Journal of Reproductive Immunology 98 (2013) 29–38

Contents lists available at SciVerse ScienceDirect

Journal of Reproductive Immunology

journal homepage: www.elsevier.com/locate/jreprimm

Preservation of human placenta facilitates multicenter studies on the local immune response in normal and aberrant pregnancies

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ARTICLE INFO

Article history:
Received 1 November 2012
Received in revised form 27 February 2013
Accepted 4 March 2013

Keywords:
Human
Decidua
Normal pregnancy
Lymphocytes
Preservation

ABSTRACT

Our standard procedure for phenotypic and functional analysis of immune cells present in the placenta is to isolate leukocytes from the decidua within five hours of the delivery. However, this results in logistical problems with deliveries at night, weekends or in other medical centers. Collecting placentas after complicated pregnancies is even more difficult owing to the low prevalence and the often unscheduled delivery. The aim was to investigate the possibility of preserving the human placenta before phenotypic and functional analysis of decidual lymphocytes. Placentas were obtained after uncomplicated pregnancy. The tissue was divided into two equal parts: decidual lymphocytes from one part were isolated within five hours according to our standard procedure, whereas the other part was preserved in either Celsior®, a storage solution for solid organ preservation, or phosphate-buffered saline (PBS) for 24 h at 4 °C before isolation. Overall, the phenotype and functional capacity of decidual lymphocytes isolated within five hours was comparable to decidual lymphocytes isolated after 24-h preservation in Celsior® or PBS. Minor differences were found between decidual lymphocytes isolated within five hours and decidual lymphocytes isolated after 24-h preservation in Celsior®. The results indicate that PBS is sufficient to preserve the placenta for 24 h for phenotypical and functional studies. The ability to preserve the placenta will simplify the procedure for the isolation of decidual lymphocytes and makes it easier to analyze tissue from women who deliver during the night, at weekends or in other hospitals, and possibly even women with complicated pregnancies.

1. Introduction

Pregnancy is an immunological paradox in which several mechanisms have been postulated to avoid a destructive immune response toward the semi-allogeneic fetus. (Billington, 2003) One of the mechanisms suggests an overall impaired maternal immune system, resulting in the acceptance of the semi-allogeneic fetus. However, the maternal immune system produces antibodies to fetal HLA in 10–30% of women during pregnancy (van Kampen et al., 2002; van Rood et al., 1958) and fetus-specific cytotoxic T lymphocytes to fetal HLA and minor histocompatibility antigens have been found (Bouma et al., 1996; van Kampen et al., 2001; Verdijk et al., 2004), indicating recognition of fetal antigens. In addition, there are no differences in the proliferative responses to fetal antigens between pregnancy and nonpregnant controls (Lashley et al., 2011). Together, these studies suggest a more local regulation

Abbreviations: cpm, counts per minute; DNase, deoxyribonuclease; FACS, fluorescence-activated cell sorter; FITC, fluorescein isothiocyanate; HLA, human leukocyte antigen; IFN, interferon; IL, interleukin; NK cell, natural killer cell; PBS, phosphate-buffered saline; PE, phycoerythrin; TNF, tumor necrosis factor.

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0165-0378
http://dx.doi.org/10.1016/j.jri.2013.03.001

of the immune response toward the semi-allogeneic fetus.

The human decidua can be divided into two parts: the decidua basalis and the decidua parietalis. The decidua basalis is located at the implantation site and is in direct contact with the invading trophoblast cells. The decidua parietalis is connected to the chorion and is therefore in direct contact with the non-invasive trophoblast cells. Both maternal and fetal mechanisms result in a dampened immune response to the semi-allogeneic fetus (Aluvihare et al., 2004; Jiang and Vacchio, 1998; Tafuri et al., 1995). Trophoblast cells only express HLA-C and the nonclassical HLA-E and HLA-G (Le Bouteiller et al., 1996; Hammer et al., 1997), which could result in local immune regulation. Furthermore, our group has shown that the decidua contains different immune cells, with altered functional capacity, compared with the maternal peripheral blood.

Previous studies have shown that activated T cells are present in decidual tissue in uncomplicated pregnancies (Sindram-Trujillo et al., 2003a) and that both early and term pregnancy decidua contain an abundance of CD4+CD25bright regulatory T cells (Sasaki et al., 2004; Tilburgs et al., 2006). In addition, Tilburgs et al. (2008) showed that fetus-specific regulatory T cells from the peripheral blood are recruited to the fetal–maternal interface. Functional regulatory T cells were only found in HLA-C mismatched pregnancies and not in HLA-C matched pregnancies (Tilburgs et al., 2009b). The percentage of CD8+CD28− T cells was increased in the decidua compared with peripheral blood (Tilburgs et al., 2006). The CD8+CD28− T cells found in the decidua did not express the cytolytic molecule perforin (Tilburgs et al., 2009a). Further investigation showed that the decidual CD8+ T cells were predominantly of the effector-memory phenotype and these cells expressed reduced levels of perforin and granzyme B compared with the peripheral effector-memory CD8+ T cells (Tilburgs et al., 2010b). Taken together, these findings suggest that maternal lymphocytes present in the placenta play an important role in the regulation of the maternal immune response toward the semi-allogeneic fetus.

Insufficient regulation of the maternal immune response may result in pregnancy complications in which placental and fetal growth are impaired. Therefore, the focus of ongoing studies is on comparing the local immunoregulation in uncomplicated pregnancies with pregnancies complicated by, for example, pre-eclampsia. However, collecting material from complicated pregnancies proved to be challenging, owing to the low prevalence of the complication and the usually unscheduled delivery or unexpected termination of pregnancy. Our standard procedure is to isolate lymphocytes from the decidua basalis and decidua parietalis within five hours of the delivery. For that reason, multi-centered studies are not feasible and unplanned emergency deliveries that take place during the night and at weekends result in logistical problems.

In the setting of organ transplantation, a diversity of preservation solutions has been used. Celsior® was originally developed as a heart preservation solution (Menasche et al., 1994). However, systematic reviews (O'Callaghan et al., 2012; Timsit and Tullius, 2011) have concluded that this solution can also be successfully used in lung (Roberts et al., 1999), kidney (Baertschiger et al., 2008; Faenza et al., 2001; Montalti et al., 2005; Pedotti et al., 2004), liver (Lopez-Andujar et al., 2009), and pancreas and islet transplantation (Baertschiger et al., 2008). Preservation of the decidual tissue prior to the isolation procedure would overcome the low prevalence of the complication and the usually unscheduled delivery or unexpected termination of pregnancy. Therefore, the aim of this study was to investigate the possibility of preservation of the human placenta before isolating the lymphocytes for phenotypic characterization and functional analysis of their immunoregulatory properties. We compared the Celsior® solution with PBS, a more commonly available solution in research facilities.

2. Materials and methods

2.1. Blood and tissue samples

Paired samples of decidua basalis, decidua parietalis, and heparinized umbilical cord blood (UCB) were collected from healthy women after uncomplicated term pregnancy. Tissue samples were obtained after delivery by elective caesarean section or uncomplicated spontaneous vaginal delivery. Both the decidua basalis and decidua parietalis were divided into two equal parts: decidual lymphocytes from one part were isolated within five hours according to our standard procedure, whereas the other part was preserved at 4 °C in either Celsior® (Genzyme Europe BV, Naarden, The Netherlands), a storage solution for solid organ preservation, or PBS (LUMC pharmacy; Leiden, The Netherlands) for 24 h after which the decidual lymphocytes were isolated. A total of 14 placentas were included in this study: five to compare decidual lymphocytes isolated within five hours to preservation in Celsior®, three to compare preservation in Celsior® with preservation in PBS, and six to compare decidual lymphocytes isolated within five hours to preservation in PBS of which three were used for the FoxP3 stainings and CD25 depletion experiments. In addition, part of the decidual tissue was embedded in paraffin and stained with H&E and antibodies directed against CD45 and CD3 to study the morphology and phenotype within five hours of delivery and after 24 h of preservation in either Celsior® or PBS.

2.2. Lymphocyte isolation

Decidual lymphocytes were isolated according to our standard protocol as described previously (Tilburgs et al., 2006). In short, the decidua basalis and decidua parietalis were macroscopically dissected from the placenta or membranes and washed in PBS. The tissue was minced and washed again to reduce contamination with blood and trophoblast cells. Decidual tissue was then incubated for an hour with 0.2% collagenase I and 0.02%DNAse I in RPMI 1640 (Gibco, Paisley, Scotland) in a water bath (37 °C, gently shaking). The decidual fragments were washed in RPMI, filtered through a 70-μm sieve (Becton Dickinson, Labware, NJ, USA) and washed again. The decidual isolates were layered on a Percoll gradient (10 ml, 1.080 g/ml; 12.5 ml,

1.053 g/ml; 20 ml, 1.023 g/ml) for density gradient centrifugation (30 min/800 g) and lymphocytes were isolated from the 1.080 g/ml–1.053 g/ml interface. Cells were washed twice and counted. Blood was layered on a Ficoll-Hypaque (LUMC Pharmacy; Leiden, The Netherlands) for density gradient centrifugation (20 min/800 g). Mononuclear cells were collected, washed twice with PBS and counted. Part of the cells were fixed with 1% paraformaldehyde and stored at 4 °C until the time of cell staining and flow cytometry analysis. The remaining cells were used for the proliferation studies.

2.3. Flow cytometry

A four-color immunofluorescence staining was performed using the following directly conjugated mouse-anti-human monoclonal antibodies: CD14-FITC, CD69-FITC, HLA-DR-FITC, CD8-PE, CD25-PE, CD3-PerCP, CD4-APC, CD28-APC, and CD45-APC (Becton Dickinson) in concentrations according to the manufacturer's instructions. The expression of FoxP3 was determined using an APC anti-human FoxP3 Staining Set (eBioscience, San Diego, CA, USA). Flow cytometry was performed on a FACS Calibur using Cellquest-Pro software (Becton Dickinson). The percentages were calculated within the lymphocyte gate, set around the viable lymphocytes and based on the expression of CD45, CD14, and CD3, as previously described (Tilburgs et al., 2006). The CD45+CD14- cells were selected in a density plot and back gated in a scatter plot. The lymphocyte gate was set around the viable lymphocytes, within which the CD3+ T cells were selected. During the analysis, the focus was on the cell subsets CD4+CD25bright, CD4+CD25dim, and CD8+CD28−, and the expression of FoxP3, HLA-D, and CD69. The percentage of CD4+CD25bright and CD4+CD25dim T cells was calculated within the CD3+CD4+ cell population and the percentage of CD8+CD28− T cells was calculated within the CD3+CD8+ cell fraction. The expression of FoxP3 was calculated for the CD3+CD4+, CD4+CD25bright, and CD4+CD25dim population, the expression of HLA-DR and CD69 was calculated for the total CD3+ T cell fraction.

2.4. Functional assays

The functional features of the isolated decidual lymphocytes were assessed in mixed lymphocyte cultures (MLC) in which decidual lymphocytes were stimulated with irradiated (3000 Rad) UCB from the own child and 3rd party UCB (3pUCB). The MLC was set up in triplicate in round-bottomed 96-well plates (Costar, Cambridge, MA, USA). A single well contained 0.5×10^5 decidual lymphocytes as responder cells in RPMI 1640 culture medium containing L-glutamine, penicillin, streptomycin (Gibco), and 10% human serum. These cells were stimulated with 0.5×10^5 irradiated (30 Gy) UCB or 0.5×10^5 irradiated (30 Gy) 3pUCB. As a positive control decidual lymphocytes were stimulated with purified phytohemagglutinin (PHA; 0.4 mg/ml; Wellcome Diagnostics, Dartford, UK) and stimulation with culture medium was used as negative control. In addition, decidual leukocytes isolated within five hours and after 24-h preservation in PBS were FACS sorted on a FACSAria SORP (Becton Dickinson). Leukocytes were stained for CD4-FITC, CD25-PE, and CD45-APC, and sorted as described before (Tilburgs et al., 2008). In short, cells within the gate set for viable lymphocytes were selected and sorted into a CD45+ population and a CD45+ population depleted for CD4+CD25bright cells. The cells were washed once in culture medium and stimulated with UCB and 3pUCB in the MLC setting described above. Proliferation was measured on day 6 by incorporation of 3H-thymidine during the last 18 h of culture. Supernatant was collected at day 5 before adding 3H-thymidine and stored at −20 °C until cytokine analysis. The results were expressed as the median cpm for each triplicate culture. The stimulation index was calculated by dividing the cpm measured when stimulator and responder are cultured together by the sum of the cpm measured when stimulator and responder are cultured separately (de Koster et al., 1992).

2.5. Cytokine analysis

The supernatants collected on day 5 of the MLC were tested for the following cytokines: IL-2, IL-4, IL-5, IL-10, IL-12(p70), IL-13, GM-CSF, IFN-γ and TNF-α, using a Bio-Plex assay (Bio-Rad Laboratories, Veenendaal, The Netherlands) following the manufacturer's instructions. Samples were analyzed using a Bio-Plex Array Reader with Bio-Plex software. The ratio of IFN-γ and IL-10 was calculated to evaluate the contribution of Th1 cytokines versus Th2 cytokines.

2.6. Statistical analysis

The paired Wilcoxon signed rank test was used to compare the percentage of cell subsets, proliferation and cytokine production between the lymphocytes isolated from both the decidua basalis and decidua parietalis within five hours and isolated after preservation for 24 h in Celsior® or PBS. P values < 0.05 are considered to denote significant differences.

3. Results

The aim of this study was to investigate the possibility of the preservation of the human placenta before isolating decidual lymphocytes for phenotypic characterization and functional analysis. Therefore, placentas were obtained from women after uncomplicated pregnancy. The characteristics of the included pregnancies are shown in Table 1. The tissue was divided into two equal parts to compare the phenotype and the functional features of decidual lymphocytes isolated within five hours with decidual lymphocytes isolated after 24-h preservation in either Celsior® or PBS. H&E staining of the decidual tissue showed similar morphology within five hours of delivery and after 24 h of preservation in either Celsior® or PBS (Fig. 1). The CD45/CD3 ratio was similar in tissues preserved in either Celsior® or PBS compared with nonpreserved tissues (data not shown). The effect of preservation on phenotypic and functional features was comparable in the decidua basalis and parietalis, and therefore the results of these two decidual tissues were combined.

Table 1
Pregnancy characteristics.

Pregnancy	
Gestational age (weeks)	39 (38–40)
Mode of delivery	
Spontaneous	2 (14%)
Caesarean section	12 (86%)
Indication for caesarean section	
Breach presentation	2 (17%)
Caesarean previous pregnancy	7 (58%)
Obstetric medical history	3 (25%)
Anesthesia (caesarean section)	
Combined spinal-epidural	9 (75%)
Spinal	2 (17%)
General anesthesia	1 (8%)
Mother	
Age (years)	34 (26–42)
Highest diastolic pressure (mmHg)	77 (70–85)
Gravidity	4 (1–6)
Parity	2 (0–4)
Spontaneous abortion	1 (0–3)
Child	
Birth weight (g)	3544 (2922–4240)
Gender (male)	10 (71%)
HLA	
HLA matches	
Class I	4 (3–5)
Class II	2 (2–3)
HLA mismatches	
Class I	2 (0–3)
Class II	2 (0–2)

3.1. Phenotypic characteristics

To compare the phenotypic characteristics of decidual lymphocytes isolated from placentas after 24-h preservation with lymphocytes isolated within five hours, we focused on the cell subsets CD4+CD25bright, CD4+CD25dim, and CD8+CD28−, and the activation markers HLA-DR and CD69.

Fig. 2 shows the percentage of the cell subsets CD4+CD25bright, CD4+CD25dim, and CD8+CD28− of the decidual lymphocytes isolated from both the decidua basalis and the decidua parietalis. Overall, the percentages of the cell subsets of interest were similar when comparing lymphocytes isolated within five hours with lymphocytes isolated after 24 h of preservation. Paired analysis of the cell subsets showed only small but significant differences in the percentage of CD4+CD25bright (7.31% vs. 9.47%) and CD8+CD28− (26.69% vs. 29.68%). T cells comparing the decidual lymphocytes isolated within five hours with decidual lymphocytes isolated after preservation for 24 h in Celsior®. There was no significant difference in the percentage of CD4+CD25dim cells between the decidual lymphocytes isolated within five hours with decidual lymphocytes isolated after preservation for 24 h in Celsior®. There were no significant differences in the percentages of all three cell subsets analyzed comparing the decidual lymphocytes isolated within five hours with decidual lymphocytes isolated after preservation for 24 h in PBS. In addition, the phenotype of the decidual lymphocytes showed no difference when isolated after 24-h preservation in Celsior® compared with 24-h preservation in PBS.

The expression of activation markers within the total CD3+ T cell population of the decidual lymphocytes isolated from both the decidua basalis and the decidua parietalis is

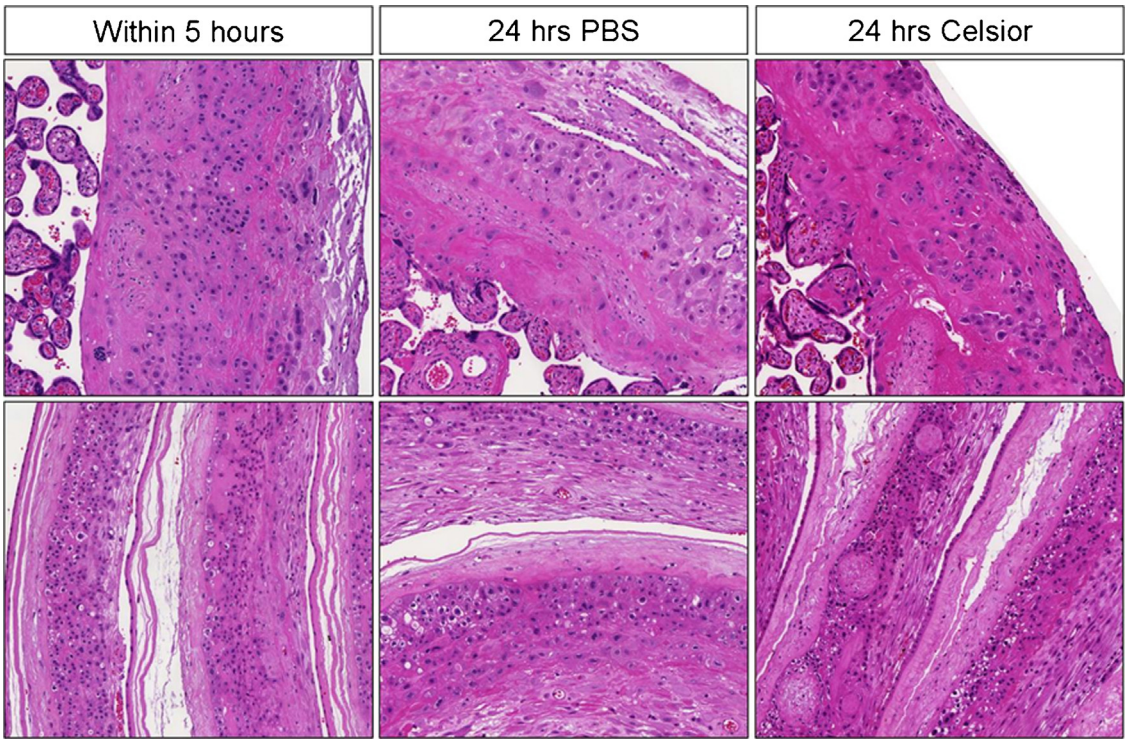

Fig. 1. H&E staining of decidual tissue. The decidua basalis (upper panel) and decidua parietalis (lower panel) were stained with H&E to investigate the morphology of the decidual tissue within five hours of delivery and after 24 h of preservation in either PBS or Celsior®.

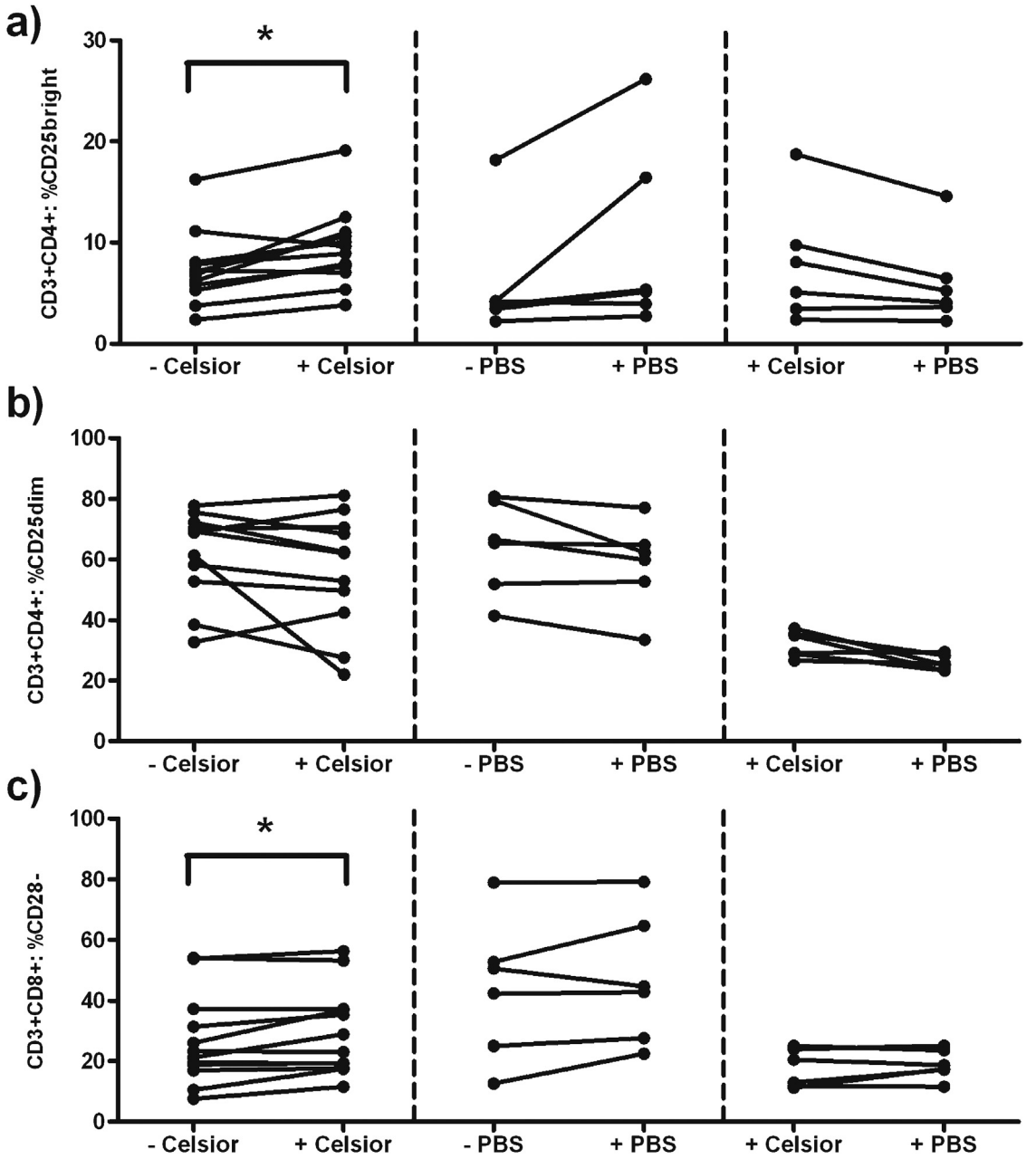

Fig. 2. The percentage of cell subsets in decidual lymphocytes isolates. The percentages of the cell subsets (a) CD4+CD25bright, (b) CD4+CD25dim, and (c) CD8+CD28− are shown for the decidual lymphocytes isolated from both the decidua basalis and decidua parietalis within five hours compared with decidual lymphocytes isolated after 24-h preservation in either Celsior® (−Celsior vs. +Celsior; $n = 5$) or PBS (−PBS vs. +PBS; $n = 3$). In addition, the percentages are shown comparing the two preservation fluids (+Celsior vs. +PBS; $n = 3$). * $p < 0.05$.

shown in Fig. 3. The percentage of CD3+ T cells expressing HLA-DR was found to be significantly different for decidual lymphocytes isolated within five hours compared with decidual lymphocytes isolated after preservation for 24 h in Celsior® (48.67% vs. 41.27% respectively). In contrast, there were no differences in the expression of CD69 for decidual lymphocytes isolated within five hours compared with decidual lymphocytes isolated after preservation for 24 h in Celsior®. There were no differences in the expression of both HLA-DR and CD69 for decidual lymphocytes isolated within five hours compared with decidual lymphocytes isolated after preservation for 24 h in PBS. In addition, the expression of activation markers of the decidual lymphocytes showed no difference when isolated after 24-h preservation in Celsior® compared with 24-h preservation in PBS.

Fig. 4 shows the expression of FoxP3 in the CD3+CD4+, CD4+CD25bright and CD4+CD25dim T cell populations for decidual lymphocytes isolated within five hours compared with decidual lymphocytes isolated after 24-h preservation

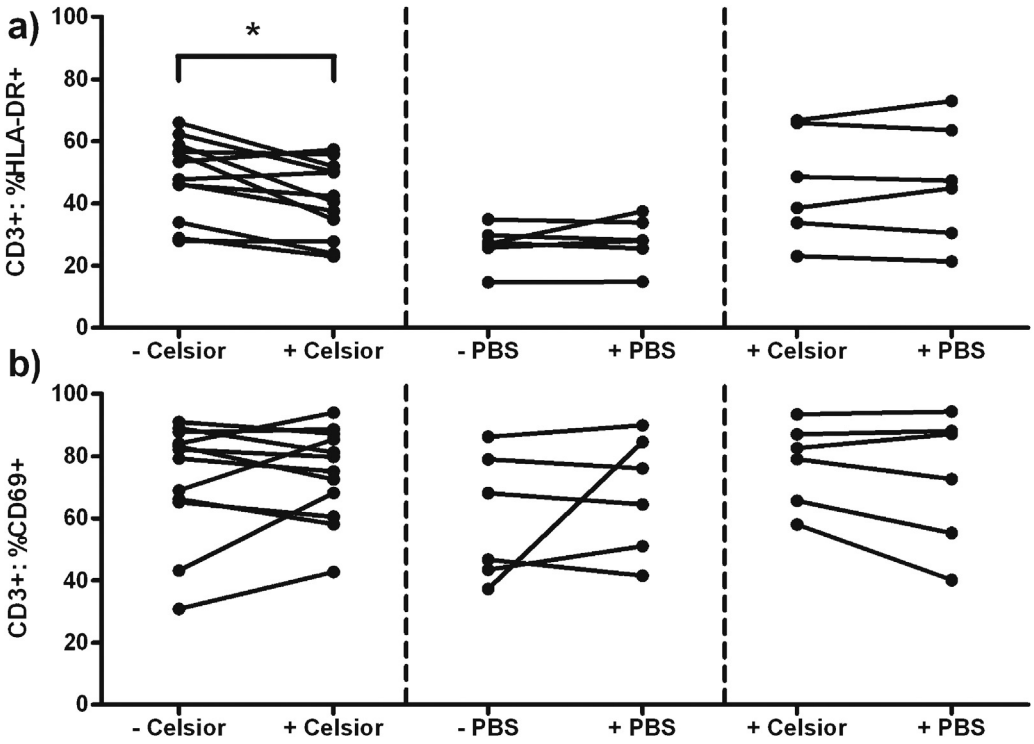

Fig. 3. The expression of activation markers in decidual lymphocyte isolates. The percentages of the activation markers (a) HLA-DR and (b) CD69 are shown for the decidual lymphocytes isolated within five hours (−Celsior and −PBS) compared with decidual lymphocytes isolated from both the decidua basalis and decidua parietalis after 24-h preservation in either Celsior® (−Celsior vs. +Celsior; $n = 5$) or PBS (−PBS vs. +PBS; $n = 3$). In addition, the percentages are shown comparing the two preservation fluids (+Celsior vs. +PBS; $n = 3$). * $p < 0.05$.

in PBS. There was no difference in the mean fluorescence intensity (MFI) of FoxP3 for either cell population comparing decidual lymphocytes isolated within five hours with decidual lymphocytes isolated after 24-h preservation in PBS. Furthermore, the FoxP3 expression was higher in the CD4+CD25bright T cell population compared with the CD4+CD25dim cells.

3.2. Functional features

The functional features of the isolated decidual lymphocytes were assessed in mixed lymphocyte cultures in which decidual lymphocytes were stimulated with irradiated UCB from the own child and 3pUCB. Table 2 shows the HLA-DR typing of the responders and stimulators used for

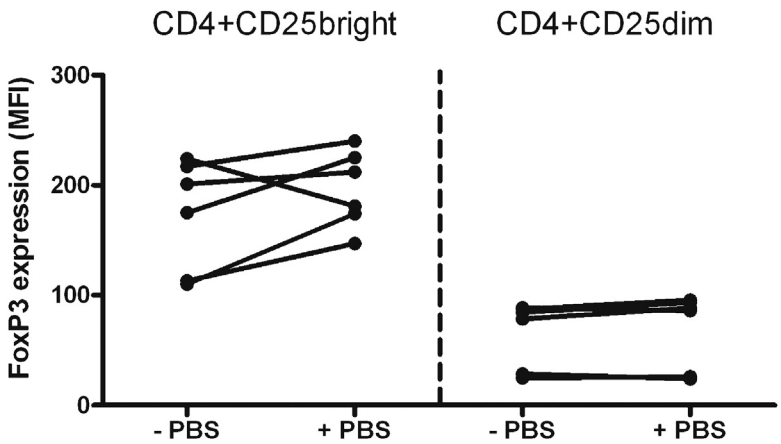

Fig. 4. The FoxP3 expression in CD3+CD4+, CD4+CD25bright, and CD4+CD25dim T cells. The mean fluorescence intensity (MFI) of FoxP3 is shown for the CD3+CD4+, CD4+CD25bright and CD4+CD25dim T cell populations of the decidual lymphocytes isolated from both the decidua basalis and decidua parietalis within five hours and the lymphocytes isolated after preservation for 24 h in PBS (−PBS vs. +PBS; $n = 3$).

Table 2
HLA-DR typing of mother, UCB, and 3pUCB.

Pregnancy	Mother	UCB	3pUCB
1	DR4, DR8	DR8, DR15	DR1, DR13
2	DR3, DR13	DR13, DR15	DR1, DR13
3	DR13, DR14	DR1, DR13	DR1, DR11
4	DR1, DR8	DR1, DR15	DR1, DR13
5	DR3, DR4	DR3, DR13	DR7, DR11
6	DR1, DR3	DR3, DR4	DR3, DR15
7	DR3, DR7	DR3, DR4	DR8, DR13
8	DR4, DR15	DR3, DR15	DR8, DR13
9	DR7, DR10	DR4, DR7	DR11
10	DR11, DR15	DR15	DR8, DR13
11	DR7, DR11	DR3, DR7	DR8, DR13
12	DR4, DR7	DR7, DR15	DR1, DR13
13	DR3, DR4	DR3, DR15	DR4, DR14
14	DR1, DR4	DR4, DR7	DR1, DR7

the functional assays. Fig. 5 a shows the calculated stimulation index of the decidual lymphocytes isolated from both the decidua basalis and the decidua parietalis. The mixed lymphocyte cultures showed no significant difference in stimulation index for the decidual lymphocytes isolated within five hours and isolated after preservation for 24 h in Celsior® or PBS. In addition, the proliferation of the decidual lymphocytes showed no difference when isolated after 24-h preservation in Celsior® compared with 24-h preservation in PBS.

Supernatant was collected at day 5 of the mixed lymphocyte culture to investigate the Th1:Th2 cytokine profile. The ratio of IFN-γ and IL-10 produced in mixed lymphocyte cultures by the decidual lymphocytes isolated from both the decidua basalis and the decidua parietalis is shown in Fig. 5b. The mixed lymphocyte cultures showed no significant difference in Th1:Th2 cytokines produced by the decidual lymphocytes isolated within five hours and isolated after preservation for 24 h in Celsior® or PBS. In addition, the cytokine production by the decidual lymphocytes showed no difference when isolated after 24-h preservation in Celsior® compared with 24-h preservation in PBS.

The mixed lymphocyte cultures indicated that PBS is already sufficient to preserve the placenta for 24 h. Therefore, the ability to preserve the placenta in PBS was further investigated by sorting the decidual lymphocytes into a

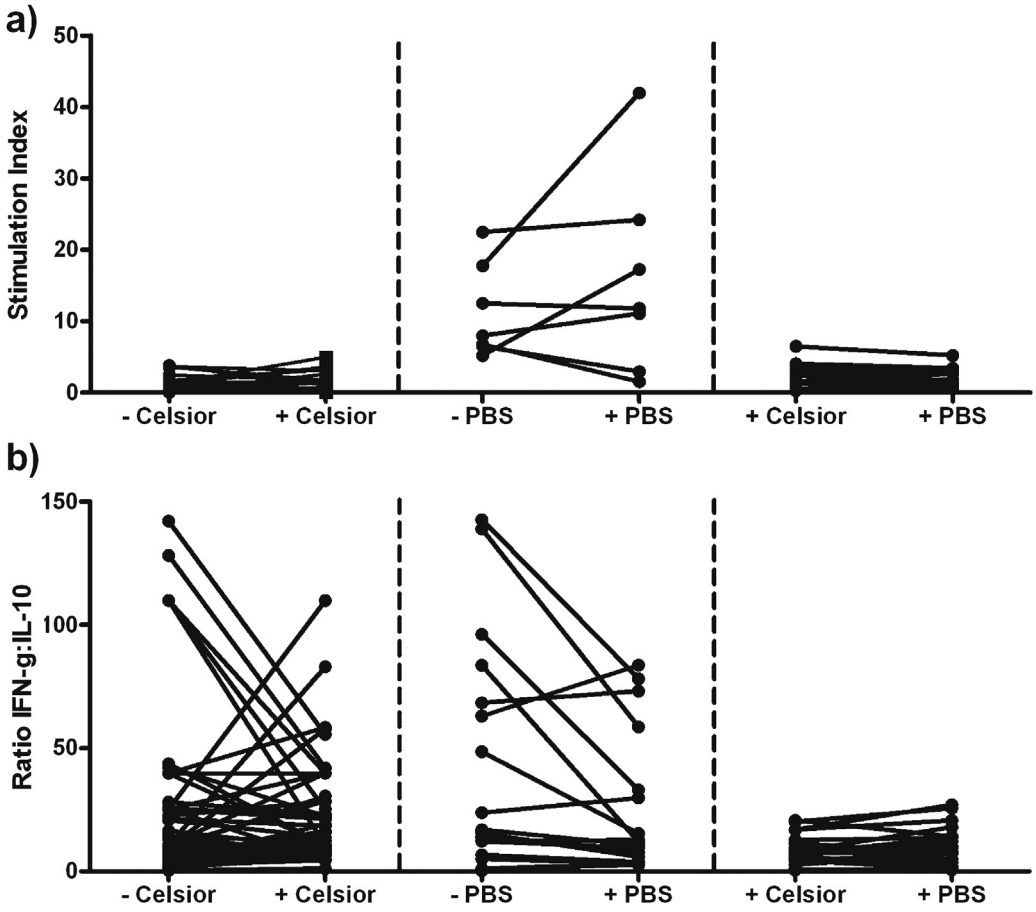

Fig. 5. The proliferative response of the decidual lymphocytes. (a) The stimulation index of the decidual lymphocytes isolated from both the decidua basalis and decidua parietalis within five hours was compared with the lymphocytes isolated after preservation for 24 h in Celsior® (−Celsior vs. +Celsior; $n = 5$) or PBS (−PBS vs. +PBS; $n = 3$). In addition, the stimulation index is shown comparing the two preservation fluids (+Celsior vs. +PBS; $n = 3$). (b) The ratio of IFN-γ and IL-10 produced in mixed lymphocyte cultures by the decidual lymphocytes isolated within five hours was compared with the lymphocytes isolated after preservation for 24 h in Celsior® (−Celsior vs. +Celsior; $n = 5$) or PBS (−PBS vs. +PBS; $n = 3$). In addition, the INF-γ:IL-10 ratio is shown comparing the two preservation fluids (+Celsior vs. +PBS; $n = 3$).

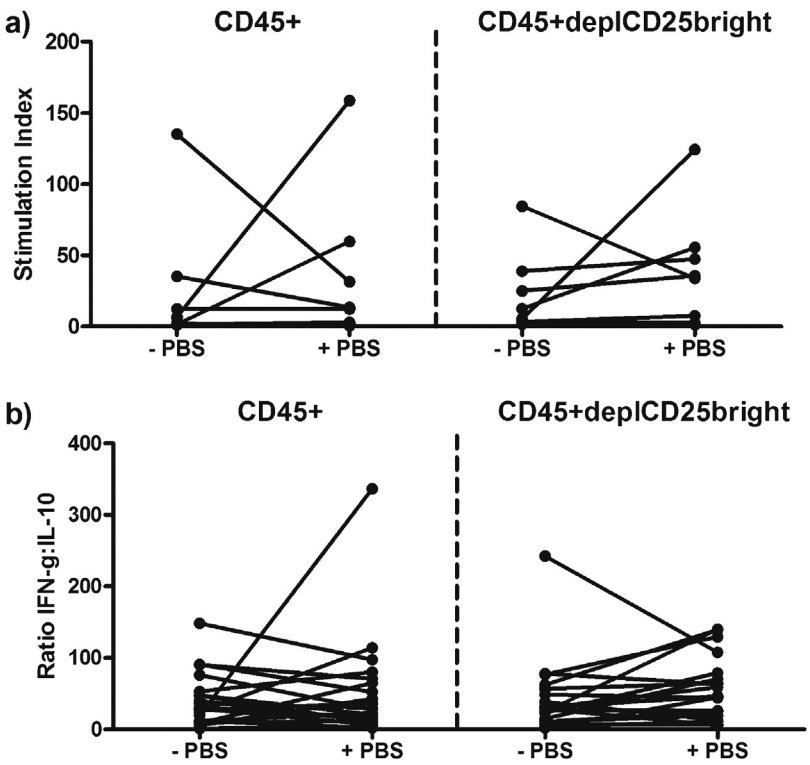

Fig. 6. The proliferative response after depleting CD4+CD25bright lymphocytes. (a) The proliferation of the decidual lymphocytes isolated within five hours was compared with the lymphocytes isolated after preservation for 24 h in PBS (−PBS vs. +PBS; $n = 3$). The stimulation index is shown for cultures with the total leukocyte fraction (CD45+) and the fraction in which the CD4+CD25bright cells were depleted (CD45+deplCD25bright). b) The ratio of IFN-γ and IL-10 produced by the decidual lymphocytes isolated within five hours was compared with the lymphocytes isolated after preservation for 24 h in PBS (−PBS vs. +PBS; $n = 3$). The ratio of IFN-γ and IL-10 is shown for cultures with the total leukocyte fraction (CD45+) and the fraction in which the CD4+CD25bright cells were depleted (CD45+deplCD25bright).

CD45+ population and a CD45+ population depleted for CD4+CD25bright cells before adding the cells to the mixed lymphocyte culture. As expected the proliferation was increased in cultures with CD45+ lymphocytes depleted for CD4+CD25bright cells compared with cultures with the total CD45+ population (data not shown). Furthermore, the stimulation index of the decidual lymphocytes isolated within five hours was similar to the stimulation index of decidual lymphocytes isolated after 24-h preservation in PBS for both the total CD45+ population and the CD45+ population depleted for CD4+CD25bright cells. (Fig. 6 a) The ratio of IFN-γ and IL-10 produced in mixed lymphocyte cultures by the decidual lymphocytes showed no significant difference when isolated within five hours and isolated after preservation for 24 h in PBS (Fig. 6b).

3.3. 48-h preservation

The possibility of preserving the human placenta for more than 24 h was explored by dividing one placenta into two parts. The decidual lymphocytes from one part were isolated within five hours according to our standard procedure, whereas the other part was preserved at 4 °C in Celsior® for 48 h after which the decidual lymphocytes were isolated. The percentage of viable CD3+ lymphocytes decreased by more than 50% when isolated after 48 h of preservation compared with isolation within five hours, both for the decidua basalis and for the decidua parietalis. In addition, the proliferative response of decidual lymphocytes isolated after 48 h of preservation toward several stimulators, including PHA, was greatly diminished compared with the decidual lymphocytes isolated within five hours (data not shown).

4. Discussion

The aim of this study was to investigate the possibility of preserving the human placenta before isolating the lymphocytes for phenotypic characterization and functional analysis of their immunoregulatory properties. The results indicated that PBS is sufficient to preserve the placenta for 24 h in immunoregulation studies.

Modifications in decidual lymphocyte isolation procedures can induce controversial results in studies investigating decidual lymphocyte phenotype and function. In addition, the results of these studies might be influenced by clinical parameters, making it hard to compare material collected from different women. Therefore, we divided the placentas collected for this study into two equal parts and isolated the decidual lymphocytes from one part within five hours according to our standard procedure, whereas the other part was preserved at 4 °C in either Celsior® or PBS for 24 h, after which the decidual lymphocytes were isolated. This strategy allows paired analysis

of the results, eliminating the possible effect of clinical parameters on the outcome.

The percentages of CD4+CD25bright, CD4+CD25dim, and CD8+CD28− cells found in previous studies (Tilburgs et al., 2006, 2009b, 2010a) were comparable to the percentages found in the present study. Tilburgs et al. (2008) already described CD4+CD25bright T cells having a higher expression of FoxP3 than the CD4+CD25dim population. Furthermore, the authors showed that depleting CD4+CD25bright regulatory T cells results in increased proliferation. The present study confirms these findings, indicating that the preservation method did not change the phenotype of effector and regulatory T cell populations. In addition, the present study showed similar percentages of HLA-DR compared with previously found percentages (Sindram-Trujillo et al., 2003a, 2004).The percentage of CD69-expressing cells was increased compared with studies by Sindram-Trujillo et al. (2003a, 2004) However, in these studies the decidual cells were isolated mechanically, possibly explaining this difference.

Although this study focused on the phenotype and function of T cells, other cell types are present in the decidual tissue. Sindram-Trujillo et al. (2003b) showed a high percentage of CD56brightCD16- decidual NK cells in term pregnancies. There was no difference in the percentage of NK cells (CD45+CD16-CD56+) comparing lymphocytes isolated within five hours with 24-h preservation in either Celsior® or PBS (data not shown). Previous studies have shown that decidual macrophages were activated during early/mid pregnancy rather than term pregnancy (Repnik et al., 2008), indicating that these cells are more important in early pregnancy. In the present study, decidual isolates contained barely any macrophages and therefore the phenotype of these cells was not investigated in this study.

The present study did not include placentas from complicated pregnancies and therefore the results do not necessarily apply to placentas collected after complicated pregnancies. An activated immune response has been shown to be a feature of complicated pregnancy, for example, pre-eclampsia. (Saito et al., 2007) Previously, we have demonstrated that the majority of the decidual T cells already have an activated phenotype in uncomplicated pregnancies (Sindram-Trujillo et al., 2003a; Tilburgs et al., 2006, 2008) and preliminary data showed that the activation status of decidual T cells is not very different when comparing pre-eclamptic pregnancies with uncomplicated term pregnancies (Fig. S1). In line with these recent findings, others have also shown that there were no differences in the expression of CD69 on decidual T cells and the expression of CD25 and HLA-DR on decidual leukocytes comparing pre-eclampsia with control pregnancies (Wilczyński et al., 2002; Rieger et al., 2009). We are confident that the present 24-h preservation method is also suitable for analysis of placentas of complicated pregnancies, as the current study shows that this method preserves all naive and activated cell populations in normal pregnancies. Complicated pregnancies will include the same populations, although they may be in different proportions. It is to be expected that the proportions will not change as they are not affected by the preservation method in normal pregnancy. However, before starting large multicenter studies comparing complicated and uncomplicated pregnancies, it would be worthwhile to repeat these experiments with complicated pregnancies.

Supplementary material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.jri.2013.03.001.

5. Conclusion

In conclusion, preservation of the human placenta prior to decidual lymphocyte isolation is feasible up to 24 h. The ability to preserve the placenta after uncomplicated pregnancies for 24 h in PBS will simplify the procedure for the isolation of decidual lymphocytes and make it easier to collect tissue from women who deliver during the night, at weekends or in other hospitals, and possibly even women with complicated pregnancies.

Acknowledgements

The authors thank the midwives, residents, and others involved in collecting the material for this study.

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