 7(a), D; p value < 0.05). Similar results were obtained for the expression of the transcriptional factor RunX-2, (Figure 7(b), A–C) which is a key transcription factor associated with osteoblast differentiation (Figure 7(b), D) (p value < 0.05). Negative controls are shown in Supplemental Figure 5.

3.4. Section 3: Endochondral Differentiation in Bone Substitute Granules. At 3 weeks of culture, deposition of a cartilaginous matrix was observed in the samples cultured with the chondrogenic induction medium, while there was

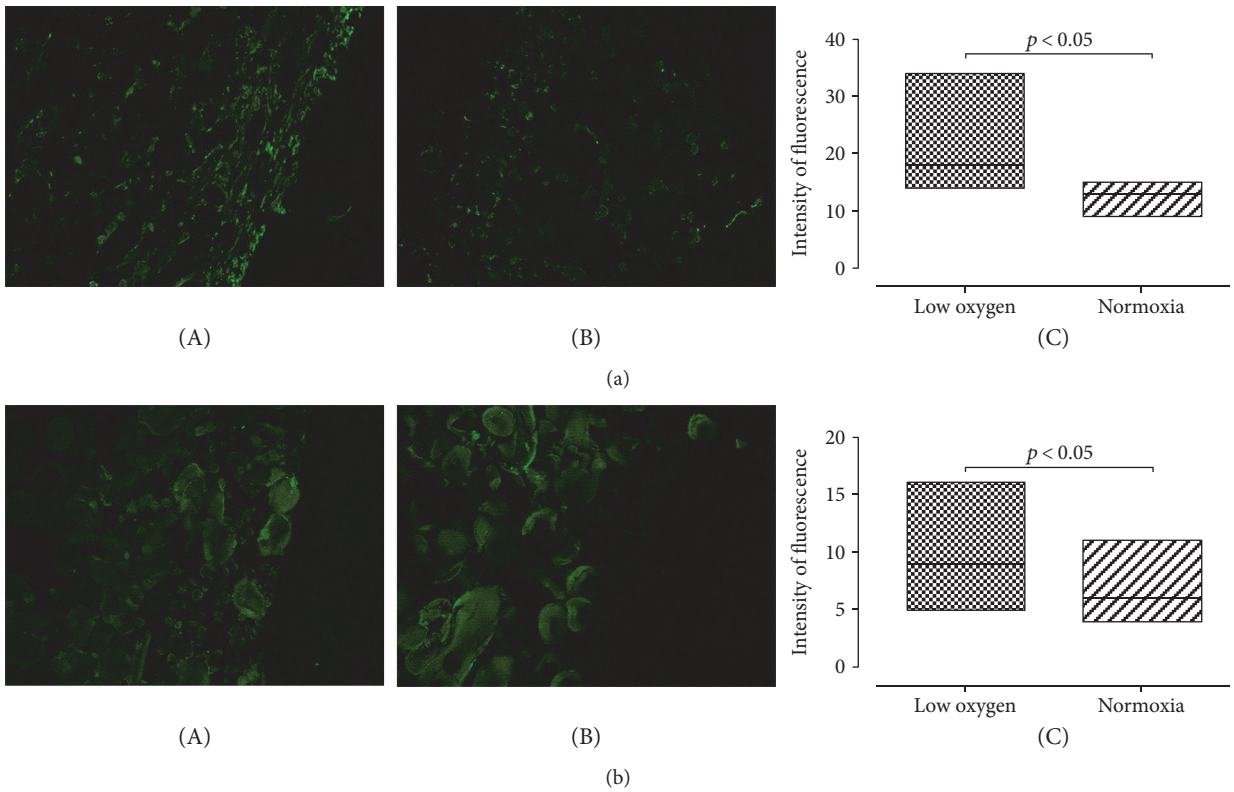


Figure 4: Chondrogenic differentiation of UC-MSCs seeded on Hyaff. (a) A representative image of immunofluorescence staining for sox-9 in Hyaff scaffold at low oxygen tension (A) and normoxic (B) conditions. Quantification of the signal is shown in (C): Fluorescence intensity is significantly higher in constructs grown at low oxygen tension (median value and standard deviation: 18 ± 5194; 13 ± 1917 for low oxygen tension and normoxia, resp.). p value < 0.05. Magnification 20x; n of samples $= 3$. (b) A representative image of immunofluorescence staining for collagen type II in Hyaff scaffold at low oxygen tension (A) and normoxic (B) conditions. Signal quantification is shown in (C): Fluorescence intensity is significantly higher in constructs grown at low oxygen tension (median value and standard deviation: 9 ± 2601; 6 ± 1594 for low oxygen tension and normoxia, resp.). p value < 0.05. Magnification 20x; n of samples $= 3$.

no evidence of osteogenic differentiation in specific differentiation medium (Figure 8(a)). At 5 weeks of culture, we observed a stronger safranin o staining in samples cultured in the chondrogenic hypertrophic medium, as well as the production of bone matrix in samples cultured in the osteogenic differentiation medium (Figure 8(b)).

Real-time PCR analysis showed a mild upregulation of sox-9 expression in samples cultured for 3 weeks in the chondrogenic medium. Also, the osteogenic marker Cfba-1 was upregulated in samples cultured both in chondrogenic and in the osteogenic medium. After 5 weeks of culture, sox-9 expression was downregulated in samples cultured with the chondrogenic hypertrophic medium, whereas Cfba-1 was upregulated. The osteogenic medium induced a strong Cfba-1 upregulation (Figure 9).

4. Discussion

The main finding of this study is that UC-MSCs collected in a straightforward and simple procedure from minced umbilical cord fragments may be committed toward chondrogenic and osteogenic lineages, when cultured in scaffolds, and they may also be addressed toward hypertrophy when cultured in bone substitute in the presence of the chondrogenic and hypertrophic chondrogenic medium. Thus, UC-MCs may represent an allogeneic cell population with a promising value for on-demand cell delivery in single-stage cartilage repair and bone regeneration.

Nowadays, the use of allogeneic cells for cartilage and bone repair is an ongoing frontier due to the increasing need of cells for better specific tissue repair.

Indeed, the simple use of bone marrow stimulating techniques, such as microfracture, has shown some limitations linked to the restricted durability of the repair and the lesser quality of the tissue obtained, compared to that achieved by more complex reconstructive techniques as autologous chondrocyte implantation [18–20] or scaffold-driven repair enriched by autologous bone marrow MSCs [21]. Even the use of bone substitutes has shown lesser results when compared with techniques combining bone substitute with biological stimuli as autologous bone marrow MSCs or autologous stem cell mobilization through growth factors as G-CSF (granulocyte colony stimulating factor) [22, 23]. However, the use of an autologous cell source has several disadvantages mainly due to the morbidity of the harvesting procedure, the individual variability in the cell number, and the limited number of cells available by each harvesting procedure. Moreover, the use of selected precursor cells (i.e., precultured autologous stem cells, chondrocytes) is necessarily associated with multiple

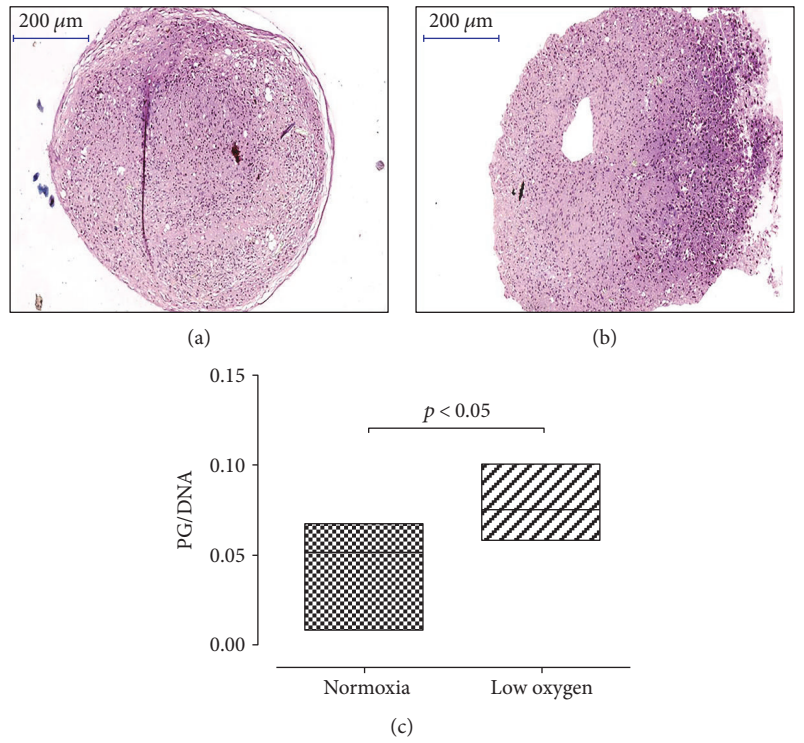


Figure 5: UC-MSC chondrogenic differentiation in pellet culture. A representative image of safranin o staining of UC-MSCs in pellet culture (a and b). Stronger safranin o staining is observed in UC-MSC pellets cultured at low oxygen tension (a) when compared to normoxic conditions (b). Higher PG/DNA ratio in pellets cultured at low oxygen tension is shown in (c) (median value and standard deviation: 0.05166 ± 0.02240; 0.07468 ± 0.01687 for and normoxia and low oxygen tension, resp.). p value < 0.05. Magnification 20x. Scale bar: 200 μm. n of samples = 3.

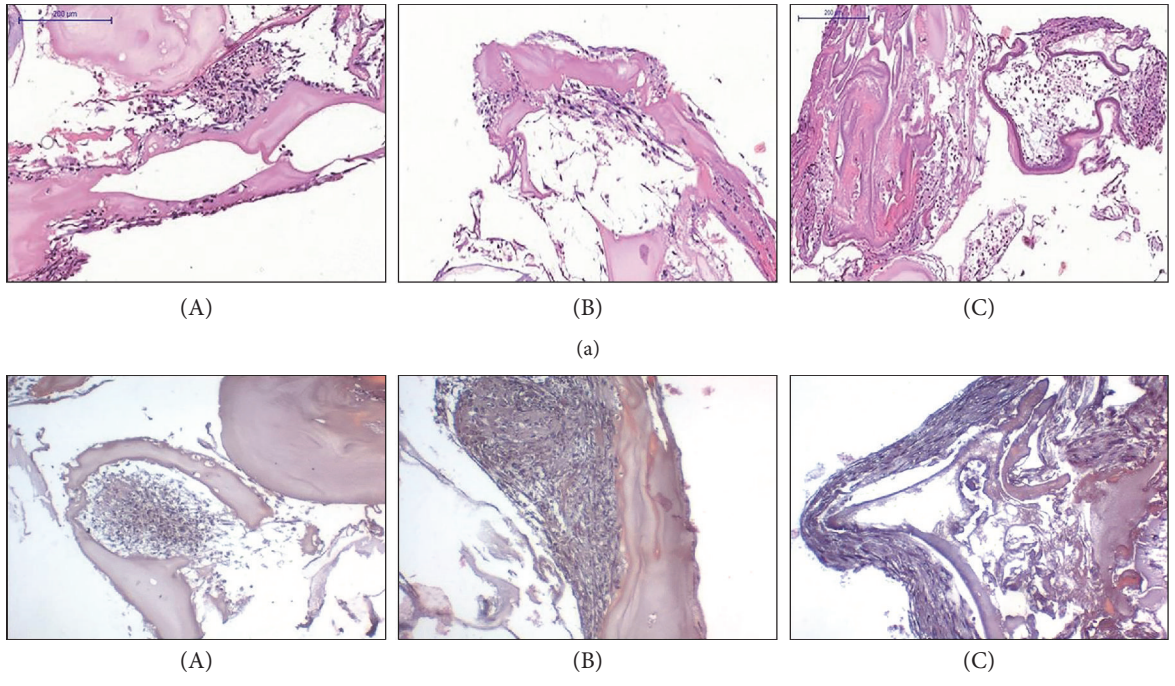


Figure 6: UC-MSC osteogenic differentiation on the Orthoss scaffold. (a) A representative image of H&E staining of UC-MSCs loaded onto Orthoss scaffold at 10 (A), 20 (B), and 30 (C) days of in vitro culture. Magnification 20x. Scale bar: 200 μm. (b) A representative image of Alizarin red staining of UC-MSCs loaded onto Orthoss scaffold at 10 (A), 20 (B), and 30 (C) days of in vitro culture. Magnification 20x. Scale bar: 100 μm.

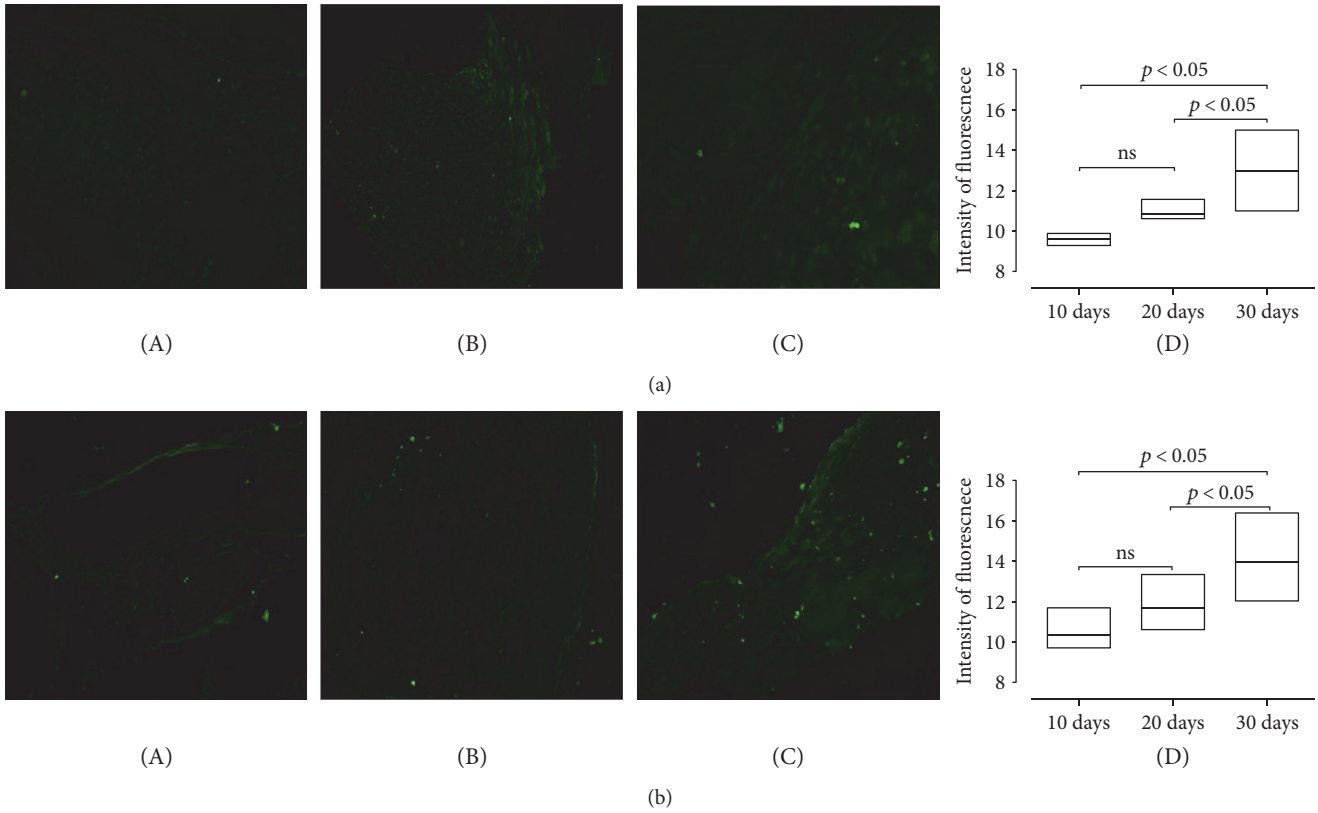


Figure 7: Expression of osteogenic markers in UC-MSCs seeded onto Orthoss cubes. (a) A representative image of immunofluorescence staining for osteocalcin in Orthoss at 10 (A), 20 (B), and 30 (C) days of *in vitro* culture. Quantification of the signal is shown in (D): Fluorescence intensity is significantly higher in samples cultured for 30 days (median value and standard deviation: 9604 ± 0.2584; 10.85 ± 0.3990; 13 ± 1030 in samples cultured for 10, 20, or 30 days, resp.). p value < 0.05. Magnification 20x; n of samples $= 5$. (b) A representative image of immunofluorescence staining for RunX-2 in Orthoss at 10 (A), 20 (B), and 30 (C) days of *in vitro* culture. Quantification of the signal is shown in (D): Fluorescence intensity significantly increases in the longer experimental time points (median value and standard deviation: 7 ± 1340; 11 ± 1712; 18 ± 3090 in samples cultured for 10, 20, or 30 days, resp.). p value < 0.05. Magnification 20x; n of samples $= 5$.

procedures to obtain the primary autologous source, to culture the cells and to reimplant the cells at the lesion site. An ongoing solution proposed in literature is the "one-step procedures" in which autologous unselected sources of cells, as bone marrow concentrate [24], cartilage fragments [1, 25], or stromal vascular fraction from lipoaspirates [5] are added at the lesion site (cartilage or bone defect) obtaining interesting results, even if in these instances, a noncommitted cell population is used to enhance the repair.

In line with the concept of "one-step procedure," the evolving technologies in cell storage and cell culture allow for hypothesizing the use of allogeneic cells as an attractive choice for cartilage and bone repair, due to the greater bioavailability of allogeneic sources compared to the autologous ones. Thus, the concept of allogeneic stem cell therapy is becoming an ongoing reality. Indeed, in several clinical fields, such as neurology, gastroenterology, or hematology, these principles are used in experimental studies to treat different diseases like cerebral palsy [26], autoimmune encephalomyelitis [27], perianal fistulas in Crohn's disease [28], liver failure [29], and aplastic anemia [30]. The main advantages in the use of allogeneic cells in orthopedics are a greater "on-demand" availability of cell precursors, the absence of harvesting morbidity, the possibility to obtain a selected cell population, and even the possibility to use cells from younger donors for lesions in older recipient, further optimizing the quality of the repair. The latest aspect has recently found a preclinical application in the study of Bonasia et al. [1], in which allogeneic juvenile cartilage fragments have been used to improve the quality of cartilage repair in a rabbit model obtaining positive results. This work suggests the possibility to use juvenile fresh allogeneic tissue grafts as a source for the repair. This concept is certainly an option for bone and cartilage reconstruction, but it implies several drawbacks as the prompt bioavailability of the specific tissues and the costs of tissue preservation, posing some concerns about a widespread application of these principles.

A different solution is to use allogeneic candidates from different anatomical sites as bone marrow and adipose tissue. In the *in vivo* study by de Windt et al. [31], cartilage defects are treated by combining allogeneic bone marrow MSCs with autologous chondrons obtained by digesting minced cartilage fragments. Result of this phase I study seems promising, though in that case, the length of the entire procedure (i.e., obtaining autologous chondrons + combination of the 2

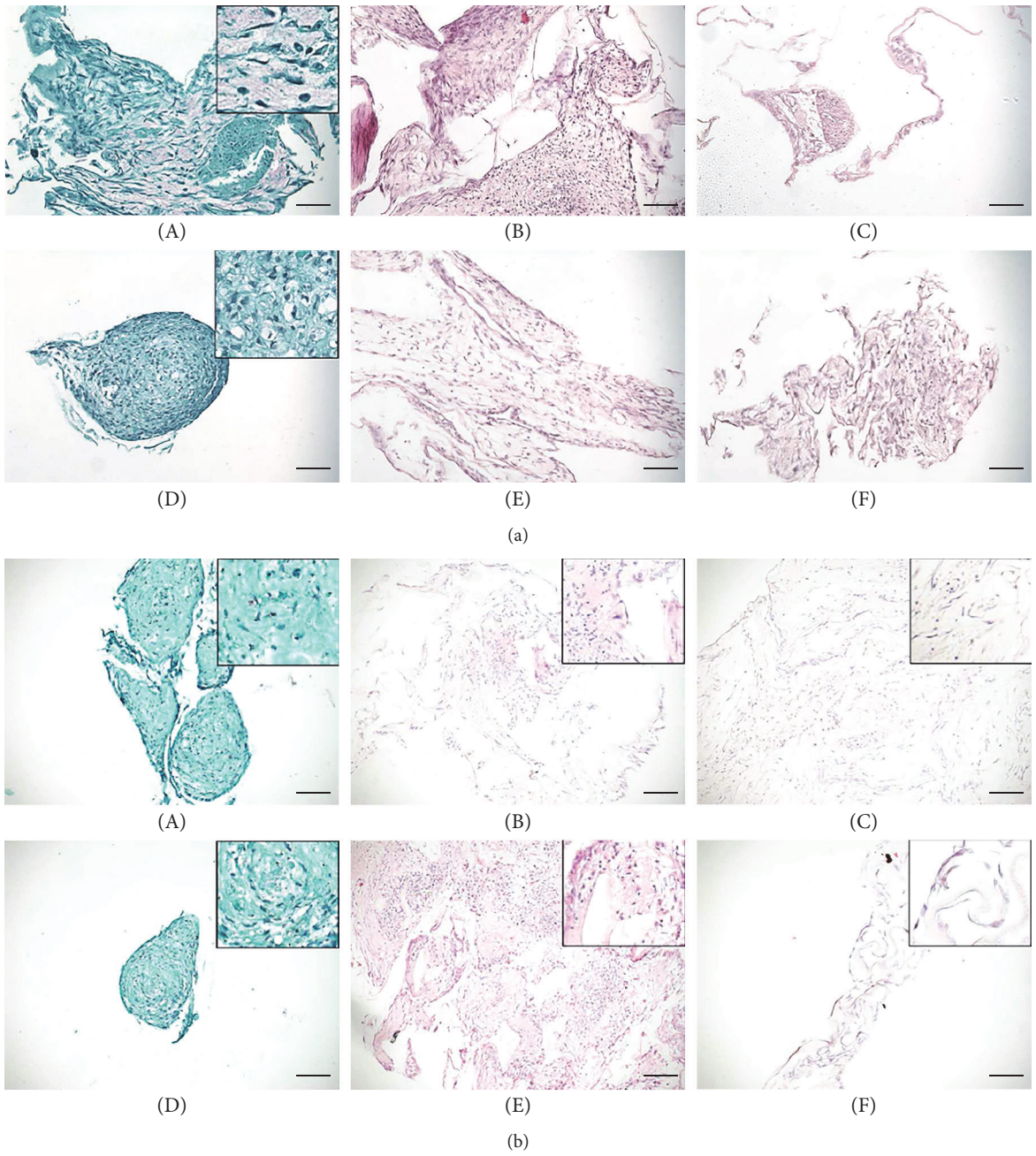


Figure 8: Hypertrophic differentiation of UC-MSCs seeded onto Orthoss granules. (a) A representative image of safranin o and H&E staining of UC-MSCs seeded onto Orthoss granules and cultured for 3 weeks in the hypertrophic medium (Safranin O stain, A and D), osteogenic medium (H&E stain, B and E) or basal medium (H&E stain, C and F), respectively. Scale bar: 100 μm; n of samples = 5. (b) A representative image of safranin o and H&E staining of UC-MSCs seeded onto Orthoss granules and cultured for 5 weeks in the hypertrophic medium (Safranin O stain, A and D), osteogenic medium (H&E stain, B and E), or basal medium (H&E stain, C and F), respectively. Scale bar: 100 μm; n of samples = 5.

sources of repair + surgical implantation) may represent a drawback, if compared to more "straightforward" procedures in which a cellularized scaffold is directly implanted at the lesion site. In preclinical rabbit and minipig models, allogeneic bone marrow-derived MSC implantation has been proposed for the treatment of knee osteochondral defects with promising results [32, 33]. Bone regeneration has been obtained in a study by Kang et al. [34] loading allogeneic bone marrow MSCs onto allogeneic cancellous bone granules in a rabbit radial defect model, with a quality of repair comparable to that with autologous BM-MSCs. In several preclinical studies, allogeneic adipose-derived MSCs (ASCs) have also improved bone and cartilage repair. In the study conducted by Wen et al. [35], ASCs have been combined with demineralized bone matrix (DBM) have been applied in ulnar bone defects in rats with promising results. Even the intra-articular injection of allogeneic, ASCs have led to satisfying outcomes when combined with hyaluronic acid

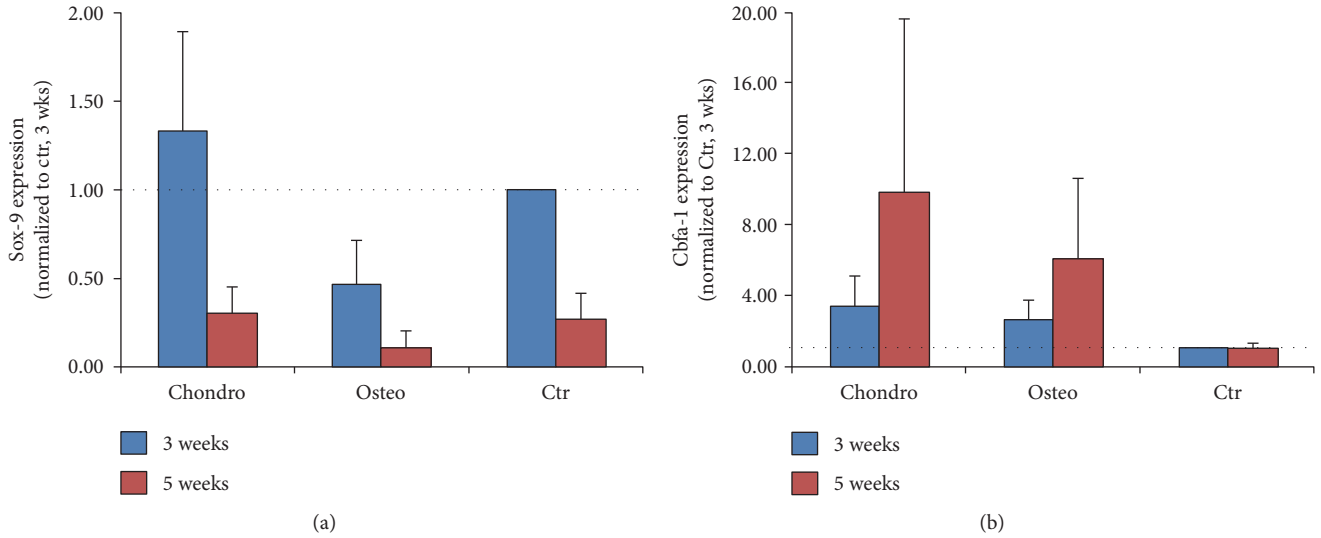


Figure 9: Expression of osteogenic markers in UC-MSCs undergoing hypertrophy. Quantification of sox-9 (a) and Cfba-1 (b) gene expression by RT-PCR analysis at 3 or 5 weeks of culture in the three experimental groups. GAPDH used as gene of reference; *n* of samples = 5.

in preclinical models of dog arthropathy [36, 37] and sheep osteoarthritis [38].

The umbilical cord-derived MSCs (UC-MSCs) may represent a novel interesting alternative in this field [39]. The umbilical cord is considered a discarded material, and its use implies fewer ethical and legal issues than the other embryonic structures (i.e., embryonic stem cells). Furthermore, it has a virtually "unlimited availability," as recently defined by Kalaszczynska and Ferdyn [8]. Moreover, the low costs and the absence of morbidity related to the collecting procedure are a "nonnegligible" advantage compared to the other common allogeneic cell sources, such as bone marrow and adipose tissue.

In literature, UC-MSCs have shown a favorable *in vitro* potential as they can differentiate toward both the chondrogenic and the osteogenic lineage similarly to the bone marrow MSCs [40, 41]. Indeed, like the bone marrow counterpart, UC-MSCs react to low oxygen tension conditions [42] or to pulsed electromagnetic fields [43] showing an improved chondrogenic commitment. The promising multidifferentiation potential may be due to the peculiar site of origin leading to a "more embryonic" feature than the bone marrow counterpart [14, 44, 45]. Indeed, they have been recently used *in vivo* as described by Sadlik et al. [46] involving the dry arthroscopic treatment of cartilage lesions with UC-MSCs embedded in a porcine type I/II collagen matrix. Dilogo et al. [47] also used UC-MSCs for the treatment of a critical-sized bone defect; however, literature suggests caution before a widespread clinical application, claiming for a better understanding of the functional characteristics of these cells [10]. Nonetheless, a recent evidence has added a further promising opportunity in the field of UC-MSCs. In recent studies, Mennan et al. [48] and Hendijani et al. [49] have demonstrated that a mixed cell population obtained by processing the whole umbilical cord seems to have the same differentiation potential than cells derived from single areas of the cord (Wharton's jelly, artery, vein, or cord lining). These findings might suggest the possibility to obtain optimal precursor cells without any concern about the separation of specific population, simplifying any theoretical use of the cord as a source of cells for future hypothetical widespread clinical application.

In line with these perspectives, our study has analyzed the *in vitro* chondrogenic and osteogenic commitment of a mixed UC-MSC population, obtained with a very simple and economic protocol without any enzymatic digestion. No cell selection has been performed during the MSC extraction from cord stroma, but the adherent properties of UC-MSCs and the simple mincing of the cord fragments represented the essential steps of our cell isolation method. This choice is in line with the aforementioned evidences in literature [48, 49], and it allows for greatly simplifying the cell harvesting without reducing the efficiency of the protocol, as recently outlined by Yoon et al. [50].

Both human platelet lysate obtained from healthy donors and fetal bovine serum (FBS) were used for culture, as described in a previously published paper [15], in order to optimize the broth conditions for the cells. For a human hypothetical application, the FBS may arise ethical concerns [51]. Thus, the choice of using human platelet lysate only seems to be preferable for future human trials. Further study might compare the efficacy of this method with and without FBS to ensure that an adequate number of cells may be obtained in both conditions.

Indeed, our protocol led to a consistent number of cells per gram of tissue, thus suggesting a possible future large-scale nonexpensive cell storage for clinical purposes, as Kalaszczynska and Ferdyn envisioned in a recent review [8].

UC-MSCs obtained with our method have shown stemness properties in terms of markers and telomere preservation, the latter suggesting a maintenance of cell viability, as recently outlined in literature [8]. Moreover, UC-MSCs showed a low expression of HLA-I and no expression of

HLA-II, consistent with a possible safe allogeneic use. This concept is confirmed by a recent study by Liu et al. [44] in which the authors demonstrated that the immunoprivileged status of UC-MSCs seems to be maintained even during the differentiation process toward a specific mesenchymal (i.e., chondrogenic) lineage. Furthermore, the finding of an "HLA-I and HLA-II double negative" cell subpopulation suggests peculiar immunoprivileged properties of these cells that may deserve future studies to verify the possible isolation of this cellular subtype, their specific differentiation potentials, and their hypothetical elective use as preferential candidate for allogeneic therapies.

To test UC-MSC chondrogenic commitment in a tridimensional environment, two widely used scaffolds have been compared (Chondro-gide and Hyaff). We have observed cell growth on both membranes, with differences in cell distribution depending on the tridimensional scaffold structures. Nevertheless, in both experimental groups, cells showed a chondrogenic phenotype and they stained positive for the chondrogenic markers sox-9 and collagen type II. Notably, the low oxygen tension exerted an influence on UC-MSCs, similar to bone marrow-derived MSCs [52]. Specifically, low oxygen tension did not hamper UC-MSC proliferation potential, but it led to an increased matrix production in UC-MSC pellet cultures and to an enhanced chondrogenic marker expression in UC-MSC scaffold cultures, consistent with previous studies [42]. This increased chondrogenic differentiation might be the result of hypoxia-inducible factor-1 alpha and factor-2 alpha stabilization and the subsequent sox-9 induction [53–56]. Further in vitro analyses are necessary to better clarify this specific aspect. Overall, a clear chondrogenic commitment has been observed with both scaffolds, with a slight greater evidence of collagen II production when UC-MSCs were grown on a hyaluronic acid membrane. This is consistent with several evidences in literature that showed a possible and efficient chondrogenic differentiation of mesenchymal stem cells derived from Wharton's jelly in different conditions, as the high-density cell cultures on rotatory systems [57], the culture in collagen hydrogels [58], or the cell growth on polycaprolactone/collagen nanoscaffolds [40].

Like the chondrogenic differentiation, the osteogenic commitment was obtained using a commercial organic bone substitute used for clinical applications, to closely mimic the clinical application. Indeed, the common bovine-derived bone matrix used in this study is widely accepted in clinical scenarios as gap filling in the presence of bone defects. We have observed an osteogenic potential similar to other studies where cells from umbilical cord were grown in monolayer [15] or in tridimensional scaffold as collagen I/III gels [59] or even when loaded onto bone scaffold and subcutaneously implanted in nude mice [60]. Our results, along with the available literature, suggest that UC-MSCs might represent a possible source allogeneic cells for bone tissue engineering.

These observations are further confirmed by the last section of our experiments. The idea of these experiments has been inspired by Scotti et al. [61], who conceived a complex preclinical model to demonstrate the feasibility of engineering a functional bone organ by preconditioning bone marrow-derived MSCs, embedded in type I collagen meshes, toward hypertrophic chondrocytic phenotype and subsequently implanting the scaffolds in the dorsal subcutaneous tissue of nude mice. The dramatic originality of Scotti's study and the potential for bone engineering are unquestionable. Indeed, bone repair mainly occurs through endochondral ossification and the possibility to recapitulate this process may allow for conceiving a "second generation" cell-seeded bone substitutes with increased efficiency to strongly facilitate bone repair and regeneration. In our study, we verify *in vitro* the UC-MSC ability to execute an endochondral program similar to BM-MSCs and we applied the same conditions previously described by Scotti et al. [61]. We seeded UC-MSCs onto the previously mentioned commercial bone substitutes, and we cultured the cell-seeded constructs in chondrogenic conditions and subsequently in the hypertrophic medium. We obtained encouraging results in terms of matrix synthesis and expression of osteogenic markers (Cbfa1). This certainly represents one of the most original features of our study. Moreover, our work represents one of the first studies describing the use of UC-MSCs in a "developmental engineering" paradigm. Indeed, the positive results observed by "pushing" UC-MSCs through endochondral ossification may be exploited by future experiments verifying the attitude of these cells to generate a tissue similar to cancellous bone after *in vivo* preclinical implantation in small animals, in order to offer a valid use of allogeneic cell-seeded bone substitutes.

The main limitation of this study is the *in vitro* nature of the experiment. This certainly claims for further studies to verify the intuitions derived from the obtained data in preclinical animal models before moving to any clinical applications. Albeit this drawback is implied in any *in vitro* study, the preclinical model is essential when dealing with allogeneic cells to assess the immune privilege of this category of stem cells before any *in vivo* human use. However, literature offers several evidences of UC-MSC immunoprivileged status. These cells express HLA-G, which is involved in immune tolerance during pregnancy IL-6 and VEGF, which are linked to MSC immunosuppressive capability. Moreover, UC-MSCs are able to suppress T-cell proliferation [14]. All these aspects seem to confirm the potential for an allogeneic use of UC-MSC in orthopaedic tissue engineering.

Nevertheless, a critical issue when dealing with allogeneic MSCs remains the potential for immune reaction to be elicited in the host tissue. Evidences in literature are present outlining the immunogenicity of MSCs from an allogeneic source and the fact that this reaction may hamper dramatically the tissue regeneration process in the same cells were supposed to be enhanced. The study of Eliopoulos et al. in 2005 [62] showed the alarming reject of allogeneic MSCs implanted subcutaneously in mice. A similar fate of these cells has been observed by Huang et al. [63] in a preclinical rat model of myocardial infarction. In their study, the authors showed that allogeneic cells were rejected from the host cardiac tissues by 5 weeks after implantation. This may be partially explained by a transient immunoprivileged status of MSCs that may later acquire immunogenicity during their

differentiation and trophic process in the host environment, thus eliciting an immune reaction by the native immune system. Immune reactions and short engraftment time have also been described by Tano et al. [64] in a model of allogeneic MSC transplantation on the epicardial surface of rat hearts. A similar paradigm has been confirmed recently by Oliveira et al. [65] in a preclinical model of MSC transplantation in the murine kidney. They observed lymphocytic infiltration and allogeneic MSC rejection no later than 28 days after transplantation. As a consequence, they suggested the fascinating concept of "preactivation" of cells, by treatment with INF-gamma and TNF-alpha, in order to reduce immunogenicity and prolong their engraftment period and their trophic properties. A potential immune reaction in human has been recently hypothesized by de Windt et al. [31] in their clinical trial of cartilage repair by means of allogeneic MSCs and chondrons. Despite their encouraging results, no allogeneic cells were found at the repair site at 1 year, suggesting a time-limited trophic effect of MSCs that would have been gradually removed by the host immune system during their differentiation processes. This agrees with a previous in vitro observations of Mukonoweshuro et al. [66], who have outlined the immunosuppressive properties but not the immunoprivileged status of allogeneic chondrogenic-committed MSCs in a mouse model. The potential immune reaction elicited by MSCs may also lead to detrimental effect in the repair setting, as shown preclinically by Sbano et al. [67] in a mouse model of skin graft transplantation and by Seifert et al. [68] in a rat kidney transplantation setting. Overall, these evidences claim for a cautious approach in a potential clinical application of allogeneic MSCs, despite the recent literature is outlining, as a counterpart, the safety and some promising results. Indeed, the studies of Garcia-Sancho et al. [69] showed a possible application in knee osteoarthritis and degenerative disc disease with few safety concerns, probably due to the peculiar "insulated" recipient site of the cells, and Wang et al. [70] have recently proposed the injection of allogeneic MSCs during ACl reconstruction to improve symptoms and structural outcome. With a longer follow-up, Park et al. [71] have shown the promising effect of the implantation of a composite made by allogeneic UC blood-derived MSCs and hyaluronate hydrogel to improve cartilage healing in osteoarthritic patients. Similar evidences have been proposed by Vega et al. [72] and by Gupta et al. [73], who treated knee osteoarthritic patients by a direct intra-articular injection of allogeneic human MSCs. In a different setting, Morrison et al. [74] have recently demonstrated the feasibility of cranial bone reconstruction by means of allogeneic mesenchymal stromal cells (MSCs) on a ceramic carrier and polymer scaffold, similarly to the preclinical observations of Todeschi et al. [75] regarding UC-MSC transplantation in mice calvarial defects. So far, the controversy of allogeneic MSC therapy is still a debated issue in literature. However, a common element seems to be present in all these studies. Indeed, a different role of MSCs is emerging from the preclinical and clinical recent evidences that may lead to reconsider the use of allogeneic MSCs as "replenishing cells for injured tissues," assuming their immune system recognition. Far from being a "building block" submitted to the host immune system (and, thus, with a limited viability of 2–4 weeks), the allogeneic MSCs may otherwise represent the transient paracrine catalyzers that work well for a short period of time with a "hit and run effect" [65]. This new paradigm implies the lack of the "need of persistence" in the host tissue, as the therapeutic effects of MSCs are independent of a direct differentiation, but they can be exerted locally by promoting the resident population to hasten the repair process for a limited period of time, until the allogeneic MSCs are cleared by the host immune system. In this perspective, it can be easily understood the greater effect of implanting allogeneic MSCs in a "confined environment" (i.e., in the joint or embedded in a bioscaffold); as in these settings, the immune system may be hampered in rapidly eliminating the MSCs. Furthermore, the various regenerative properties of allogeneic MSCs observed in literature may coexist with a reduced survival time due to the fact that they depend on the factors secreted by the cells in the first days after transplantation, also known as the "secretome." Indeed, together with the concept of MSC preactivation to prolong the cell survival time, the secretome surely represents one of the future frontiers in allogeneic MSC tissue engineering research.

In conclusion, the present study confirms the chondrogenic and osteogenic commitment of UC-MSCs when cultured in tridimensional scaffold and it suggests the possible involvement of UC-MSCs for the generation of endochondral scaffolds to improve bone regeneration by recapitulating endochondral ossification process. These observations offer a solid perspective for future preclinical studies aiming to improve cartilage repair and bone regeneration. Furthermore, UC-MSCs derive from a previously discarded material as the umbilical cord; thus, they represent a stem cell population with several advantages as a greater bioavailability and lower ethical implications than other cell sources. All in all, UC-MSCs may be reasonably considered an attractive opportunity for orthopaedic allogeneic stem cell therapy.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Research Article

Human Chorionic Plate-Derived Mesenchymal Stem Cells Restore Hepatic Lipid Metabolism in a Rat Model of Bile Duct Ligation

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In cholestatic liver diseases, impaired bile excretion disrupts lipid homeostasis. We investigated changes of lipid metabolism, including mitochondrial β-oxidation, in a rat model of bile duct ligation (BDL) in which chorionic plate-derived mesenchymal stem cells (CP-MSCs) were transplanted. Serum cholesterol level, which was elevated after BDL, was significantly decreased following CP-MSC transplantation. The expression levels of genes involved in intracellular lipid uptake, including long-chain fatty acyl-CoA synthetases and fatty acid transport proteins, were decreased in rats after BDL; however, they were not significantly changed by subsequent CP-MSC transplantation. Carnitine palmitoyltransferase 1A (CPT1A), a rate-limiting enzyme in mitochondrial β-oxidation, was upregulated after BDL and then was downregulated after CP-MSC transplantation. CPT1A expression was changed via microRNA-33—a posttranscriptional regulator of CPT1A—in a peroxisome proliferator-activated receptor α-independent manner. Cellular adenosine triphosphate production—an indicator of mitochondrial function—was reduced after BDL and was restored by CP-MSC transplantation. Expression levels of heme oxygenases also were significantly affected following BDL and CP-MSC transplantation. Lipid metabolism is altered in response to chronic cholestatic liver injury and can be restored by CP-MSC transplantation. Our study findings support the therapeutic potential of CP-MSCs in cholestatic liver diseases and help in understanding the fundamental mechanisms by which CP-MSCs affect energy metabolism.

1. Introduction

Cholestatic liver injury, which is caused by accumulation of bile acids and lipids, comprises a wide spectrum ranging from acute transient hepatitis to cirrhosis with portal hypertension [1–3]. The liver controls central processes of lipid metabolism including fatty acid synthesis, mitochondrial β-oxidation, and phospholipid transport. Impaired bile excretion, caused by biliary obstruction or liver damage, disrupts cholesterol and phospholipid metabolism [4]. In a rat model of bile duct ligation (BDL), serum levels of very low-density lipoprotein cholesterol and low-density lipoprotein (LDL) cholesterol are drastically elevated, whereas hepatic lipid concentrations are unchanged [5]. However, alterations in mitochondrial function in chronic cholestatic liver diseases have not been elucidated.

Mesenchymal stem cells (MSCs) are multipotent adult stem cells that can differentiate into various cell types of the three germ layers (i.e., the ectoderm, mesoderm, and endoderm) [6]. The human placenta is an abundant source of MSCs. Placenta-derived MSCs (PD-MSCs) which originate from the fetus possess great potential for self-renewal, proliferation, and differentiation [7, 8]. We previously found that full-term placenta harbors several types of PD-MSCs,

including chorionic plate-derived MSCs (CP-MSCs), chorionic villus-derived MSCs, and Wharton's jelly-derived MSCs [9]. CP-MSCs are highly capable of differentiating into various lineage cells, including hepatocytes. Moreover, CP-MSCs have been demonstrated to have anti-inflammatory, antifibrotic, and proregenerative properties in the damaged liver [10, 11].

We therefore used a BDL rat model of chronic cholestatic liver injury to clarify the alterations in hepatic lipid homeostasis—focusing on mitochondrial dysfunction—and the impact of CP-MSC transplantation restoring the alterations in hepatic lipid metabolism.

2. Materials and Methods

2.1. Cell Culture. Collection of placenta samples for research purposes was approved by the Institutional Review Board of CHA Gangnam Medical Center, Seoul, Korea (IRB 07-18). All participants provided written informed consent prior to sample collection. Placentas were obtained from women who were free of any medical, obstetrical, or surgical complications and who delivered at term (38 ± 2 gestational weeks). CP-MSCs were isolated as described previously [10] and were cultured in Dulbecco's modified Eagle medium/Ham's F-12 medium (DMEM/F12; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich), 1% penicillin/streptomycin (Sigma-Aldrich), 1 μg/mL heparin (Sigma-Aldrich), and 25 ng/mL human fibroblast growth factor-4 (hFGF-4; Peprotech Inc., Rocky Hill, NJ, USA) at 37°C in a 5% CO_2 incubator containing 20% O_2.

2.2. BDL Rat Model and Transplantation of CP-MSCs. Male 7-week-old Sprague-Dawley rats (Orient Bio Inc., Seongnam, Korea) were maintained in an air-conditioned animal facility. The common bile duct was ligated under general anesthesia with Avertin (2,2,2-tribromoethanol, Sigma-Aldrich) as described previously [12, 13]. One week after BDL, CP-MSCs (2×10^6 cells, 8–10 passages) were injected intravenously via tail vein in the transplanted group. The CP-MSC number was determined based on the previous dose-determining experiments [10, 14]. Liver tissues and blood samples were collected 1, 2, 3, and 5 weeks posttransplantation in the transplanted group and 1, 2, 3, and 5 weeks post-BDL in the nontransplanted group. The experimental protocols were approved by the Institutional Animal Care and Use Committee of CHA University, Seongnam, Korea (IACUC-140009).

2.3. Histological Analysis. Liver tissue samples were fixed in 10% formalin, embedded in paraffin, and sectioned at 5 μm thickness. Sections then were stained with hematoxylin and eosin and observed under light microscopy at 200x magnification (Axioskop2, Carl Zeiss Micro-Imaging, Oberkochen, Germany).

2.4. Immunofluorescence Staining. To analyze the expression of carnitine palmitoyltransferase 1A (CPT1A) in liver tissues, 6 μm thick cryostat sections were incubated with protein blocking solution (Dako, Glostrup, Denmark) for 40 minutes at room temperature. Then, a mouse anti-CPT1A antibody (1 : 100, Abcam, Cambridge, MA, USA) was treated, and sections were incubated at 4°C overnight. After washing with phosphate-buffered saline (PBS), samples were incubated with an Alexa 488-conjugated secondary antibody (1 : 150, Invitrogen, Carlsbad, CA, USA) for 1 hour at room temperature. Sections then were stained with 4′,6-diamidino-2-phenylindole (DAPI) for nuclear counterstaining and were observed under fluorescence microscopy at 400x magnification (Nikon, Tokyo, Minato, Japan).

2.5. Blood Chemistry. The serum concentrations of total cholesterol, high-density lipoprotein (HDL) cholesterol, LDL cholesterol, triglyceride, albumin, total bilirubin, alkaline phosphatase (ALP), aspartate transaminase, alanine transaminase, and C-reactive protein (CRP) were measured enzymatically by an automated analyzer (Hitachi 747, Hitachi, Tokyo, Japan).

2.6. Fatty Acyl-CoA Synthetase Activity Assay. Long-chain fatty acyl-CoA synthetase (ACSL) activity was assessed by the enzyme-linked immunosorbant assay (ELISA). Liver tissues were homogenized in cold PBS with a glass homogenizer on ice. ACSL activity was measured using a Rat Fatty acyl-CoA synthetase ELISA Kit (MyBioSource, San Diego, CA, USA) in strict accordance with the manufacturer's instructions and detected using a microplate reader (BioTek, Winooski, VT, USA) at 450 nm.

2.7. Quantitative Real-Time Polymerase Chain Reaction. Rat liver tissues were homogenized and lysed, and total RNA was isolated with the TRIzol reagent (Invitrogen). Reverse transcription was performed with 500 ng of total RNA and Superscript III reverse transcriptase (Invitrogen). Real-time polymerase chain reaction (PCR) was performed with SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). The cDNA subsequently was amplified by PCR using the following thermal conditions: 5 minutes at 95°C, 40 cycles of 95°C for 5 seconds, and 60°C for 30 seconds. The sequences of the primers are listed in Table 1. GAPDH or β-actin was used as an internal control for normalization.

2.8. Isolation and Quantification of MicroRNA-33. Total RNA was isolated with the TRIzol reagent (Invitrogen) and reverse-transcribed with a Mir-X miRNA First-Strand Synthesis Kit (Clontech, Mountain View, CA). Then, real-time PCR for microRNA-33 (miR-33) was performed using the following primer: 5′-GTG CAT TGT AGT TGC ATT GCA-3′ (forward). The expression of miR-33 was normalized to U6 snRNA expression.

2.9. Western Blot Analysis. Liver tissues were homogenized and lysed on ice with RIPA buffer containing protease inhibitor cocktail (Roche, Branchburg, NJ, USA) and a phosphatase inhibitor (Sigma-Aldrich). Protein lysates were separated by 8% to 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA, USA), and then blocked in blocking buffer (0.1% Tween20 and 8% bovine serum albumin [BSA] in Tris-buffered saline [TBS]) for 1 hour. Membranes

Table 1: Primer sequences.

Gene		Sequence
ACSL 1	Forward	5′-AAG CTC TGG AGG ATC TTG GA-3′
	Reverse	5′-GGG TTG CCT GTA GTT CCA CT-3′
ACSL 3	Forward	5′-TAA AGG CTG ACG TGG ACA AG-3′
	Reverse	5′-CCT TTG GAA TTC CTG TGG AT-3′
ACSL 4	Forward	5′-ATC TCC CAA AGC TGG AAC AC-3′
	Reverse	5′-CTG GTC CCT TAA CGT GTG TG-3′
ACSL 5	Forward	5′-TGT AGG GAT TGA GGG AGG AG-3′
	Reverse	5′-CAC AGC AAG TCC TCT TTG GA-3′
FATP 1	Forward	5′-CCC TGG ATG AGA GAG TCC AT-3′
	Reverse	5′-GCA GGA GAA ACA CCT GAA CA-3′
FATP 2	Forward	5′-CTC TTT CAG CAC ATC TCG GA-3′
	Reverse	5′-CCT CTT CCA TCA GGG TCA CT-3′
FATP 3	Forward	5′-CTG GGA CGA GCT AGA GGA AG-3′
	Reverse	5′-GCT GAG GCC AGA GGT CTA AC-3′
FATP 4	Forward	5′-CGC TGC TGT TCT CCA AGC TGG-3′
	Reverse	5′-GAT GAA GAC CCG GAT GAA ACG-3′
FATP 5	Forward	5′-GAA GGA ACC TGG AAG CTC TG-3′
	Reverse	5′-AGT GTC GAT TTC CGA TTT CC-3′
FATP 6	Forward	5′-CAG TAC CAC CAA GCC ATC AC-3′
	Reverse	5′-TGG AAC TGG CTA ATC ACA GC-3′
PPARα	Forward	5′-AGC CAT TCT GCG ACA TCA-3′
	Reverse	5′-CGT CTG ACT CGG TCT TCT TG-3′
CPT1A	Forward	5′-GCT TCC CCT TAC TGG TTC C-3′
	Reverse	5′-AAC TGG CAG GCA ATG AGA CT-3′
HO-1	Forward	5′-TGC ACA TCC GTG CAG AGA AT-3′
	Reverse	5′-CTG GGT TCT GCT TGT TTC GC-3′
HO-2	Forward	5′-AGG GCA GCA CAA ACA ACT CA-3′
	Reverse	5′-TCT GGC TCA TTC TGT CCT AC-3′
β-Actin	Forward	5′-GGG ACC TGA CTG ACT ACC TCA T-3′
	Reverse	5′-ACG TAG CAC AGC TTC TCC TTA AT-3′
Gapdh	Forward	5′-TCC CTC AAG ATT GTC AGC AA-3′
	Reverse	5′-AGA TCC ACA ACG GAT ACA TT-3′

subsequently were incubated with mouse anti-CPT1A (1 : 1000, Abcam), rabbit anti-peroxisome proliferator-activated receptor α (PPARα) (1 : 1000, Abcam), and rabbit anti-GAPDH (1 : 3000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibodies at 4°C overnight. After the reaction, membranes were treated with a horseradish peroxidase-(HRP-) conjugated secondary antibody (anti-rabbit IgG [1 : 25000, Bio-Rad Laboratories] or anti-mouse IgG antibody [1 : 25000, Bio-Rad Laboratories]) for 1 hour at room temperature. The bands were detected using an enhanced chemiluminescence reagent (Bio-Rad Laboratories).

2.10. Adenosine Triphosphate Assay. Adenosine triphosphate (ATP) concentrations of homogenized liver tissue samples were measured using an ATP assay kit (Abcam), according to the manufacturer's instructions, and were assessed using a microplate reader (BioTek) at 570 nm.

2.11. Statistical Analysis. All experiments were conducted in duplicate or triplicate. Data are expressed as mean ± standard deviation. Student's t-tests were performed for groupwise comparisons, and $P < 0.05$ was considered statistically significant. Statistical analyses were performed using PASW version 22.0 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. CP-MSC Transplantation Ameliorates Inflammation in the BDL Rat Liver. To assess the effect of transplantation of CP-MSCs on cholestatic liver injury, BDL rats were divided

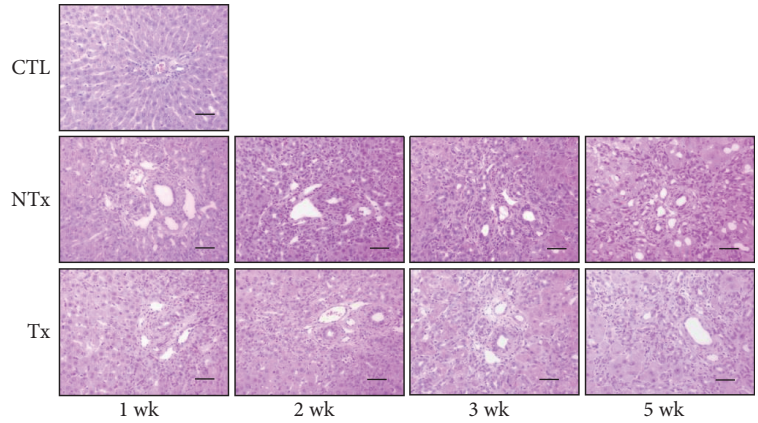

Figure 1: Inflammatory response induced by chronic cholestasis and the effect of CP-MSC transplantation. Histological analysis with hematoxylin and eosin staining (scale bar = 50 μm; original magnification, ×200). CTL: control group; NTx: nontransplanted group; Tx: transplanted group.

into 2 groups: rats in the transplanted group were injected with CP-MSCs, and rats in the nontransplanted group were injected with the culture medium. As shown in Figure 1, we observed the infiltration of inflammatory cells around bile ducts and bile duct proliferation in portal areas in both nontransplanted and transplanted groups 1 week after BDL. Two weeks after BDL, portal areas were expanded as a result of extensive bile duct proliferation and concentric periductal fibrosis, and disorganization of normal lobular structures was observed in the nontransplanted group. Bile duct proliferation was less prominent, and the lobular pattern was preserved in the transplanted group compared to the nontransplanted group (Figure 1). Hepatic steatosis was not observed in the control, nontransplanted, or transplanted groups.

3.2. CP-MSC Transplantation Attenuates BDL-Induced Hypercholesterolemia but Does Not Affect Fatty Acid Uptake. Obstruction of bile excretion induced by BDL results in overflow of biliary phospholipids in the circulation [4]. Therefore, we explored the effect of transplantation of CP-MSCs on cholesterol metabolism by measuring the cholesterol concentrations in serum. Total cholesterol was markedly elevated in the nontransplanted group 2 weeks after BDL compared to the control group, whereas it was significantly reduced in the transplanted group compared to the nontransplanted group ($P < 0.05$; Figure 2(a)). Results similar to those for total cholesterol were found for the concentrations of serum LDL cholesterol and triglyceride (Figure 2(a)). Increases in serum levels of total bilirubin, ALP, and CRP were shown to be attenuated after transplantation of CP-MSCs ($P < 0.05$; Figure S1 in Supplementary Material available online at https://doi.org/10.1155/2017/5180579).

Because hypercholesterolemia is induced by chronic cholestasis, we hypothesized that fatty acid uptake into hepatocytes may be altered in BDL rats. ACSLs and fatty acid transport proteins (FATPs) are thought to be essential for the intracellular uptake and transport of fatty acids [15, 16]. Therefore, we determined the activity of ACSLs and the expression levels of ACSLs and FATPs in rat liver tissues. ACSL activity—measured by ELISA—was increased significantly in the transplanted group compared to the nontransplanted group ($P < 0.05$; Figure 2(b)). The expression levels of ACSL1, which is highly expressed in the normal liver [17], were decreased in BDL rats; however, they were not increased significantly by CP-MSC transplantation (Figure 2(c)). The expression levels of ACSL4 and ACSL5, which are located in rat liver peroxisomes and mitochondria, respectively [18], declined drastically after BDL and were not restored by CP-MSC transplantation (Figure S2). The expression levels of FATP2 and FATP5, which are expressed in hepatocytes [19, 20], were decreased in BDL rats and were not increased significantly by CP-MSC transplantation (Figures 2(c) and S3). Collectively, these findings indicate that cholestasis and hypercholesterolemia induced by BDL are ameliorated by CP-MSC transplantation. However, transplantation of CP-MSCs does not appear to restore processes of fatty acid import into hepatocytes.

3.3. CPT1A Expression Is Changed via MiR-33 in BDL Rats. CPT1A is a rate-limiting enzyme located in the mitochondrial outer membrane that catalyzes β-oxidation of free fatty acid [21]. PPARα regulates mitochondrial and peroxisomal fatty acid oxidation by controlling downstream genes, such as CPT1A [22]. We investigated whether the expression of genes associated with fatty acid oxidation is altered in BDL rats and restored by transplantation of CP-MSCs. The mRNA levels of PPARα and CPT1A were remarkably decreased after BDL (Figures 3(a) and 3(b)). PPARα mRNA levels were similar in the nontransplanted and transplanted groups (Figure 3(a)); however, CPT1A mRNA expression was significantly augmented 2 weeks after CP-MSC transplantation ($P < 0.05$; Figure 3(b)). On the contrary, the increased protein expression levels of CPT1A by BDL were reinstated to near-control levels 3 and 5 weeks after transplantation of CP-MSCs ($P < 0.05$; Figure 3(c)). These results were confirmed by immunofluorescence staining (Figure 3(d)). MiR-33 represses its target genes, which

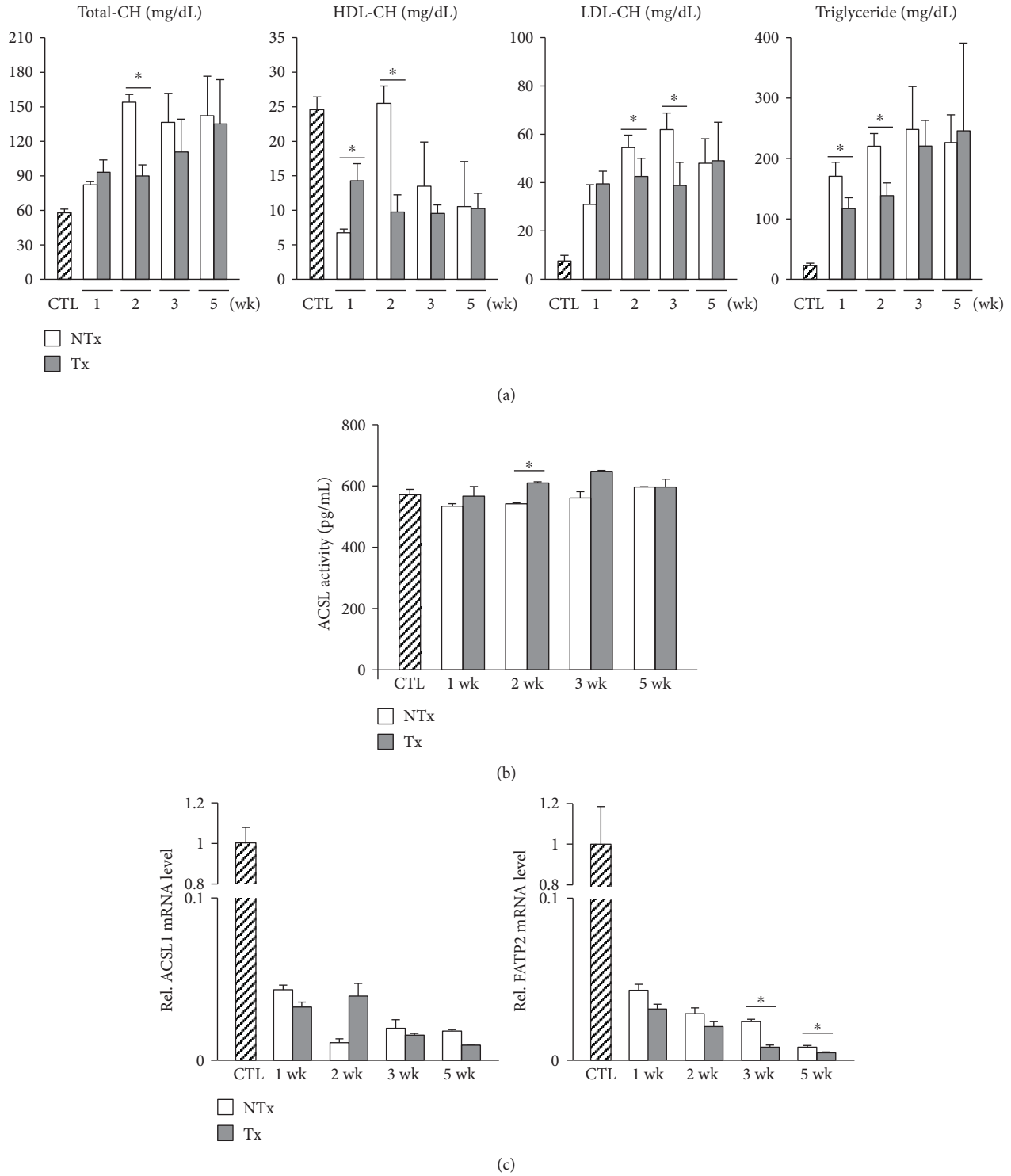

Figure 2: Changes in serum lipid profiles and expression levels of genes associated with intracellular uptake of fatty acids after BDL and/or CP-MSC transplantation. (a) Serum levels of total cholesterol, HDL cholesterol, LDL cholesterol, and triglyceride. (b) Activities of ACSL, as measured by ELISA. (c) mRNA expression levels of ACSL1 (left) and FATP2 (right). β-Actin was used as an internal control for normalization. Data are expressed as a fold change related to the control group. $^*P < 0.05$ (compared to the nontransplanted group). CTL: control group; NTx: nontransplanted group; Tx: transplanted group.

are involved in free fatty acid oxidation, such as CPT1A [23]. To evaluate whether miR-33 is a posttranscriptional regulator of CPT1A in BDL rat liver, we analyzed the expression levels of miR-33. As expected, we determined that miR-33 expression was reduced in BDL rats and was restored by transplantation of CP-MSCs (Figure 3(e)). Taken together, these results suggest that CPT1A may be regulated posttranscriptionally by miR-33 in a PPARα-independent manner.

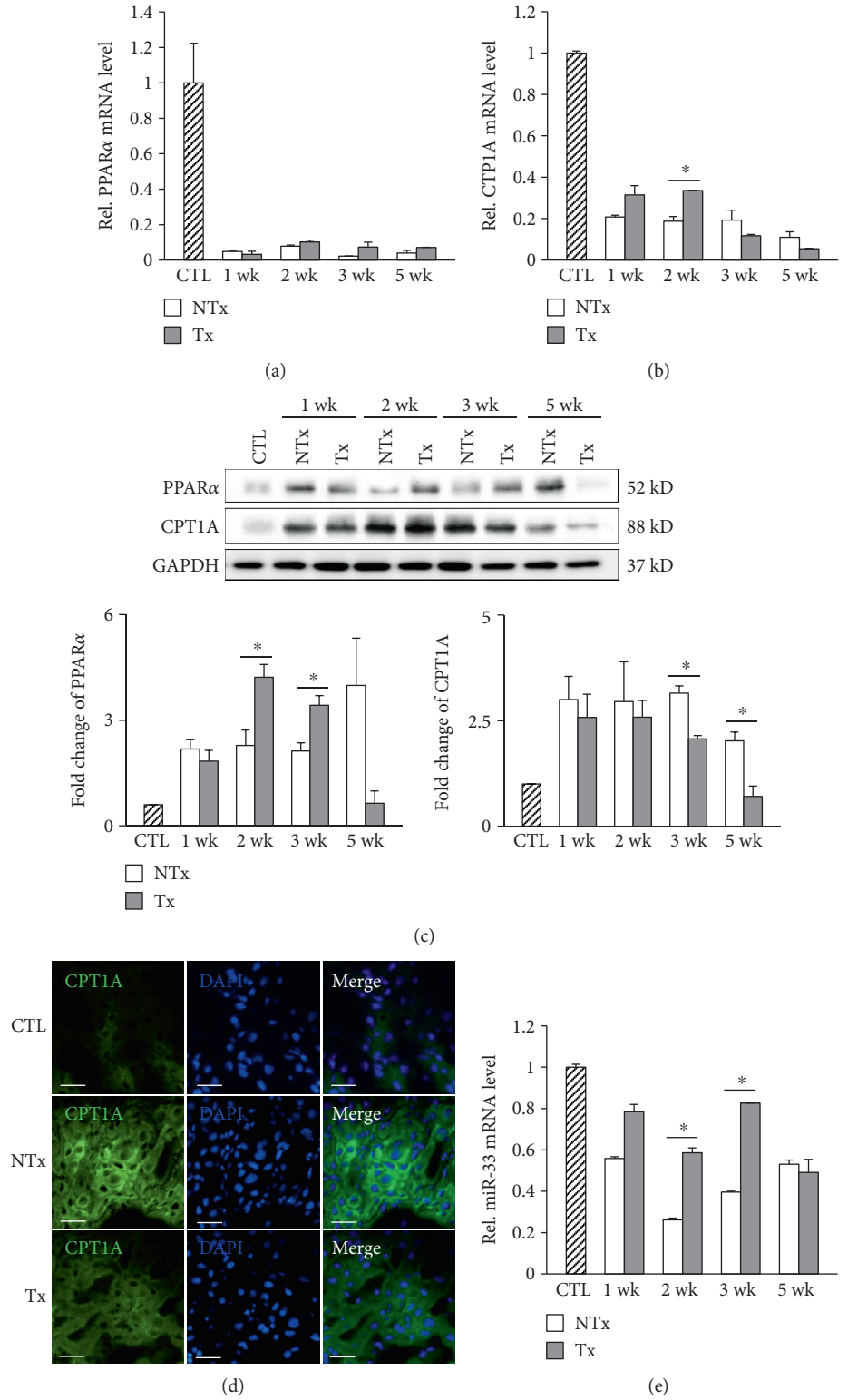

Figure 3: Expression of genes associated with fatty acid oxidation after BDL and/or CP-MSC transplantation. mRNA expression levels of PPARα (a) and CPT1A (b) by real-time PCR. β-Actin was used as an internal control for normalization. Data are expressed as a fold change related to the control group. (c) Protein expression levels of PPARα and CPT1A. GAPDH was used as a loading control, and quantification by densitometry of Western blots was normalized to GAPDH. Data are expressed as a fold change related to the control group. (d) Analysis of CPT1A expression with immunofluorescence staining (scale bar = 200 μm; original magnification, ×400). Liver tissues, which were collected at 3 weeks posttransplantation in the transplanted group and post-BDL in the nontransplanted group, were used in immunofluorescence staining. (e) mRNA expression levels of miR-33. U6 snRNA was used as an internal control for normalization. $^*P < 0.05$ (compared to the nontransplanted group). CTL: control group; NTx: nontransplanted group; Tx: transplanted group.

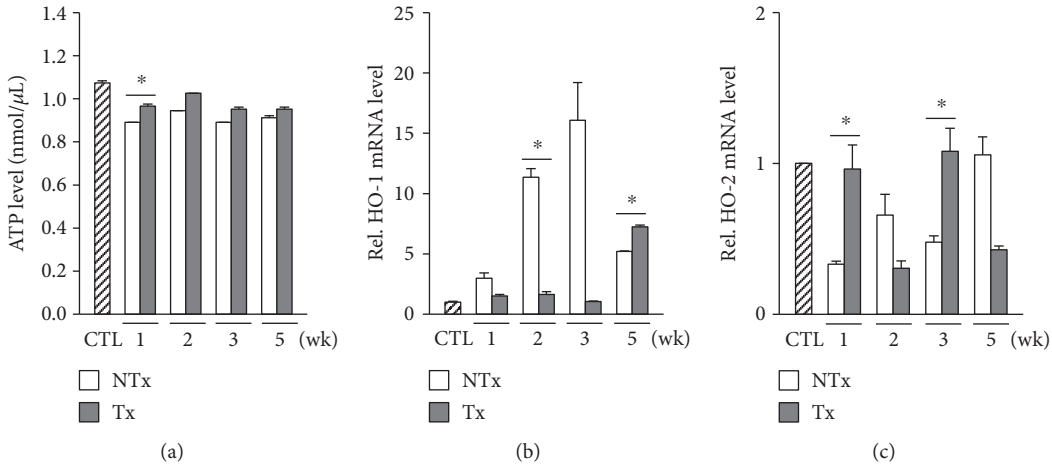

Figure 4: Changes in cellular ATP production and expression levels of HOs after BDL and/or CP-MSC transplantation. (a) Analysis of ATP levels in the liver tissues by the ATP assay. mRNA expression levels of HO-1 (b) and HO-2 (c), assessed by real-time PCR. GAPDH was used as an internal control for normalization. Data are expressed as a fold change related to the control group. $^*P < 0.05$ (compared to the nontransplanted group). CTL: control group; NTx: nontransplanted group; Tx: transplanted group.

3.4. CP-MSC Transplantation Restores Cellular ATP Production by Regulating Heme Oxygenases. To demonstrate alterations in cellular energy production after BDL, we measured the ATP levels in BDL rat liver. ATP production was decreased after BDL but was augmented 1 week after CP-MSC transplantation (Figure 4(a)). Heme oxygenases (HOs) are suggested to be involved in regulating mitochondrial function [24]. Therefore, we assessed the expression levels of HOs in liver tissues. We determined that HO-1 expression was increased substantially in a time-dependent manner post-BDL until week 3. However, the augmented expression of HO-1 reverted to near-control levels 2 weeks after transplantation of CP-MSCs ($P < 0.05$; Figure 4(b)). The HO-2 expression pattern was inversely related to that of HO-1 (Figure 4(c)). These findings implicate that CP-MSC transplantation may ameliorate cellular ATP production via alternative expressions of HO-1 and HO-2.

4. Discussion

In this study, we demonstrated that alterations in lipid metabolism in BDL rats might be ameliorated by transplantation of CP-MSCs. Chronic cholestasis, resulting from BDL, led to massive inflammation, hypercholesterolemia, and a drastic decrease in intracellular fatty acid transport; these changes were partially reverted by CP-MSC transplantation. Regarding mitochondrial β-oxidation, the expression of CPT1A was changed following BDL and CP-MSC transplantation via miR-33, which is known as a posttranscriptional regulator of CPT1A, independent of PPARα. Decreased cellular ATP production after BDL, which reflects mitochondrial dysfunction, was increased by CP-MSC transplantation via regulation of HO-1 and HO-2.

Stem cell therapy with MSCs has been tried for the treatment of various liver diseases, including cirrhosis and hepatic failure, as an alternative to liver transplantation. We previously reported that CP-MSCs had anti-inflammatory, antifibrotic, and proregenerative effects in a chronic liver injury model induced by carbon tetrachloride (CCl_4) [10, 11]. Liver fibrosis and increased expression of type I collagen and α-smooth muscle actin in CCl_4-treated rats were reduced after CP-MSC transplantation, which suggested that CP-MSCs have antifibrotic effects [10]. Transplantation of CP-MSCs also showed anti-inflammatory effects of attenuating leukocyte infiltration and augmenting anti-inflammatory cytokine interleukin 10 in liver tissues. In addition, CP-MSC transplantation promoted liver regeneration through activating autophagy [11]. In our present study, we demonstrated a novel effect of CP-MSCs as modulators of hepatic lipid metabolism in a BDL rat model. Alterations in serum cholesterol profiles and hepatic fatty acid oxidation, which resulted from BDL, were ameliorated after CP-MSC transplantation.

Because bile acids play a key role in lipid and energy homeostasis, alterations in lipid metabolism are inevitable in cholestatic liver diseases [4, 5, 25]. De Vriese and colleagues reported the results of lipid analysis of BDL rats and identified hypercholesterolemia and changes in the serum phospholipid profile, in proportion to serum levels of total bilirubin and ALP; however, a decrease in liver fat content in BDL rats was also observed [4]. In a more recent study, a high-cholesterol diet was not found to cause hepatic steatosis in BDL mice [25]. Our study findings of hypercholesterolemia without hepatic steatosis in BDL rats are consistent with those of these previous studies. Also, we demonstrated that intracellular fatty acid transport was markedly suppressed after BDL. The absence of hepatic steatosis, despite hypercholesterolemia, might be explained by intestinal lipid malabsorption via bile acids combined with the suppression of fatty acid import into hepatocytes.

Because the previous studies, which reported the changes in lipid metabolism in cholestatic liver diseases, focused on lipid malabsorption and cholesterol profiles, alterations in fatty acid oxidation have not been elucidated so far. Mitochondrial β-oxidation is a catabolic process that yields acetyl-CoA from long-chain acyl-CoA; acetyl-CoA then

serves as a substrate in ATP generation [26]. Fatty acids, in the form of acyl-CoA, enter mitochondria by CPT1A, a rate-limiting enzyme that catalyzes mitochondrial β-oxidation [21]. Moreover, PPARα has been identified as an upstream regulator of CPT1A [22]. We demonstrated that protein expression of CPT1A was upregulated in BDL rats and was downregulated after CP-MSC transplantation, independent of PPARα. In contrast, mRNA expression of CPT1A exhibited an opposite pattern to CPT1A protein expression. Therefore, we explored the possibility of post-transcriptional regulation of CPT1A and verified that CPT1A is changed via alternative expression of miR-33 [23]. Because mitochondrial β-oxidation is a major source of ATP production in the liver [26], we further analyzed ATP production as an estimation of mitochondrial function. We revealed that decreased ATP production in BDL rat liver was restored by transplantation of CP-MSCs. HOs are thought to be mediators by which CP-MSCs correct mitochondrial dysfunction. Although HO-1 has been suggested to play a role in regulating mitochondrial function [24, 27], further studies are warranted to ascertain whether mitochondrial fatty acid oxidation is regulated by HOs. We have failed to demonstrate a consistent therapeutic effect on ATP production over time after CP-MSC transplantation. It may be worthwhile to transplant CP-MSCs repeatedly to overcome these limitations and to augment the therapeutic effect.

5. Conclusions

In our present study, we delineated perturbed lipid homeostasis in a model of chronic cholestatic liver injury. We demonstrated the therapeutic effect of CP-MSC transplantation to ameliorate alterations in lipid metabolism involving mitochondrial fatty acid oxidation. These results provide a novel insight into the mechanisms of stem cell therapy and support the therapeutic potential of CP-MSC transplantation in chronic cholestatic liver diseases.

Abbreviations

ACSL: Long-chain fatty acyl-CoA synthetase
ALP: Alkaline phosphatase
ATP: Adenosine triphosphate
BDL: Bile duct ligation
BSA: Bovine serum albumin
CCl4: Carbon tetrachloride
CP-MSCs: Chorionic plate-derived MSCs
CPT1A: Carnitine palmitoyltransferase 1A
CRP: C-reactive protein
DAPI: 4′,6-Diamidino-2-phenylindole
DMEM/F12: Dulbecco's modified Eagle medium/Ham's F-12 medium
ELISA: Enzyme-linked immunosorbant assay
FATP: Fatty acid transport protein
FBS: Fetal bovine serum
HDL: High-density lipoprotein
hFGF-4: Human fibroblast growth factor-4
HO: Heme oxygenase
HRP: Horseradish peroxidase
LDL: Low-density lipoprotein
miR: MicroRNA
MSCs: Mesenchymal stem cells
PBS: Phosphate-buffered saline
PCR: Polymerase chain reaction
PD-MSCs: Placenta-derived MSCs
PPARα: Peroxisome proliferator-activated receptor α
SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBS: Tris-buffered saline.

Conflicts of Interest

The authors declare that they have no competing interests.

Authors' Contributions

Yun Bin Lee and Jong Ho Choi contributed equally to this work.

Acknowledgments

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Research Article

Mesenchymal Stromal Cells Are More Immunosuppressive *In Vitro* If They Are Derived from Endometriotic Lesions than from Eutopic Endometrium

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Endometriosis is an inflammatory disease with predominance of immunosuppressive M2 macrophages in the pelvic cavity that could be involved in the pathology through support and immune escape of ectopic lesions. Mesenchymal stromal cells (MSC) are found in ectopic lesions, and MSC from nonendometriosis sources are known to induce M2 macrophages. Therefore, MSC were hypothesized to play a role in the pathology of endometriosis. The aim was to characterize the functional phenotype of MSC in ectopic and eutopic endometrium from women with endometriosis. Stromal cells from endometriotic ovarian cysts (ESC_{cyst}) and endometrium (ESC_{endo}) were examined if they exhibited a MSC phenotype. Then, ESC were phenotypically examined for protein and gene expression of immunosuppressive and immunostimulatory molecules. Finally, ESC were functionally examined for their effects on monocyte differentiation into macrophages. ESC_{cyst} and ESC_{endo} expressed MSC markers, formed colonies, and differentiated into osteoblasts and adipocytes. Phenotypically, ESC_{cyst} were more immunosuppressive, with significantly higher expression of immunosuppressive molecules. Functionally, ESC_{cyst} induced more spindle-shaped macrophages, with significantly higher expression of CD14 and CD163, both features of M2 macrophages. The results suggest that ESC_{cyst} may be more immunosuppressive than ESC_{endo} and may promote immunosuppressive M2 macrophages that may support growth and reduce immunosurveillance of ectopic lesions.

1. Introduction

Endometriosis is an inflammatory disease where the endometrium grows in ectopic sites, most commonly in the pelvic cavity [1]. The major symptoms of endometriosis are chronic pelvic pain and infertility [2]. Although medical and surgical treatments are available, they are not sufficient as recurrence of ectopic lesions and symptoms is common [3]. The mechanism behind disease development, progression, and recurrence is not fully known. Therefore, there is a need for improved understanding of the pathology of endometriosis.

Sampson's theory of retrograde menstruation is the most widely accepted theory for the pathophysiology of endometriosis [2] and states that reflux of menstrual debris during menstruation implants in the pelvic cavity and causes endometriosis [4]. Almost all women have retrograde menstruation, but only approximately 10% develop this disease [5], indicating that endometriosis has a multifactorial pathogenesis with other factors involved, such as reduced immunosurveillance in the pelvic cavity of women with endometriosis and involvement of stem/stromal cells [2, 6]. The latter factor refers to the stem cell theory, which postulates that a putative stem/stromal cell population such as

mesenchymal stromal cells (MSC) is refluxed back into the pelvic cavity via retrograde menstruation and then gives rise to ectopic lesions [6].

MSC are multipotent cells that are known to exist in both ectopic lesions and in the endometrium [6]. They have attracted much attention in the last decade mainly for their capacity to modulate immune responses [7]. Accordingly, they have been suggested as a potential treatment for inflammatory diseases such as graft versus host disease, multiple sclerosis and type 1 diabetes, among others [8]. Moreover, it would be expected that MSC are found in ectopic lesions through retrograde menstruation.

High levels of proinflammatory cytokines such as interferon-γ (IFN-γ) and tumour necrosis factor-α (TNF-α) are found in the pelvic cavity of women with endometriosis [9]. Moreover, ectopic lesions behave like eutopic endometrium in the menstrual cycle stimulated to grow and then shed with fluctuations in the levels of steroid hormones furthermore promoting inflammation [2, 10]. MSC can sense and respond to inflammation in their microenvironment [11–13]. In fact, it has been suggested that high levels of inflammation polarize them into an immunosuppressive phenotype expressing high levels of immunosuppressive molecules such as indoleamine 2,3-dioxygenase 1 (IDO1), cyclooxygenase 2 (COX2), and heme oxygenase 1 (HO-1) leading to immunosuppression via promotion of immunosuppressive M2 macrophages [13]. In contrast, low levels of inflammation have been suggested to polarize them into an immunostimulatory phenotype expressing high levels of proinflammatory cytokines and chemokines such as interleukin 8 and C-X-C motif chemokine 12 (CXCL12) which cause immunostimulation via immune cell recruitment and promotion of immunostimulatory M1 macrophages [13]. Studies show that M2 macrophages, immature dendritic cells, and T helper 2 (TH2) responses predominate in ectopic lesions [14–16]. In addition, cytotoxic functions of natural killer and CD8 T cell activities are inhibited and regulatory T cell activities are induced [17–19]. Moreover, MSC from nonendometriosis sources are known to promote these processes [20–25] which suggest that MSC in ectopic lesions may be immunosuppressive.

Accordingly, the aim of this study was to characterize the functional phenotype of MSC in ectopic and eutopic endometrium isolated from women with endometriosis. We hypothesized that immunosuppressive MSC may predominate in ectopic lesions contributing to their reduced immunosurveillance and growth. Stromal cells from ectopic (ESC_{cyst}) and eutopic (ESC_{endo}) endometrium were examined if they exhibited MSC characteristics. Their phenotypes were examined by determining their expression of several immunosuppressive and immunostimulatory markers, and finally, their functional effects on differentiation of monocytes into macrophages were examined. Both sources of stromal cells were found to be MSC, but ESC_{cyst} displayed more phenotypically and functionally immunosuppressive characteristics. The data suggest that ESC_{cyst} may promote immunosuppressive M2 macrophages that may support and reduce immunosurveilance of ectopic lesions allowing their growth.

2. Materials and Methods

2.1. Human Tissue Samples. Two types of tissues were collected: (i) endometriotic ovarian cysts (ectopic endometrium) and (ii) endometrium from women with endometriosis (eutopic endometrium). The endometriotic ovarian cysts and endometrium were collected from women aged from 31 to 42 years (mean ± SD, 36.3 ± 5.8 years, $n = 4$) undergoing laparoscopic surgery for confirmation or treatment of endometriosis. All women were histologically confirmed to have endometriosis by a pathologist. Only one woman underwent hormonal treatment. Moreover, two of the biopsies were from the proliferative phase: one was unknown and one had amenorrhea. Informed oral and written consent was obtained from each participant, and ethical approval was obtained from The Regional Ethical Review Board in Stockholm (2013/1094-31/2).

2.2. Isolation of Stromal Cells from Eutopic and Ectopic Endometrium. Human endometrial and endometriotic ovarian cyst tissues were digested to a single cell suspension using 1 mg/mL collagenase type I (Sigma, Missouri, United States) diluted in Hank's balanced salt solution (Life Technologies, Paisley, UK) (90 minutes for endometriotic tissue and 30 minutes for endometrial tissue) at 37°C with shaking every 10 minutes. The tissue digests were filtered twice through 100 μm cell strainers (Corning, New York, United States), and eventually the stromal cells were filtered through a 40 μm cell strainer (Corning), with undigested tissue and epithelial cells being removed at each of the steps. The cell suspension was washed twice with phosphate-buffered saline (PBS) (Life Technologies), by centrifugation at 500 ×g for 10 minutes. Finally, the cell pellet was resuspended in complete growth medium containing Dulbecco modified essential medium low glucose (DMEM-LG) (Life Technologies) + 10% MSC certified fetal calf serum (FCS) (Life Technologies) + 1% antibiotic and antimycotic (Life Technologies). Viable cells were counted in 1% Eosin (Merck KGaA, Darmstadt, Germany) and cultured at 4000 cells/cm^2 in tissue culture flasks at 37°C with 5% CO_2. After two days, the growth medium was changed and thereafter every three to four days. When the cells reached 70–90% confluency, they were trypsinised using 0.05% trypsin/EDTA (Life Technologies) and cultured as described above. At passage 2, the stromal cells were cryopreserved in 10% dimethyl sulfoxide (DMSO) (Sigma) in complete growth medium. To ensure that we were working with a pure population of cells, ESC_{endo} and ESC_{cyst} were used at passages three to six, as earlier passages may be contaminated with other cell types.

2.3. MSC Characterization Using Flow Cytometry. ESC_{endo} and ESC_{cyst} were stained with antibodies against CD73 PE (Becton-Dickinson, New Jersey, United States), CD90 PerCP Cy5 (BioLegend, California, United States), CD105 FITC (Ancell, Minnesota, United States), HLA class I PE (Agilent, Stockholm, Sweden), HLA class II FITC (Agilent), CD14 FITC (Becton-Dickinson), CD45 APC (BioLegend), and CD31 APC (BioLegend) for 20 minutes at room temperature (RT). Then, they were washed twice with PBS

by centrifugation at 500 ×g for 10 minutes. Finally, the cells were resuspended in PBS with 0.1% bovine serum albumin (Sigma) and analyzed with BD FACSCalibur (Becton-Dickinson). Unstained controls were used to set gates and voltages. The data was analyzed using the software Flow-Jo (Tree Star Inc., Ashland, United States). When the percentage of cells expressing a particular marker was ≥95% or ≤5%, then they were termed positive or negative for that marker, respectively.

2.4. Colony-Forming Units Fibroblasts (CFU-F). Colony-forming efficiency of ESC_{endo} and ESC_{cyst} was assessed using CFU-F in which cells were seeded in six-well plates at optimized densities of 200 cells/well in complete DMEM-LG growth medium. The growth medium was changed every 4 days. Following 21-day culture in 37°C with 5% CO_2, the growth medium was removed and the cells were washed twice with PBS. The cells were then fixed and permeabilized with 100% cold methanol for 5 minutes at RT. After washing the cells twice with PBS, they were stained with 1% Eosin in PBS for 20 minutes at RT, then rinsed twice with milliQ water, and visualized at 4x magnification under an Olympus CKX41 inverted microscope (Olympus, Tokyo, Japan). Colonies of cell aggregates of ≥50 cells were scored in the whole wells.

2.5. Osteogenic Differentiation. For differentiation into osteoblasts, stromal cells at a density of 5×10^3 cells/cm^2 were seeded in 12-well plates and cultured at 37°C with 5% CO_2 until they reached 50–70% confluency. After removing the growth medium, cells were washed twice with PBS and then induced to differentiate into osteoblasts using osteoblast differentiation medium containing complete DMEM-LG, 10 nM dexamethasone (Sigma), 10 mM β-glycerophosphate (Sigma), and 0.05 mM ascorbic acid (Sigma). After 14–21 days culture, the growth medium was removed, cells were then washed twice with PBS, and fixed with 4% paraformaldehyde (PFA) (Sigma) for 30 minutes at RT. Then cells were washed twice with PBS and stained with 2% of Alizarin red S (Sigma) at pH 4.1–4.3 for 10 minutes at RT with gentle rotation. After washing the cells 5 times with milliQ water, then 15 min with PBS, they were visualized under an Olympus CKX41 inverted microscope, and images were then captured. For quantitation of the calcium salts stained by Alizarin red S, the Alizarin red S dye was eluted with 10% cetylpyridinium chloride (CPC) (Sigma) in milliQ water for 15 minutes at RT with gentle rotation. The absorbance was measured using the Infinite F200 PRO Tecan spectrophotometer (Tecan, Mannedorf, Switzerland) at 570 nm. 10% CPC was used as a blank.

2.6. Adipogenic Differentiation. For adipocyte differentiation, stromal cells at a density of 2×10^4 cells/cm^2 were seeded in 12-well plates and cultured at 37°C with 5% CO_2 until they reached 100% confluency. After removing the growth medium, cells were washed twice with PBS and then were induced to differentiate into adipocytes using an induction medium of complete growth medium containing DMEM high glucose (DMEM-HG) (Life Technologies), 10% FCS, 1% A/A, 1 μM dexamethasone, 0.2 mM indomethacin (Sigma), 0.5 mM 3-isobutyl-1-methylxanthine (Sigma), and 0.01 mg/mL insulin (Life Technologies). After 3 days, support medium containing DMEM-HG, 10% FCS MSC, 1% A/A, and 0.01 mg/mL insulin was added for another 1–3 days. This induction and support medium cycle was repeated 3 times, and then the cells were cultured for 7 days in support medium. Following differentiation, cells were washed twice with PBS and fixed with 4% PFA for 60 minutes at RT. Then, the cells were washed twice with milliQ water, and 60% isopropanol (Sigma) was added for 5 minutes at RT. Afterwards, the cells were stained with Oil red O (Sigma) for 10 minutes at RT. Finally, the cells were washed 4 times with milliQ water, visualized under an Olympus CKX41 inverted microscope, and images were captured. For quantitation of the lipid vacuoles staining, the Oil red O dye was eluted with 100% isopropanol. The absorbance was measured using the Infinite F200 PRO Tecan spectrophotometer at 492 nm. 100% isopropanol was used as a blank.

2.7. Phenotypic Characterization of ESC by Flow Cytometry. The protein expression of IDO1, COX2, HO-1, and CXCL12 was determined by flow cytometry. ESC_{endo} and ESC_{cyst} were cultured at 1×10^4 cells/cm^2 until ~90% confluency, and then they were harvested using 0.05% trypsin/EDTA. For CXCL12, 5 hours before the end of culture and harvesting, cells were treated with golgi plug (Becton-Dickinson). Then the cells were fixed with 4% PFA for 10 minutes at RT, washed twice with PBS, and permeabilized using 0.1% saponin (USB, Buckinghamshire, UK) for 15 minutes at RT. Cells were then washed twice with PBS and stained with IDO PE (Bio-Techne, Minnesota, United States), COX2 Alexa fluor 488 (Cell Signaling Technologies, Massachusetts, United States), HO-1 APC (US Biological, Massachusetts, United States), and CXCL12 Alexa Fluor 488 (Novus Biologicals, Colorado, United States) in 0.1% saponin for 20 minutes in the dark at RT. Afterwards, the cells were washed twice with 0.1% saponin, resuspended in PBS with 0.1% BSA, and then run on the BD LSR Fortessa (Becton-Dickinson). Unstained cells were used to set gates and voltages. The data was analyzed using the software Flow-Jo.

2.8. Phenotypic Characterization of ESC by Quantitative Polymerase Chain Reaction (qPCR). To determine the gene expression of the IDO1, COX2, HO-1, and CXCL12 (genes listed in Table 1), qPCR was performed. Beta-actin (β-actin) was used as a housekeeping gene control. Forward and reverse primers were designed as instructed by Eurofins genomics and used according to the manufacturer's instructions. Cells previously stored in RNA*later* (ThermoFisher Scientific, Massachusetts, United States) at −80°C were thawed, diluted with an equal volume of PBS, and centrifuged at 500 ×g for 10 minutes, and the supernatant was then carefully removed. The resulting cell pellets were used to isolate total RNA using the RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Then, the RNA purity and concentration were measured using the nanodrop 2000c spectrophotometer

Table 1: Genes used in this study with the forward and reverse primers.

Gene	Forward primer	Reverse primer
β-Actin	AGCTACGAGCTGCCTGAC	AAGGTAGTTTCGTGGATGC
IDO1	GCATTTTTCAGTGTICTTCGCATA	TCATACACCAGCCGTCTGATAGC
COX2	ATCATAAGCGAGGGCCAGCT	AAGGCGCAGTTTACGGGTC
HO-1	CTTCTTCACCTTCCCCAACA	AGCTCCTGCAACTCCTCAAA
CXCL12	TTGACCCGAAGCTAAAGTGG	CCCTCTCACATCTTGAACCTCT

(ThermoFisher Scientific). 100 ng/μL RNA was used to synthesize cDNA using the High capacity cDNA reverse transcription kit (Applied Biosystems, Vilnius, Lithuania) according to the manufacturer's instructions. Finally, gene expression was quantified using the Fast SYBR green master mix (Applied Biosystems) according to the manufacturer's instructions, and the reactions were carried out in triplicate on the qPCR CFX384 real-time system C1000 touch thermal cycler (Bio-Rad, Stockholm, Sweden). Then, the data was analyzed using the software CFX manager (Bio-Rad). The relative expression level of the housekeeping gene β-actin was used to normalize target gene expression, and gene expression between ESC_{endo} and ESC_{cyst} was analyzed using the comparative Ct method ($\Delta\Delta$Ct method), using ESC_{endo} as the calibrator.

2.9. Isolation of Human Monocytes. Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats from healthy donors using SepMate tubes (StemCell Technologies, Cambridge, United Kingdom) and Lymphoprep gradient separation according to the manufacturer's instructions (Axis-Shield). Then, monocytes were isolated ($n = 3$) using the Monocyte isolation kit II (Miltenyi Biotech, Lund, Sweden) and a magnetic cell separation system (Miltenyi Biotech) as described previously [26]. The PBMCs were magnetically labelled with a cocktail of biotin-conjugated antibodies against CD3, CD7, CD16, CD19, CD56, CD123, and Glyocophorin A and antibiotin microbeads. Then, untouched monocytes were isolated by passing PBMCs through a column placed in a magnetic cell separator according to the manufacturer's instructions and depleting the magnetically labelled cells. The purity of the isolated monocytes was assessed by flow cytometry using an anti-CD14 monoclonal antibody (Becton-Dickinson); samples with purity $\geq$ 95% were used for experiments.

2.10. Functional Characterization of ESC. When the confluency for ESC_{endo} and ESC_{cyst} was ~70%, the growth medium was removed, the cells were washed twice with PBS, and fresh growth medium was added. After three days, the conditioned medium (CM) was collected, centrifuged at 500 ×g for 10 minutes to remove cellular debris, aliquoted, and frozen at −80°C. The CM was used for subsequent experiments (60% CM and 40% growth medium as described below). The untouched human monocytes were cultured in Roswell Park Memorial Institute (RPMI) (Life Technologies) 1640 growth medium, 10% FCS, 1% L-glutamine, 1% penicillin and streptomycin, and CM from ESC_{endo} or ESC_{cyst} from day 0 for 7 days. Then, images were acquired with the Olympus CKX41 inverted microscope at 20x magnification, and the cells were harvested for flow cytometry analysis. The monocytes were stained with CD14 FITC (Becton-Dickinson), CD163 PE (Becton-Dickinson), and CD206 FITC (Becton-Dickinson) for 20 minutes in the dark at RT. Then, the cells were washed twice with PBS by centrifugation at 500 ×g for 10 minutes, finally resuspended in PBS with 0.1% BSA, and analyzed with BD FACSCalibur. Unstained cells were used to set gates and voltages. The data was analyzed using the software Flow-Jo.

2.11. Statistical Analysis. All statistical analyses were performed using GraphPad prism 6. When data was normally distributed, the means were analyzed with Student t-test, and when it was not normally distributed, the medians were analyzed with the Mann–Whitney test. All values are shown as the mean ± standard deviations (SD). For the study, n refers to the number of biological replicates. Results were considered to be statistically significant if $P < 0.05$.

3. Results

3.1. MSC Exist in both Ectopic and Eutopic Endometrial Tissue. To verify that ESC_{endo} and ESC_{cyst} had the phenotype of MSC, we examined the cells by flow cytometry, CFU-F, and differentiation assays into osteoblasts and adipocytes. ESC_{endo} and ESC_{cyst} expressed the MSC markers CD73, CD90, CD105, and HLA class I, but did not express the non-MSC markers CD14, CD45, CD31, and HLA class II (Figures 1(a) and 1(b)). ESC_{endo} and ESC_{cyst} were both able to form colonies, albeit at a significantly lower ($P < 0.05$) efficiency for ESC_{cyst} (Figures 2(a) and 2(b)). Furthermore, stromal cells from both sources were able to differentiate into osteoblasts and adipocytes significantly ($P < 0.05$) as compared to untreated controls ($P < 0.05$) (Figures 2(c), 2(d), 2(e), and 2(f)). Taken together, the results indicate that both ESC_{endo} and ESC_{cyst} are MSC.

3.2. ESC_{cyst} Have a More Immunosuppressive Phenotype than ESC_{endo}. To characterize ESC_{endo} and ESC_{cyst} phenotypically, the gene and protein expressions of the immunosuppressive enzymes IDO1, COX2, and HO-1 and the proinflammatory chemokine CXCL12 were examined by both flow cytometry and qPCR, respectively. By flow cytometry, ESC_{cyst} expressed higher levels of IDO1, COX2, and HO-1 compared to ESC_{endo}. The percentage of ESC expressing these immunosuppressive enzymes and the level of expression or the median fluorescence intensity (MFI) in the positive cells

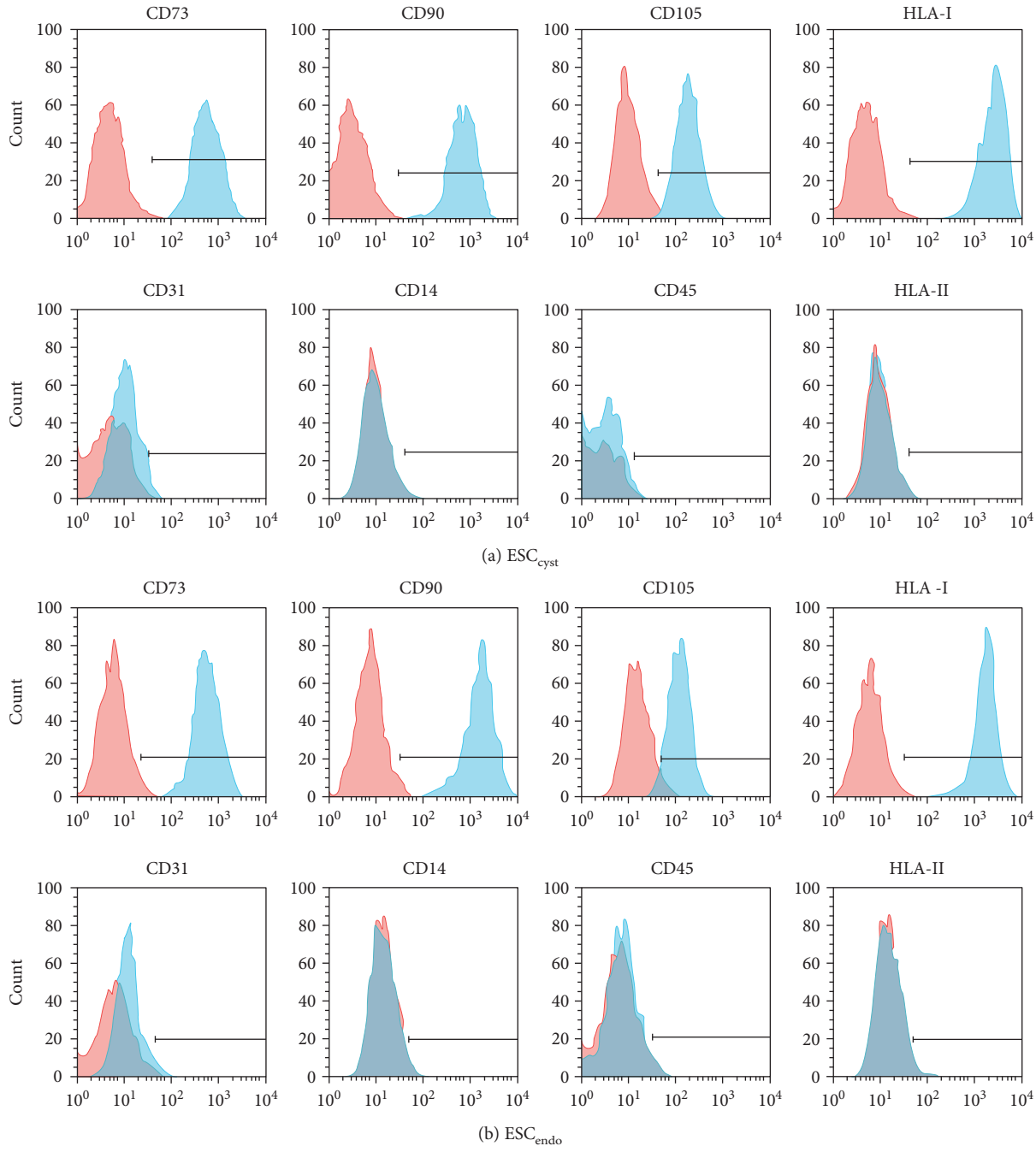

Figure 1: Representative flow cytometry histograms for ESC$_{endo}$ and ESC$_{cyst}$, which were positive (≥95%) for the MSC markers CD73, CD90, CD105, and HLA class I and negative (≤5%) for the non-MSC markers CD14, CD45, CD31, and HLA class II. Unstained control cells are in red, and stained cells are in blue. The horizontal lines on the histograms show the percentage of expression of the markers compared to the unstained control cells. Five to seven independent experiments ($n = 3$ biological replicates) were carried out.

was significantly higher ($P < 0.05$) for ESC$_{cyst}$ compared to ESC$_{endo}$ (Figure 3). Moreover, gene expression as analyzed by qPCR showed that IDO1, COX2, and HO-1 was expressed significantly higher ($P < 0.05$) in ESC$_{cyst}$ compared to ESC$_{endo}$ (Figure 3).

Flow cytometry analysis showed that ESC$_{cyst}$ expressed higher levels of CXCL12 than ESC$_{endo}$; the percentage of stromal cells expressing CXCL12 and the MFI was significantly higher ($P < 0.05$) for ESC$_{cyst}$ as compared to ESC$_{endo}$ (Figure 3). However, gene expression of CXCL12 was significantly lower ($P < 0.05$) for ESC$_{cyst}$ compared to ESC$_{endo}$ by qPCR (Figure 3).

3.3. ESC$_{cyst}$ Have a More Immunosuppressive Function than ESC$_{endo}$. To functionally characterize ESC$_{endo}$ and ESC$_{cyst}$, their effects on the differentiation of monocytes into macrophages were examined. Specifically, the morphology of the monocytes and their protein expression of CD14, CD163,

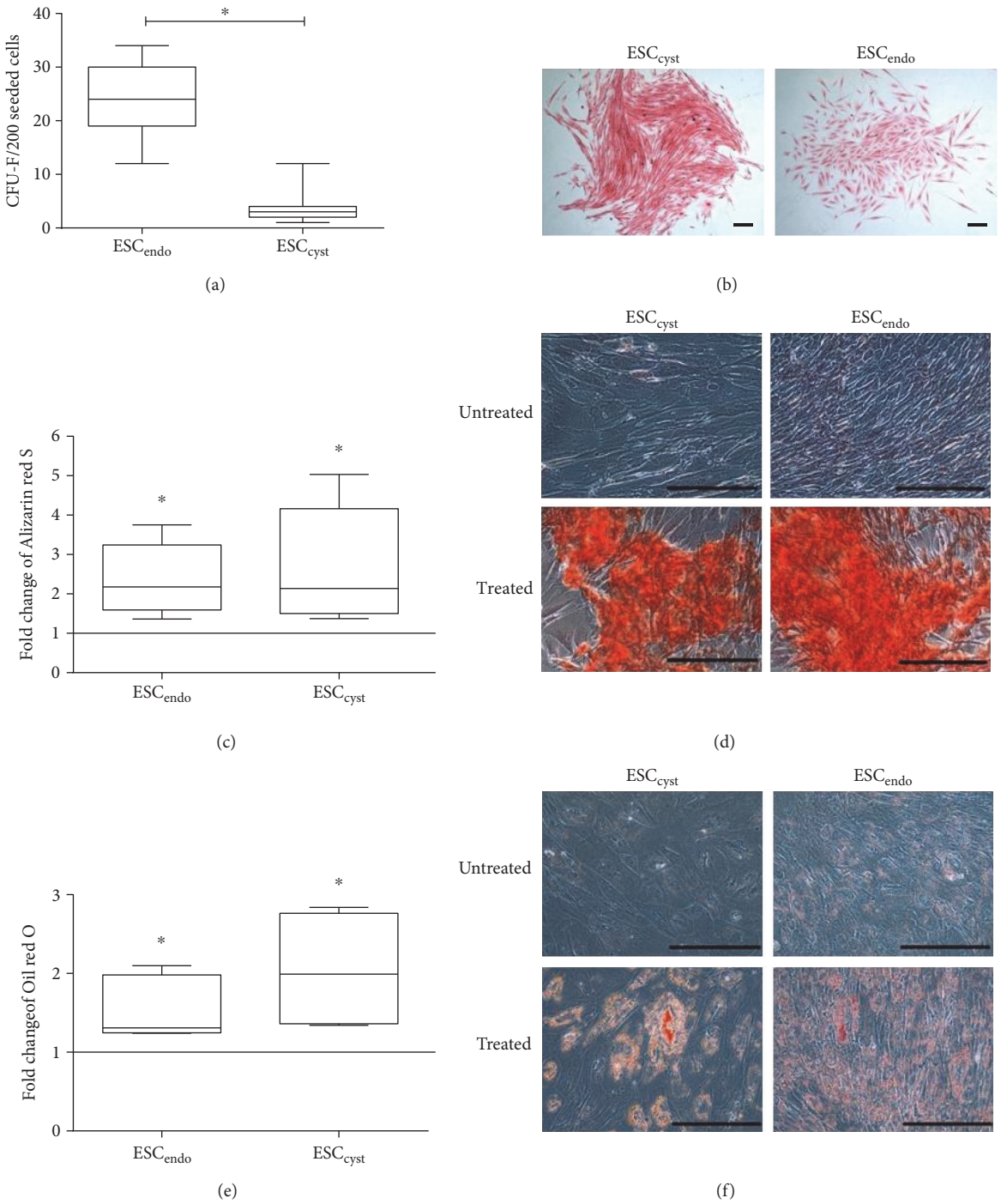

Figure 2: Colony-forming unit fibroblasts for ESC_{endo} and ESC_{cyst} after they were cultured at clonal density for 21 days. Also, osteoblast differentiation for ESC_{endo} and ESC_{cyst} following 14–21 days culture in osteoblast differentiation growth medium and adipocyte differentiation for ESC_{endo} and ESC_{cyst} following 28-day culture in adipocyte differentiation growth medium are shown. The colony-forming efficiency (a) shows that ESC_{endo} formed significantly more colonies than the ESC_{cyst} ($^*P < 0.05$). Representative phase contrast images (b) at 4x magnification following Eosin staining of ESC_{endo} and ESC_{cyst}. Colonies with ≥50 cells were counted. Quantitation of the Alizarin red S dye (c) as a mean fold change relative to the untreated controls; both types of stromal cells significantly induced osteoblast differentiation ($^*P < 0.05$). Representative phase contrast images (d) at 20x magnification following Alizarin red S staining of the calcium salts. Quantitation of the Oil red O dye (e) as a mean fold change relative to the untreated controls; both types of stromal cells significantly induced adipocyte differentiation ($^*P < 0.05$). Representative phase contrast images (f) at 20x magnification following Oil red O staining of the lipid vacuoles. Nine independent experiments ($n = 3$ biological replicates) were carried out in triplicates for (a, b). Three to four independent experiments ($n = 3$ biological replicates) were carried out in duplicates for (c, d, e, f). Mean ± SD. Scale bars represent 50 μm at 4x magnification for (a,b) and 50 μm at 20x magnification for (c, d, e, f).

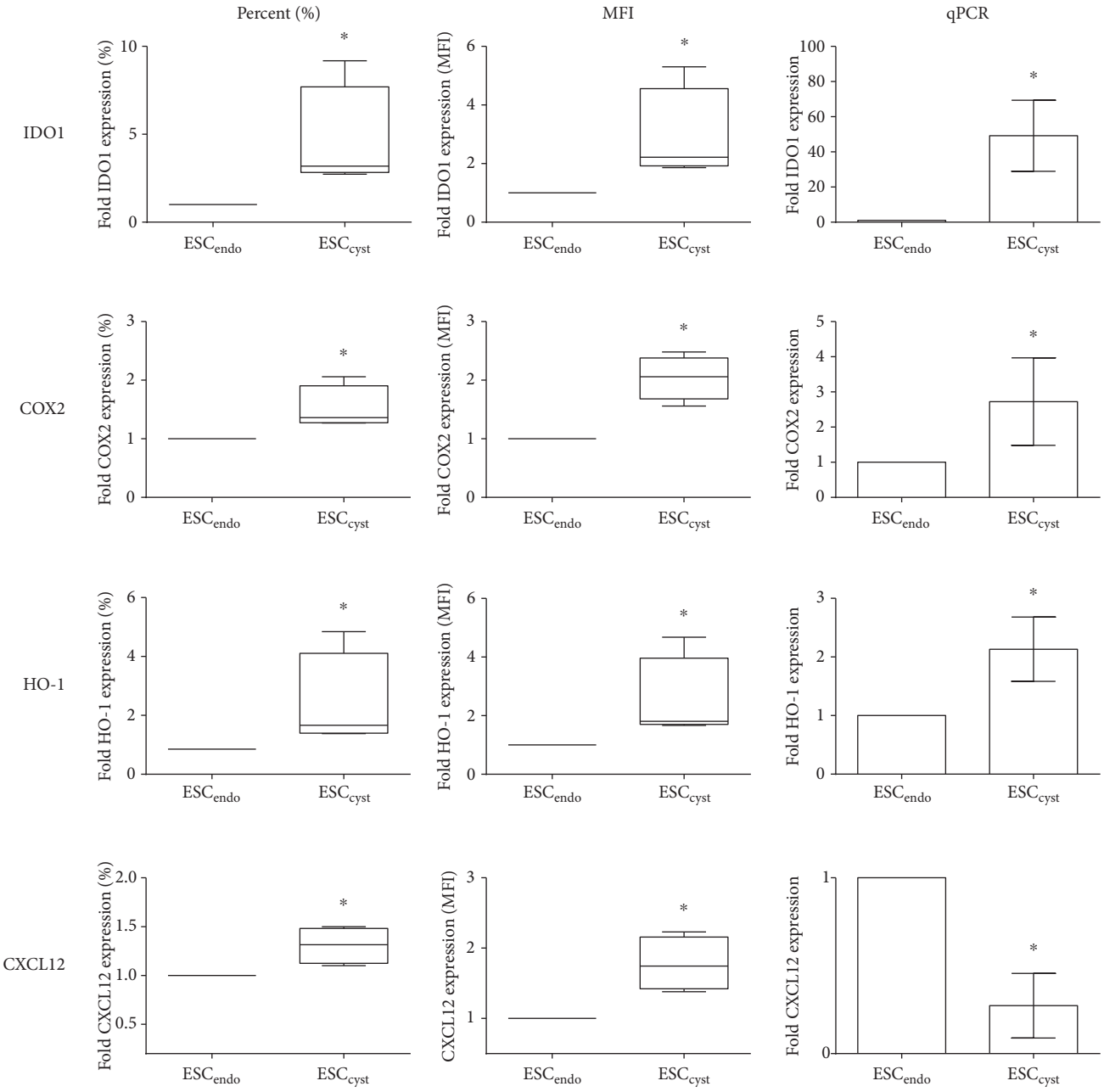

Figure 3: Protein and gene expression of the immunosuppressive enzymes IDO1, COX2, and HO-1 and the proinflammatory chemokine CXCL12 in ESC_{endo} and ESC_{cyst} by flow cytometry and qPCR, respectively. The percentage of cells expressing IDO1 is higher for ESC_{cyst} ($^*P < 0.05$). ESC_{cyst} express higher levels of IDO1 by MFI ($^*P < 0.05$). ESC_{cyst} have higher gene expression of IDO1 ($^*P < 0.05$). The percentage of cells expressing COX2 is higher for ESC_{cyst} ($^*P < 0.05$). ESC_{cyst} express higher levels of COX2 by MFI ($^*P < 0.05$). ESC_{cyst} have higher gene expression of COX2 ($^*P < 0.05$). The percentage of cells expressing HO-1 is higher for ESC_{cyst} ($^*P < 0.05$). ESC_{cyst} express higher levels of HO-1 by MFI ($^*P < 0.05$). ESC_{cyst} have higher gene expression of HO-1 ($^*P < 0.05$). The percentage of cells expressing CXCL12 is higher for ESC_{cyst} ($^*P < 0.05$). ESC_{cyst} express higher levels of CXCL12 by MFI ($^*P < 0.05$). ESC_{cyst} have lower gene expression of CXCL12 ($^*P < 0.05$). Four independent experiments ($n = 4$ biological replicates) were carried out in duplicates. Mean ± SD.

and CD206 was examined following 7-day culture in the CM from ESC_{endo} and ESC_{cyst}. Morphologically, ESC_{cyst} induced more spindle-shaped macrophages than ESC_{endo} (Figure 4(a)). Also, the percentage of macrophages expressing CD14 and CD163 and the MFI in these positive macrophages was significantly higher ($P < 0.05$) for ESC_{cyst} as compared to ESC_{endo} (Figure 4(b)). The percentage of macrophages expressing CD206 and the MFI in these positive macrophages was not significantly different ($P > 0.05$) between ESC_{cyst} and ESC_{endo} (Figure 4(b)).

Taken together, the results indicate that ESC_{cyst} may be functionally more immunosuppressive than ESC_{endo} based on their ability to induce more spindle-shaped M2 macrophages with a significantly higher ($P < 0.05$) level of

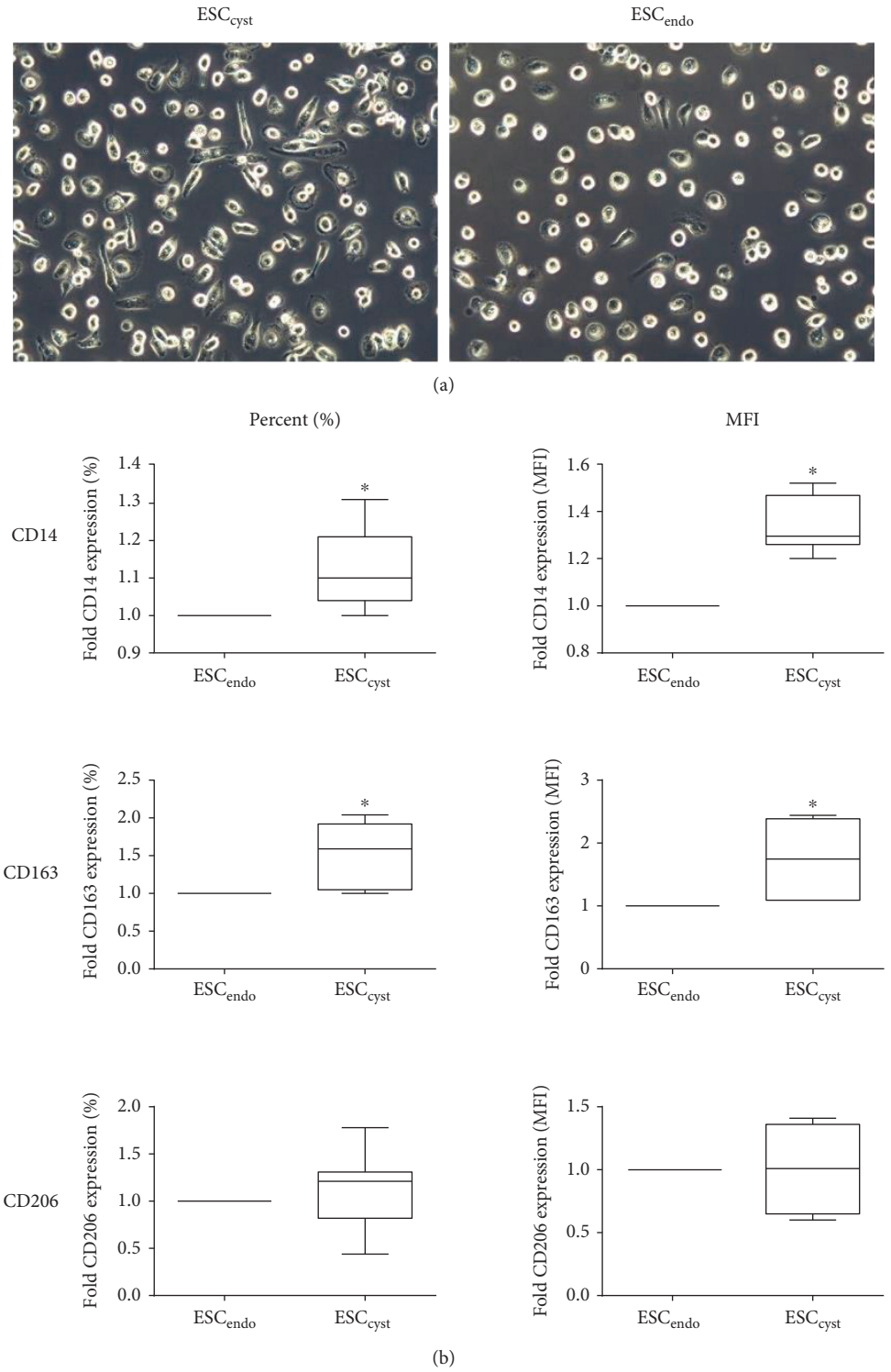

Figure 4: The effects of ESC$_{endo}$ and ESC$_{cyst}$ conditioned medium (CM) on freshly isolated untouched human monocytes. Representative phase contrast images (a) of the CM-treated monocytes after 7 days of culture at 20x magnification. The percentage of macrophages expressing CD14 and CD163 and the level of expression in these positive macrophages are greater for ESC$_{cyst}$ ($^{*}P < 0.05$) (b). The percentage of macrophages expressing CD206 and the level of expression in these positive macrophages are not different between ESC$_{endo}$ and ESC$_{cyst}$ (b). Seven independent experiments ($n = 4$ biological replicates) were carried out in duplicates. Mean ± SD. Scale bars represent 50 μm at 20x magnification.

expression of CD14 and CD163, both distinctive features of immunosuppressive M2 macrophages [20].

4. Discussion

Currently, it is unclear how endometriotic lesions avoid immunosurveillance in the pelvic cavity, and the cause of reduced immunosurveillance in the pelvic cavity is unknown. In addition, the phenotype and function of MSC in endometriotic lesions is not completely known. Herein, ESC from endometriotic ovarian cysts displayed all characteristics of MSC. We have shown that the ESC have a more immunosuppressive phenotype if located within endometriotic ovarian cysts compared to the eutopic endometrium and that they direct monocyte differentiation into immunosuppressive M2 macrophages. Taken together, ectopic MSC may contribute to reduced immunosurveillance in the pelvic cavity to allow immune escape of ectopic lesions and support their growth in endometriosis.

In agreement with previous findings, ESC_{endo} and ESC_{cyst} isolated from women with endometriosis met the criteria to be classified as MSC as they showed fibroblastic morphology, appropriate expression of surface markers, and differentiation into osteoblasts and adipocytes [27, 28]. In addition, and as shown before, ESC_{endo} formed CFU-F to a greater extent than ESC_{cyst} [29]. This could be because ESC_{cyst} grow in an ischemic microenvironment *in vivo* in the pelvic cavity, which may affect their proliferation and hence colony-forming ability.

Immunosuppressive MSC have been suggested to express high levels of immunosuppressive and low levels of immunostimulatory molecules, respectively. Therefore, we examined the expression of such molecules by ESC_{endo} and ESC_{cyst}. Both protein and gene expression of IDO1, COX2, and HO1 was significantly higher in ESC_{cyst}, suggesting that they may be more immunosuppressive phenotypically. Other studies have shown similar results, with higher gene expression of COX2 and HO-1 in ESC_{cyst} and stromal cells from peritoneal endometriotic tissue compared to ESC_{endo} [30, 31]. In contrast to these results, two studies reported that ESC_{cyst} expressed similar gene and protein levels of IDO1 as ESC_{endo} [32, 33]. It was also found that ectopic stromal cells may be more immunostimulatory than ESC_{endo} from healthy controls [34]. However, ESC_{endo} from healthy controls may introduce individual to individual variations in terms of the immunological microenvironment and endocrine factors [34]. Moreover, it is unclear if the ectopic stromal cells were from peritoneal endometriotic tissue or endometriotic ovarian cysts, which are two different types of endometriosis lesions [34]. Interestingly, in a differently designed study, IDO1 and COX2 gene expression was found to be higher in menstrual blood-derived stromal cells in women with endometriosis compared to healthy controls after culture in a transwell system with PBMCs [35]. In contrast to the latter study, herein, we used unstimulated ESC to reflect the *in vivo* environment more closely. We next examined if ESC_{cyst} were less immunostimulatory phenotypically than ESC_{endo} by studying their expression of CXCL12. Similar to a previous report [36], we found that protein expression of CXCL12 was significantly higher for ESC_{cyst} compared to ESC_{endo}, but in contrast to herein, they stimulated their ESC with estrogen or progesterone [36]. However, the gene expression of CXCL12 was lower in ESC_{cyst}. This discrepancy may be due to posttranscriptional and posttranslational processes, since weak correlations between mRNA and protein abundance have been described before [37]. Proteins, not genes, bestow cellular function, and therefore ESC_{cyst} seem to have a more immunostimulatory phenotype than ESC_{endo} [37]. CXCL12 is a ligand of the C-X-C chemokine receptor type (CXCR) 4, and through it, ESC_{cyst} may further increase levels of inflammation in the pelvic cavity by recruiting CXCR4-positive immune cells [38]. Interestingly, ESC_{endo} express CXCR4 and may hence be recruited to ectopic lesions by CXCL12, which may also possess nonimmune functions in endometriosis, such as promotion of tissue repair, angiogenesis, migration, invasion, and suppression of apoptosis [1, 38, 39], processes proposed to be involved in growth of ectopic lesions. ESC_{cyst} may be more immunostimulatory phenotypically than ESC_{endo} in response to fluctuations of levels of pathological inflammation in the pelvic cavity. In summary, these results imply that the inflamed pelvic cavity may induce ESC_{cyst} to become more immunosuppressive phenotypically to allow them to reduce inflammation and promote tissue homeostasis.

IDO1, COX2 via secretion of prostaglandin E2 (PGE2), and HO-1 have been suggested to be able to induce immunosuppressive M2 macrophages [13, 40–44]. Interestingly, endometriosis is a disease with profound macrophage involvement, with predominance of M2 macrophages in peritoneal endometriotic tissue, and endometriotic ovarian cysts that have been suggested to play a role in the pathology [14, 45–47]. Therefore, we examined the effects of ESC_{endo} and ESC_{cyst} on monocyte differentiation into macrophages. Morphologically, ESC_{cyst} were found to induce more spindle-shaped macrophages than ESC_{endo}. Furthermore, ESC_{cyst} induced a significant increase in macrophages expressing scavenger receptors CD14 and CD163 compared to ESC_{endo}. CD14 is involved in the uptake of apoptotic cells, CD163 is involved in the uptake of haptoglobin-hemoglobin complexes, and both have crucial roles in clearing up the pelvic cavity from apoptotic cells and heme-iron that accumulates by dying red blood cells, respectively [45, 48]. Elevated CD163 expression has been suggested to be a marker of M2c, a subtype of M2 macrophages involved in immunosuppression, matrix deposition, and tissue remodeling [20, 49–51]. Therefore, increased M2c levels may explain the extensive fibrosis that occurs in endometriotic lesions [52]. The macrophages expressed similar levels of the scavenger receptor CD206 after treatment with CM from ESC_{endo} and ESC_{cyst}. CD206 is involved in inactivating inflammatory signals and may have a central role in the inflamed pelvic cavity [45]. In summary, this data shows that ESC_{cyst} may be more immunosuppressive functionally in comparison to ESC_{endo}.

Two previous studies showed that ectopic stromal cells [53] or ESC_{endo} [47] polarized human macrophages or U937 monocytes stimulated with lipopolysaccharide into M2 macrophages, respectively. In the former study, macrophage expression of CD163 and CD209 and their intracellular

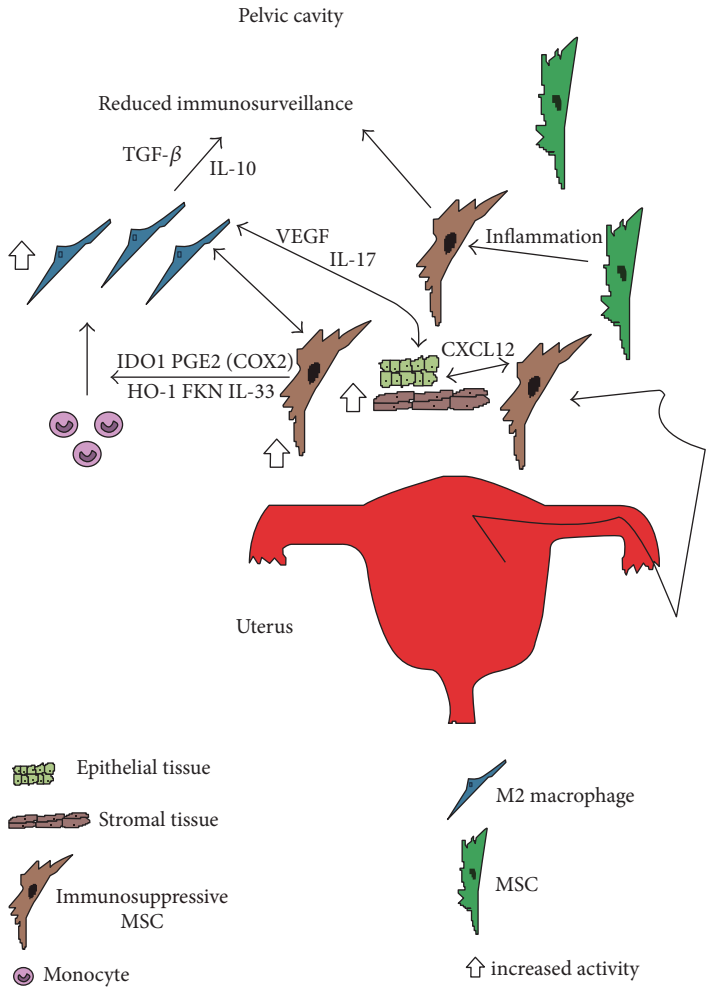

Figure 5: Schematic figure showing the role immunosuppressive ectopic MSC may be playing in the pathology of endometriosis. MSC along with stromal and epithelial endometrial tissue enter the pelvic cavity via retrograde menstruation. The highly inflammatory environment may induce MSC to become immunosuppressive to help promote tissue homeostasis. The recruited monocytes responding to the inflammatory environment and FKN, IL-33, IDO1, PGE2 via COX2, and HO-1 from immunosuppressive MSC may then polarize and differentiate into immunosuppressive M2 macrophages. Then, M2 macrophages may increase invasion of refluxed endometrial cells, repair ectopic lesions, and support their growth by inducing angiogenesis via their secretion of VEGF and IL-17 [58]. In addition, secretion of IL-10 and TGF-β by the M2 macrophages may suppress other recruited immune cells to reduce immunosurveillance in the pelvic cavity [58]. Also, ectopic MSC may directly support ectopic lesion growth by promotion of tissue repair, angiogenesis, migration, invasion, and suppression of apoptosis through CXCL12. The net effect may be large numbers of immunosuppressive MSC and M2 macrophages in the pelvic cavity that may directly support ectopic lesion growth, reduce immunosurveillance in the pelvic cavity, and reciprocally support each other's growth contributing to the development and progression of endometriosis. MSC: mesenchymal stromal cell; FKN: fractalkine; IDO1: indoleamine 2,3-dioxygenase 1; COX2: cyclooxygenase 2; HO-1: heme oxygenase 1; CXCL12: chemokine c-x-c motif chemokine ligand 12; VEGF: vascular endothelial growth factor; IL-17: interleukin 17; IL-10: interleukin 10; TGF-β: transforming growth factor-beta; IL-33: interleukin 33; PGE2: prostaglandin E2.

expression and extracellular secretion of transforming growth factor-beta 1 (TGF-β1) and interleukin-10 (IL-10) was increased compared to macrophages treated with ESC_{endo} from healthy controls [53]. However, as discussed above, ESC_{endo} from healthy controls may not be an appropriate control as it may introduce individual to individual variations in terms of the immunological microenvironment and endocrine factors [53]. Moreover, it is unclear if the ectopic stromal cells were from peritoneal endometriotic tissue or endometriotic ovarian cysts, which are two different types of endometriosis lesions [53]. In the latter study, there was an increase in immunosuppressive cytokine IL-10 and a decrease in the expression of the costimulatory molecule CD86 by the M2 macrophages [47]. These studies suggested that IDO1 via IL-33 [53] secreted by ectopic stromal cells or ESC_{endo}-derived fractalkine (FKN) [47], respectively, was driving the M2 macrophage polarization. Moreover, the M2-polarized macrophages significantly increased the viability and proliferation of ESC, decreased apoptosis of ESC [54], and enhanced the invasiveness of ESC_{endo} [47], suggesting that they may

support the growth of ectopic lesions in endometriosis. In contrast to the aforementioned studies, we examined the effects of ESC_{cyst} on the ability of primary unstimulated human monocytes to differentiate into macrophages in comparison to ESC_{endo}. To our knowledge, this is the first time that this has been performed.

Interestingly, soluble factors in the CM from unstimulated ESC_{cyst} induced the M2 macrophage differentiation, indicating that stimulation of ESC_{cyst} by monocytes through paracrine mechanisms and direct contact are not required. IDO1 and HO1 can be secreted by MSC and, along with secreted PGE2 via COX2, may have been involved in ESC_{cyst} promoting M2 macrophage differentiation [55, 56]. M2 macrophages have been suggested to play a role in the pathology of endometriosis by recognizing initial ectopic lesions as wounds and initiating "healing" [45]. The wound healing properties of M2 macrophages may be important in skin wounds; however, they may be detrimental in endometriosis [57]. Moreover, M2 macrophage secretion of IL-10 and TGF-β may suppress other immune cells leading to reduced immunosurveillance in the pelvic cavity and hence protect ectopic lesions from immune clearance [58]. Therefore, it has been suggested that redirection of M2 macrophages to M1 macrophages may be a strategy to stimulate immune responses against ectopic lesions [45]. A schematic figure illustrating our proposed hypothesis for the role of ectopic MSC in the pathogenesis of endometriosis is shown in Figure 5.

The limited number of donors and presence of hormonal treatment did not affect the consistency of data between the four women with endometriosis being studied. Significant differences were observed, and meaningful conclusions could be made. A similar number of patients have been used in other studies [35, 59]. The stromal cells herein were unstimulated and unmodified but were cultured, which may alter their functional phenotype. Therefore, it would be interesting to study native stromal cells. Nevertheless, to our knowledge, this is the first *in vitro* study showing that ESC_{cyst} may have more immunosuppressive properties than ESC_{endo}. This is an important finding that will improve our knowledge on the pathogenesis of endometriosis and may benefit development of new therapies.

5. Conclusion

In summary, immunosuppressive ectopic MSC may contribute to reduced immunosurveillance in the pelvic cavity. This may be in part by their immunosuppressive effects through M2 macrophages, which may subsequently support the growth of endometriotic ovarian cysts in endometriosis. This finding supports the retrograde menstruation and the stem cell theories by adding an immunosuppressive ectopic MSC component. Finally, we speculate that reducing the immunosuppressive effects of ectopic MSC to promote M1 macrophage and T Helper 1 responses may provide the necessary immunostimulation to remove ectopic lesions in the pelvic cavity and to potentially treat endometriosis.

Conflicts of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contributions

Fawaz Abomaray, Sebastian Gidlöf, and Cecilia Götherström contributed to the idea and design of the paper. Fawaz Abomaray contributed to the data acquisition. Fawaz Abomaray, Sebastian Gidlöf, and Cecilia Götherström contributed to the data analysis and interpretation. Fawaz Abomaray performed laboratory assays. Fawaz Abomaray, Sebastian Gidlöf, and Cecilia Götherström wrote the paper and revised it critically. All the authors read and approved the final paper.

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