Fetus specific immune recognition and regulation by T cells at the fetal-maternal inferface in human pregnancy

Tilburgs, T.

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Do’s and don’ts in isolation and purification of decidual lymphocytes and macrophages

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T. Tilburgs, U. Repnik, B.J. van der Mast, G.M. de Groot-Swings, D.L. Roelen, F.H. Claas and S.A. Scherjon

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Abstract

Human decidual tissue contains a significant number of leukocytes, phenotypically and functionally different from peripheral blood leukocytes. In vitro studies using purified human decidual leukocytes subsets will improve our understanding of the role of these cells in acceptance of the allogeneic fetus and their possible role in development of pregnancy pathology. However, decidual leukocyte isolation and purification procedures are complex and may alter leukocyte phenotype and functional activity. In this study we describe an optimized decidual leukocyte isolation procedure using enzymatic digestion, followed by percoll gradient centrifugation. Subsequently we purified leukocyte subsets using immuno-magnetic beads and autoMACS separation or immuno-fluorescence labelling and FACS sort. The procedure is suitable for isolation and purification of leukocytes from decidua basalis and decidua parietalis tissue. With this protocol highly purified, viable and functionally active decidual lymphocytes and macrophages can be obtained. The isolation and purification procedure has minimal effect on lymphocyte phenotype and functional activity. In contrast, isolation and purification may alter macrophage cell surface markers and cytokine secretion profiles. The potential pitfalls of the decidual leukocyte isolation and purification procedure are discussed including the proper controls to be included in future studies using decidual leukocyte isolates.

Introduction

During pregnancy the maternal immune system has to tolerate the persistence of fetal cells in maternal tissue. Many mechanisms including various fetal and maternal cell types have shown to contribute to the prevention of a destructive immune response at the fetal-maternal interface. However, the immune acceptance of the allogeneic fetus remains an immunologic paradox (1,2). Decidual tissue is populated by many types of maternal leukocytes like NK cells, macrophages, dendritic cells and T cells that contain many phenotypic and functional differences from circulating peripheral blood leukocytes (3-5). In vitro studies using purified leukocyte subsets from the fetal-maternal interfaces will improve our understanding of the role of these cells in acceptance of the allogeneic fetus and development of pregnancy pathology.

In human pregnancy three fetal-maternal interfaces where fetal and maternal tissues connect can be identified; 1 maternal peripheral blood that contacts the fetal syncytiotrophoblast layer during utero-placental circulation, 2 decidua basalis the maternal part of the placenta at the implantation site that connects with the invading extra villous trophoblasts and 3 decidua parietalis the maternal part of the membranes contacts the non-invading trophoblast of the chorion. Each interface contains specialized fetal trophoblast cells with distinct HLA expression profiles and unique immune modulatory and immune stimulatory capacities (6-9). In addition, many differences in presence of leukocyte subsets exist between maternal peripheral blood, decidua basalis and decidua parietalis (4,10,11). Decidual leukocyte isolation and purification procedures may alter leukocyte phenotype and functional activity 12,13. Therefore isolation protocols require constant improvement and appropriate controls must be included in all studies. We describe an optimized technique to obtain purified, viable and functionally active leucocytes from decidua basalis and decidua parietalis that is suitable for phenotypic and functional analysis. In addition, we discuss potential pitfalls during isolation and purification of decidual leukocytes and suggest controls that can be included in future studies using decidual leukocyte isolates.

Materials & Methods

Blood and tissue samples

Samples of decidua basalis, decidua parietalis and heparinised maternal peripheral blood were obtained from healthy women after uncomplicated term pregnancy (gestational age ≥37 weeks). Tissue samples were obtained after delivery by elective caesarean section or spontaneous vaginal delivery. Non pregnant control peripheral blood samples were obtained from healthy volunteer blood donors. Signed informed consent was obtained from all women, and the study received medical ethical approval by the LUMC Ethics Committee (P02-200).

Lymphocyte isolation

Lymphocyte isolation from decidua was done as described previously (4,5). Decidua basalis was macroscopically dissected from the maternal side of the placenta. After dissection, the remains of villous tissue were cut from decidua basalis with scissors. Decidua parietalis was collected by removing the amnion and delicately scraping the decidua parietalis from the chorion. The obtained tissue was washed thoroughly with firm shaking (without centrifugation) in PBS. Thereafter the tissue was finely minced between two scalpel blades. To minimize contamination with blood and villi decidual tissue fragments were washed several times in PBS (without centrifugation) until the supernatant was completely transparent. Decidual fragments were incubated with 0.2% collagenase I (Gibco-BRL, Grand Island, NY) and 0.02% DNAse I (Gibco) in RPMI-1640 medium, gently shaking in a waterbath at 37ºC for 60 min and thereafter washed once with RPMI. The resultant suspensions were filtered through a 70µm sieve (Becton Dickinson Labware; New Jersey, USA) under negative pressure and thereafter washed once in RPMI-1640. The decidual isolates were resuspended in 20 ml of Percoll (1.023 g/ml) (Amersham Biosciences, Amersham, UK) and layered on a Percoll gradient of (10ml 1.080g/ml and 12.5ml 1.053g/ml) for density gradient centrifugation for 30 min at 2000 rpm (Figure 1b). Lymphocytes were isolated from the 1.080g/ml – 1.053g/ml interface whereas macrophages were isolated from the 1.053g/ml – 1.034g/ml interface. Maternal peripheral blood and umbilical cord blood (UCB) samples were directly layered on a Ficoll Hypaque gradient (LUMC pharmacy; Leiden, The Netherlands) for density gradient centrifugation for 20 min at 2000 rpm. Mononuclear cells were collected, washed twice with PBS containing 1% FCS. All cells were fixed with 1% paraformaldehyde and stored at 4˚C until cell staining and flow cytometric analysis.

Flowcytometry

The following directly conjugated mouse-anti-human MoAbs were used for four-color immunofluorescence staining: CD14-FITC, CD27-FITC, CD56-FITC, CD69-FITC, HLA-DR-FITC, CD8-PE, CD14-PE, CD19-PE, CD25-PE, CD80-PE, CD86-PE, CD3-PerCP, CD4-APC (Becton Dickinson, Franklin Lakes, NJ, USA), CD163-PE, CD28-APC, CD45-APC (BD Pharmingen, San Diego, CA, USA) and CD66b-FITC (Serotech, UK). MoAbs are used in concentrations according to manufactures instructions. Flowcytometry was performed on a FACS Calibur using Cellquest-pro Software (Becton Dickinson). Analysis of the lymphocytes were done within the lymphocyte gate, set around the viable lymphocytes as previously described 4. The percentage of lymphocytes were analyzed as CD45+ cells within the lymphocyte gate and depicted as percentage of total events. The percentages of CD3+, CD56+ and CD19+ cells were calculated within the CD45+ cell fraction. The percentage of macrophages (CD45+CD14+) and

granulocytes (CD45+CD66b+) were calculated within total events and without using the lymphocyte gate. Expression of HLA-DR, CD80, CD86 and CD163 was analyzed within the CD45+CD14+ gate.

Lymphocyte purification and functional analysis

Lymphocyte isolates were purified using immuno-magnetic labelling and autoMACS (Miltenyi Biotec) separation or immuno-fluorescence labelling and FACS sorting. For immuno-magnetic labelling decidual lymphocytes isolates were resuspended in autoMACS buffer containing 0.2% EDTA and 0.5% FCS. Thereafter lymphocytes were stained with directly labelled CD14 microbeads (Miltenyi Biotec), CD66b (Serotech), HLA-G (Serotech) and HAI-1 (kindly provide by Professor Hiroaki Kataoka, University of Miyazaki, Japan) MoAbs for 30 min. The cells were washed once and Goat-anti-Mouse labelled microbeads (Miltenyi Biotec) were added for 30 min. Thereafter the cells were washed again and filtered through a 40µm sieve (BD Labware) and separated using the negative selection protocol in sensitive mode (depleteS) on autoMACS. Cells were washed once with PBS 1% FCS (Greiner, Bio-one BV Alphen ad Rijn, The Netherlands) and all fractions were analyzed with flowcytometry for cell purity and counted with Türk and Eosine for cell viability. For Immuno-fluorescence labelling the lymphocyte fraction was stained with CD45-APC (BD Pharmingen) for 30 min in RPMI-1640 1%FCS (Greiner, Bio-one BV). Thereafter the cells were washed in RPMI-1640 1%FCS and filtered through a 40µm sieve (BD Labware). All lymphocyte fractions were FACS sorted on a Flow sorter ARIA (Becton Dickinson) for viable CD45+ lymphocytes. The cells were sorted within the lymphocyte gate set around the viable lymphocytes avoiding granulocytes, macrophages and other contaminating cell types. After cell sorting the

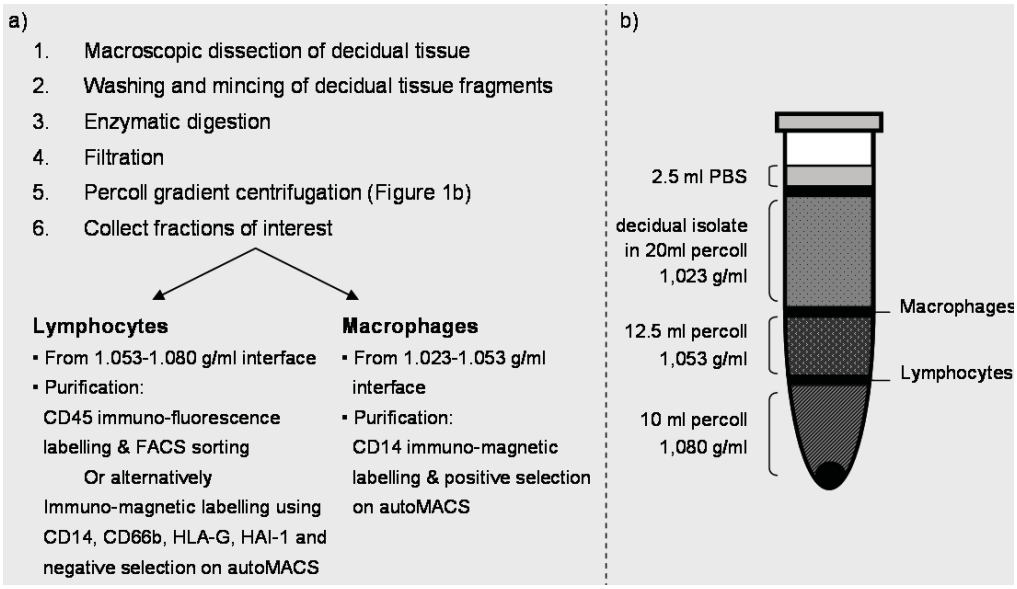

Figure 1. decidual leukocyte isolation and purification procedure
a) Shows a schematic diagram of the decidual leukocyte isolation and purification procedure and b) the properties of the Percoll gradient for separation of decidual lymphocyte and decidual macrophage fractions.

lymphocytes were incubated in RPMI-1640 supplemented with L-glutamine 2 mM, penicillin 50 units/ml en streptomycin 50 µg/ml (all obtained from Gibco Laboratories) and 10% human serum in a round-bottomed 96 well plate (Costar Cambridge, MA, USA) at a density of 50.000 cells per well in triplicate. All fractions were stimulated with pre-coated anti-CD3 using UCHT-1 (BD Pharmingen) at 5 µg/ml for 2 hours or OKT-3 (Orthoclone) at 10 µg/ml for 4 hours. Cultures were incubated at 37ºC with 5% CO2. At day 4 supernatants were collected and stored at -20 ºC until the time of analysis. Supernatants were analyzed with a Th1-Th2 Bio-plex pre mixed human cytokine panel (containing IL-2, IL-4, IL-5, IL-10, IL-12(p70), IL-13, GM-CSF, IFN-γ and TNF-α) (Biorad Laboratories; Veenendaal; The Netherlands) according to manufactures description. Proliferation was measured by [3H]thymidine (1µCi) incorporation for the last 16 hours and measured by liquid scintillation spectroscopy using a betaplate (Perkin Elmer-Wallac, Turku, Finland). Results are expressed as the median counts per minute (cpm) for each triplicate culture.

Macrophage purification and functional analysis

Macrophage fractions were purified using immuno-magnetic labelling and autoMACS (Miltenyi Biotec) separation. Herefore macrophage isolates were resuspended in MACS buffer containing 0.2% EDTA and 0.5% FCS (Greiner, Bio-one BV) and stained with directly labelled CD14 microbeads (Miltenyi Biotec) for 30 min. The cells were washed once, filtered through a 40µm sieve (BD Labware) and separated using a double positive selection protocol (Posseld2) on autoMACS. After selection macrophages were incubated in RPMI-1640 supplemented with L-glutamine 2 mM, penicillin 50 units/ml en streptomycin 50 µg/ml (all obtained from Gibco Laboratories) and 10% FCS in a 24 well plate (Costar Cambridge, MA, USA) at a density of 0.5 x106 cells per well. Macrophages were stimulated with LPS (100 ng/ml) alone, LPS (100 ng/ml) + IFN-γ (500 units) or cultured with medium alone. After 16 hours supernatants were collected and stored at -20 ºC until the time of analysis. Supernatants were analyzed with Bio-plex cytokine panel for IL-10, IL-12(p70), IL-12(p40), IL-1β and TNF-α (BioRad Laboratories; Veenendaal; The Netherlands) according to manufactures description.

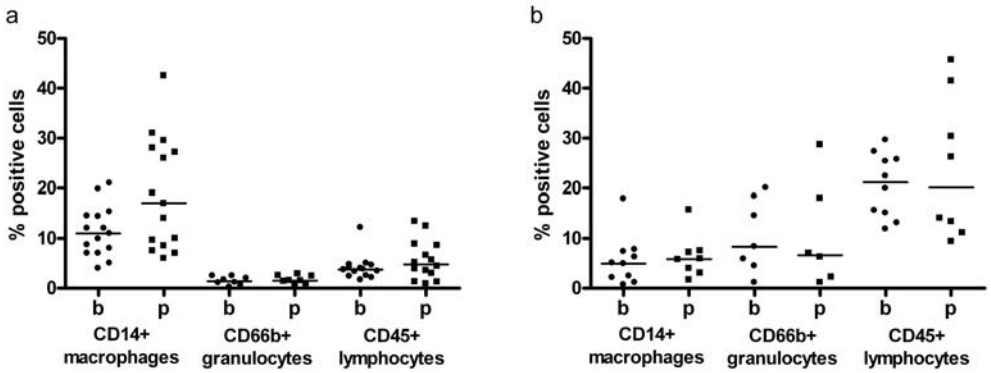

Figure 2. Leukocyte composition of decidual macrophage and decidual lymphocyte enriched fractions

a) The percentage of CD14+ macrophages, CD66b+ granulocytes and CD45+ lymphocytes in the (a) macrophage enriched fraction from the 1.023-1.053 interface and (b) the lymphocyte enriched fraction from the 1.053-1.080 interface in decidua basalis (b) and decidua parietalis (p) samples. Percentage is depicted as percentage in total events and lines indicate median percentages.

Results

Isolation of decidual macrophages and lymphocytes

The isolation procedure as depicted in figure 1a contains the following important steps: Macroscopic dissection of decidual tissue and extensive washing and mincing of decidual tissue fragments is followed by enzymatic digestion, filtration and percoll gradient centrifugation. Following percoll gradient centrifugation, the gradient contains 3 interfaces that contain different cell types (Figure 1b). The macrophage and the lymphocyte enriched fractions are harvested from the 1.023-1.053 g/ml interface and 1.053-1.080 g/ml interface respectively. FACS analysis of the macrophage enriched fraction shows 11.3% ± 5.0% and 18.9% ± 11.2% CD14+ macrophages in decidua basalis and decidua parietalis respectively whereas the percentage of granulocytes and lymphocytes are significantly less (Figure 2a). FACS analysis of the lymphocyte enriched fraction shows 20.6% ± 6.4% and 24.0% ± 14.2% CD45+ lymphocytes in decidua basalis and decidua parietalis respectively whereas the percentage of macrophages and granulocytes are significantly less (Figure 2b).

Percoll gradient optimisation

For optimisation of the percoll gradient we analysed the use of several combinations of percoll layers with different densities. Increase of the bottom layer density (1.080 g/ml) to 1.085 g/ml results in an increased percentage of contaminating granulocytes in the lymphocyte fraction whereas a decrease to 1.075 g/ml leads to an increase loss of lymphocytes to the cell pellet. Increase in the middle percoll layer density (1.053 g/ml) to 1.058 g/ml results in an increased number of macrophages in the macrophage fraction but also to a high loss of lymphocytes to the macrophage fraction. Reduction of the middle percoll layer density (1.053 g/ml) to 1.047 g/ml results in loss of macrophages from the macrophage fraction to the lymphocyte fraction. A small increase in number of lymphocytes in the lymphocyte fraction is observed although the percentage of lymphocytes in the lymphocyte fraction is reduced in comparison with the 1.053 g/ml layer, which may complicate FACS analysis and further purification steps (data not

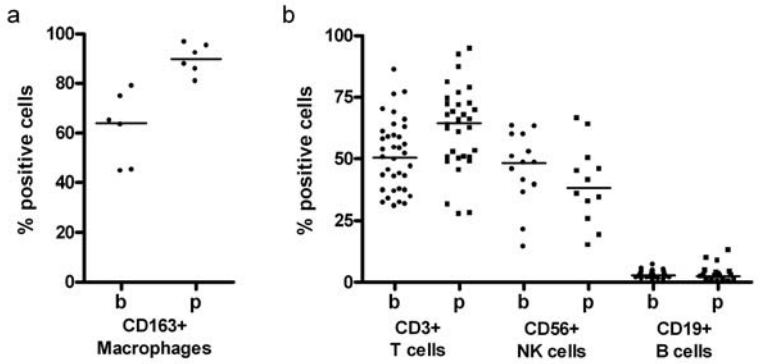

Figure 3. Different macrophage and lymphocyte subsets in decidua basalis and decidua parietalis

a) The percentage of CD163+ cells within the CD45+CD14+ macrophage fraction in decidua basalis (b) and decidua parietalis (p) samples. b) Shows the percentage of CD3+ T cells, CD56+ NK cells and CD19+ B cells within the CD45+ lymphocyte fraction in d.basalis (b) and d.parietalis (p) samples. (Lines indicate median percentages)

shown). Furthermore, the lymphocyte and T cell composition from 1.023-1.053 g/ml interface and 1.053-1.080 g/ml interface were compared and showed no differences indicating that no specific selection of particular lymphocyte and T cell subsets to the 1.023-1.053 g/ml interface and 1.053-1.080 g/ml interface occurs during percoll gradient centrifugation (data not shown).

Different macrophage and lymphocyte subsets in decidua basalis and decidua parietalis

Although a high variation in leukocyte composition is found in different isolates, no significant differences are present in the percentages of macrophages, granulocytes and lymphocyte between decidua basalis and decidua parietalis isolates (Figure 2). However, analysis of the macrophage and lymphocyte subsets, show significant differences between decidua basalis and decidua parietalis. Decidua parietalis contains mainly CD163+ macrophage type 2 cells, whereas decidua basalis contains both CD163- type 1 and CD163+ type 2 macrophages (Figure 3a). In addition, decidua parietalis contains an increased percentage of CD3+ T cells compared to the percentage of CD56+ NK cells whereas decidua basalis contains equal percentages of CD3+ T cells and CD56+ NK cells (Figure 3b).

As enzymatic digestion may influence expression of cell surface markers, we analyzed the influence of the enzymatic digestion protocol on lymphocyte and monocyte markers by adding enzymes to peripheral blood samples and incubating the samples for 1 hour at 37°C similar to the decidual isolation protocol. Enzymatic digestion does not influence cell surface expression of CD45, CD19, CD3, CD25, CD69, HLA-DR, CD8, CD27 and CD28 on peripheral blood lymphocytes (Figure 4a, b). Although the expression of CD4 and CD56 was reduced after enzymatic treatment, the CD4+ and CD56+ population were clearly visible and percentages CD4+ and CD56+ cells remained unchanged (figure 4a, b). In contrast, analysis of enzyme treated monocytes and percoll gradient centrifugation modifies expression of monocyte activation markers like HLA-DR and CD80 while expression of CD14 and CD163 is not affected (Figure 4c).

Lymphocyte purification and functional analysis

In order to perform functional tests with decidual lymphocytes, further purification of the decidual lymphocyte enriched fraction was performed using FACS sort or autoMACS separation. For the FACS sort purification CD45+ fluorescence labelling was used and lymphocytes were selected for viable CD45+ cells within the lymphocyte gate. For autoMACS separation a negative selection protocol to deplete contaminating CD14+ macrophages, CD66b+ granulocytes, HLA-G+ trophoblasts and HAI-1+ trophoblasts was used. FACS analysis of the purified lymphocyte isolates shows that after FACS sort the highest lymphocyte purity is achieved in decidua basalis and decidua parietalis isolates (figure 5a). In addition cell counting using either Türk in a Burker-Türk chamber or MGG stained cell smears shows that contamination in lymphocyte isolates after FACSsort is mainly cell debris, whereas after autoMACS purification also non-lymphocyte cell types are visible (data not shown). The number of lymphocytes was determined in the unsorted, autoMACS and FACS sorted fractions. There are no significant differences in lymphocyte gain (figure 5b) or sorting efficiency between autoMACS and FACS sorting (data not shown). In addition, of all fractions the lymphocyte viability was determined using Eosine staining and shows >98% viability of lymphocytes in both purification protocols (data not shown).

To analyze the functional capacity of the purified lymphocytes FACS sorted lymphocyte fractions of decidua basalis and decidua parietalis, were stimulated with plate bound anti-CD3 using the OKT-3 and UCTH-1 clones. In addition, CD45+ lymphocyte isolates from maternal PBL and non-pregnant control PBL were included. For control of the isolation procedure, control PBL samples were incubated with enzymes, enriched for lymphocytes using a percoll gradient and thereafter FACS sorted for CD45+ lymphocytes similar to the decidual lymphocyte isolation protocol. Upon stimulation with OKT-3 and UCTH-1 all CD45+ lymphocyte fractions show high proliferative responses (Figure 6a). No significant differences in proliferative capacity are observed between peripheral blood and decidual lymphocytes.

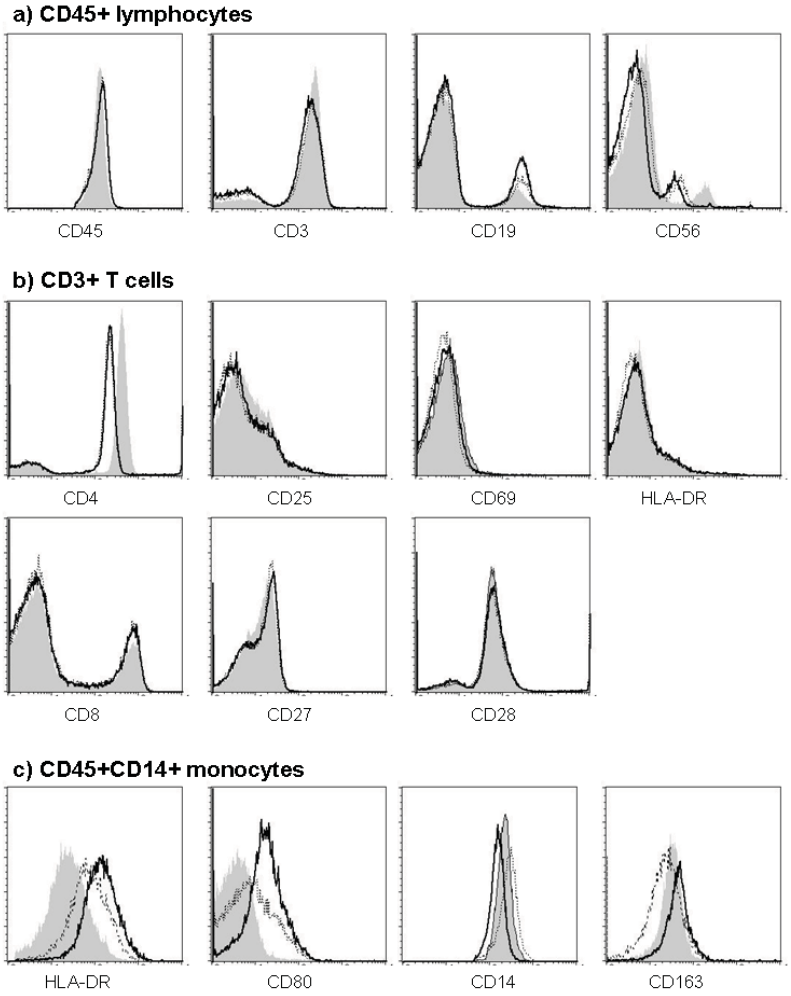

Figure 4. Influence of enzymatic digestion and percoll gradient centrifugation on leukocyte cell surface marker expression
Representative histograms showing Mean Fluorescence Intensity of a) CD45, CD3, CD19 and CD56 in CD45+ lymphocytes, b) CD4, CD25, CD69, HLA-DR, CD8, CD27 and CD28 on CD3+ T cells and c) HLA-DR, CD80, CD14 and CD163 in CD45+CD14+ monocytes from untreated PBL combined with a ficoll gradient (grey histogram), enzymatic digestion and ficoll gradient (dotted line) and enzymatic digestion with percoll gradient (black line)

Furthermore no significant differences in proliferative capacity are observed between the control PBL and the control PBL treated with the decidual lymphocyte isolation protocol (Figure 6a). Upon stimulation with OKT-3 a significant increase of IFNγ, IL-10 and CM-CSF is observed in decidual lymphocyte cultures in comparison with peripheral blood lymphocytes. However lymphocytes from control PBL samples treated with the decidual isolation protocol also show a comparable increase in IFNγ, IL-10 and CM-CSF. Upon stimulation with UCTH-1 a significant decrease of IL-2 is observed in decidual lymphocyte cultures in comparison with peripheral blood lymphocytes (Figure 6d). However lymphocytes from control PBL samples treated with the decidual isolation protocol also show a comparable decrease in IL-2. Upon UCTH-1 stimulation no significant differences in IFNγ, IL-10, IL-4, IL-5, IL-13, TNFα and GM-CSF production are observed between peripheral blood and decidual lymphocytes or between the control PBL and control PBL treated with the isolation procedure (Figure 6b-i).

Macrophage purification and functional analysis

In order to perform functional tests with decidual macrophages further purification of the decidual macrophage enriched fraction was required and performed using CD14 double positive selection on autoMACS. The macrophage purity of all sorted and unsorted fractions were assessed using flowcytometry and show a high macrophage purity in decidua basalis (83.2% ± 8.6%) and decidua parietalis (86.0% ± 24.2%) fractions after autoMACS sorting (Figure 7a). The macrophage yield in unsorted fractions could not be determined due to high contamination with other non macrophage cell types and cell debris. After autoMACS separation the number of macrophages were determined and show high macrophage yield in decidua basalis (median: 3.6x106 ± 2.0 x106; n=9) and decidua parietalis (median: 2.6x106 ± 1.7 x106; n=9) fractions (Figure 7b).

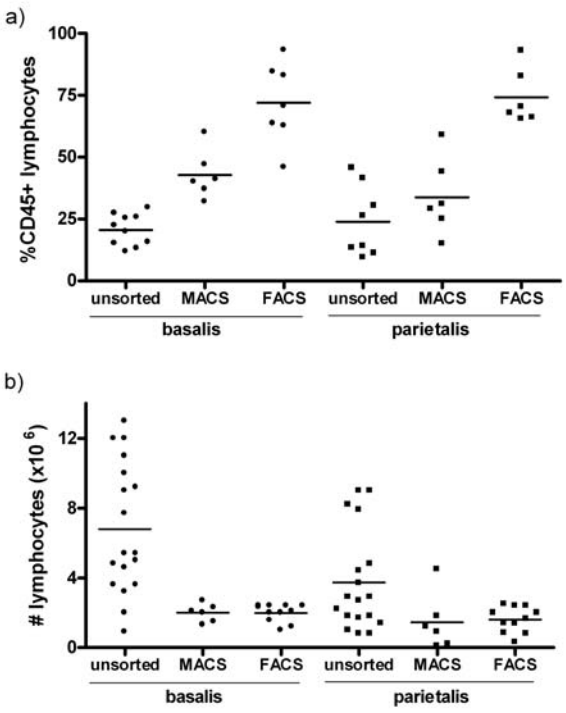

Figure 5. Decidual lymphocyte purity (a) and lymphocyte yield (b) before and after autoMACS or FACSsort purification in decidua basalis and decidua parietalis isolates. Lines indicate median percentages

To determine the functional capacity of decidual macrophages after the isolation and purification procedure, macrophages were stimulated with medium, LPS or LPS and IFN-γ and incubated for 16 hours at 37°C at 5% CO2. In addition, CD14+ monocytes isolated from control PBL and maternal PBL were included. For control of the isolation procedure, control PBL samples were incubated with enzymes and enriched for monocytes using a percoll gradient similar to the decidual isolation protocol. Supernatants were collected from all fractions and analyzed for IL-10, IL-1β, TNF-α, IL-12(p70) and IL-12(p40). LPS and LPS+IFN-γ stimulation induces IL-10 production in all peripheral blood and decidual macrophage fractions. IL-10 production in decidua basalis macrophages stimulated with medium, LPS and LPS+IFN-γ is significantly increased in comparison to peripheral blood monocytes stimulated with medium, LPS and LPS+IFN-γ. However monocytes from control PBL samples treated with the decidual isolation protocol also produce substantially increased levels of IL-10 after stimulation with medium, LPS and LPS+IFN-γ (Figure 8a). Upon LPS and LPS+IFN-γ stimulation monocytes from cPBL and mPBL produce increasing levels of IL-1β, TNF-α, IL-12(p70) and IL-12(p40). Macrophages derived from decidua basalis and decidua parietalis produce significantly lower levels of IL-1β, TNF-α, IL-12(p70) and IL-12(p40) upon LPS and LPS+IFN-γ stimulation in comparison to monocytes from cPBL and mPBL (Figure 9b-e). Monocytes from peripheral blood samples treated with the decidual isolation protocol produce similar or higher levels of IL-1β and IL-12(p70) and lower levels of TNF-α and IL-12(p40) compared to untreated PBL samples (Figure 8b-e).

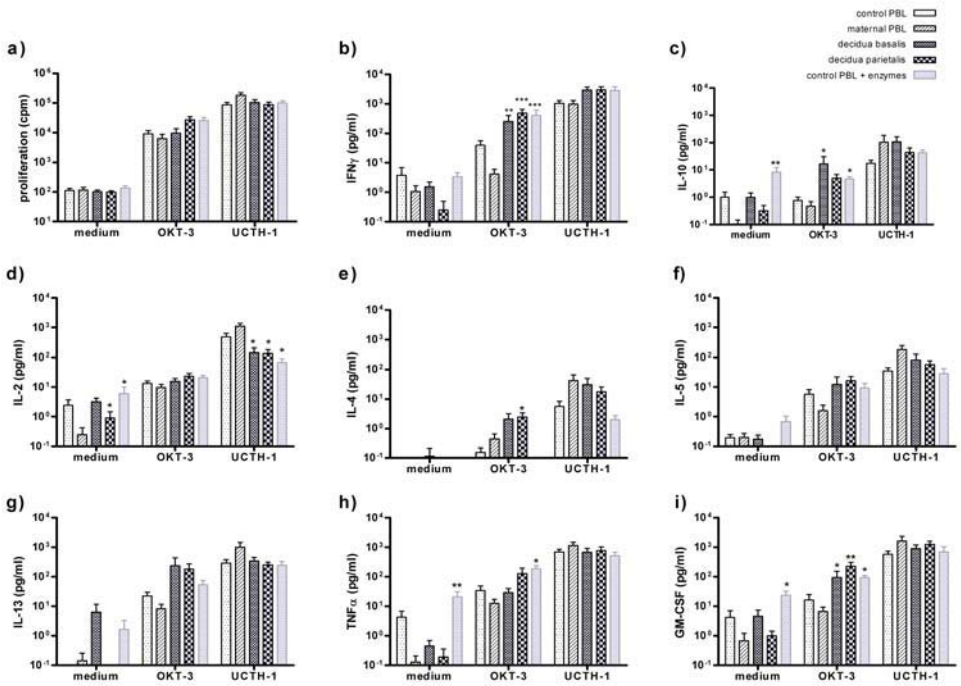

Figure 6. Proliferation and cytokine production by decidual lymphocytes
a) proliferation, b) IFNγ, c) IL-10, d) IL-2, e) IL-4, f) IL-5, g) IL-13, h) TNFα and i) GM-CSF production by purified CD45+ lymphocytes from control PBL (n=16), maternal PBL (n=11), decidua basalis (n=10), decidua parietalis (n=10) and enzyme treated control PBL (n=6) upon medium, OKT-3 and UTCH-1 stimulation. Bars indicate mean and standard deviation (* $p<0.05$; **$p<0.01$; *$p<0.001$).

Discussion

Modifications and disparity in decidual leukocyte isolation and purification procedures can induce controversial results of experiments performed by individual laboratories. In addition, decidual leukocyte isolation and purification procedures can also modify decidual leukocyte phenotype and functional capacity. Therefore decidual cell isolation procedures require constant improvement and the use of appropriate controls for proper interpretation of experiments using decidual leukocyte isolates. Previous studies have already suggested improvements at different phases of decidual leukocyte isolation procedure. The use of enzymatic digestion above mechanical disaggregation has shown to increase leukocyte viability and cell yield (14,15). However, enzymatic treatment may disrupt cell surface molecules and different combinations of enzymes may be favoured for specific cell subsets (12,14-16). In addition, the use of percoll gradient centrifugation as a replacement of ficoll gradient centrifugation has shown to increase cell viability and lymphocyte purity (13). However, optimization of the decidual leukocyte isolation protocol may be influenced by gestational age, decidual type and cells of interest. The aim of this study was to optimize the decidual leukocyte isolation and purification procedure so that highly purified, viable decidual lymphocytes and macrophages can be obtained from decidua basalis and decidua parietalis tissue which can be used in phenotypic and functional studies. The protocol is developed using term pregnancy samples, however the protocol has previously shown to be applicable for use in 1st and 2nd trimester samples (4,11).

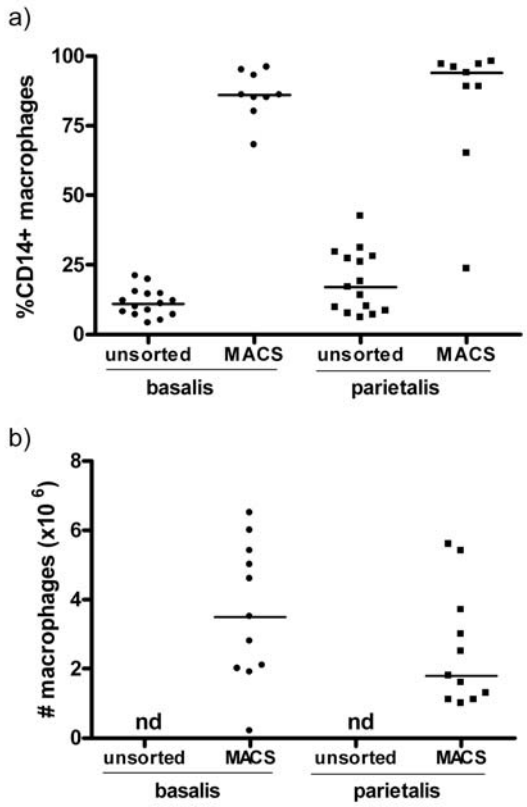

Figure 7. Decidual macrophage purity (a) and macrophage yield (b) before and after autoMACS separation in decidua basalis and decidua parietalis isolates. Lines indicate median percentages.

As previously described (15) phenotypic analysis of lymphocyte subsets shows a minimal influence of enzymatic digestion and percoll gradient centrifugation procedures on lymphocyte cell surface molecules. In addition, purified decidual lymphocytes contain comparable capacity to proliferate upon anti-CD3 stimulation to peripheral lymphocytes. In addition, treatment of peripheral blood lymphocytes with the decidual lymphocyte isolation procedure does not influence the proliferative capacity. However the increase in IFNγ, IL-10 and CM-CSF upon OKT-3 stimulation and the decrease of IL-2 upon UCTH-1 stimulation by decidual lymphocytes is also observed in the treated peripheral blood lymphocytes cultures. Therefore the changes in production by decidual lymphocytes can be a factor of decidual lymphocytes themselves or may be related to the isolation procedure. Analysis of monocyte cell surface molecules upon enzymatic digestion and percoll gradient centrifugation induces modification of the monocyte cell surface molecules HLA-DR, CD80 and CD86 whereas CD163 expression remains unchanged. In addition, enzymatic digestion and percoll gradient centrifugation alters cytokine secretion profiles of peripheral monocytes by significantly increasing IL-10 and IL-1β secretion while TNF-α and IL-12(p40) secretion is reduced. Therefore the observed increase in IL-10 production by decidua basalis macrophages can be either intrinsic factor of these macrophages or may be related to the isolation

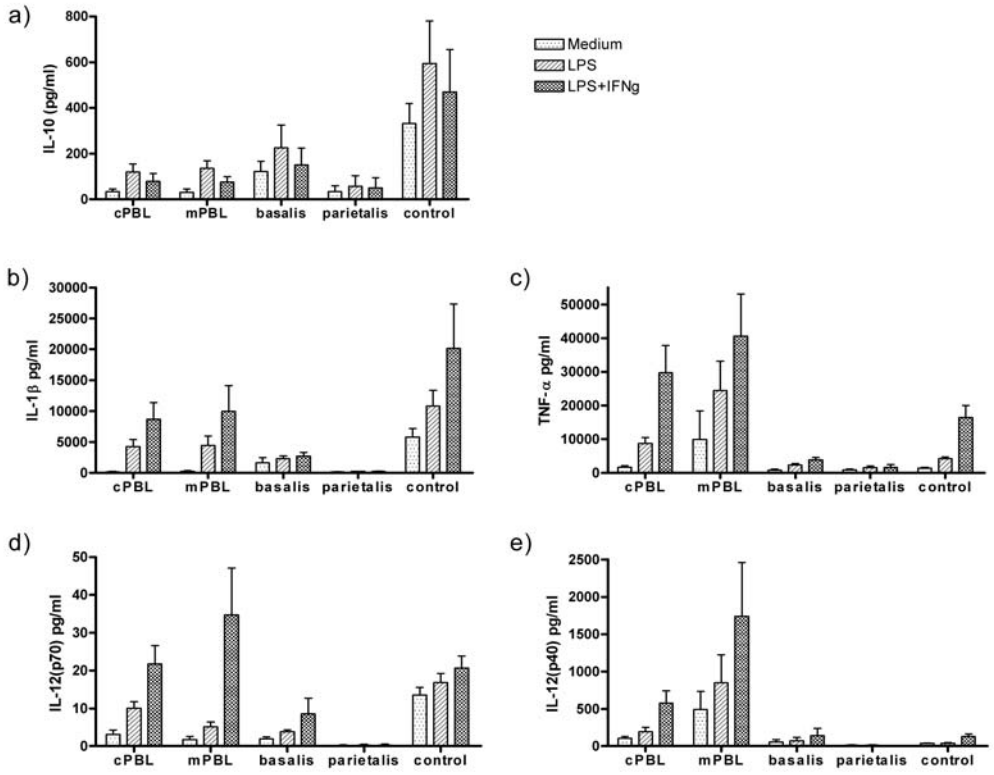

Figure 8. Cytokine production by purified decidual macrophages
a) IL-10, b) IL-1β, c) TNF-α, d) IL-12(p70) and e) IL-12(p40) production by purified CD14+ monocytes/macrophages from control PBL (cPBL) (n=14) maternal PBL (mPBL) (n=11), decidua basalis (n=10), decidua parietalis (n=9) and enzyme treated PBLs (control) (n=8) upon medium, LPS and LPS+IFN-γ stimulation. Bars indicate means and standard deviation.

procedure. Similarly the reduced secretion of IL-1β, TNF-α, IL-12(p70) and IL-12(p40) in both decidua basalis and decidua parietalis macrophages can be a feature of decidual macrophages or a side effect of the isolation procedure. Further refinements in the macrophage isolation protocol are required to perform reliable functional experiments and exclude the influence of the isolation protocol on decidual macrophages. The use of other enzyme cocktails may diminish macrophage modifications. In addition, the use of Immuno Histochemistry (IHC) can provide a good control for confirmation of phenotypic analysis of the macrophage subsets done by flowcytometry.

For purification of lymphocytes we isolated CD45+ lymphocytes by FACS sort and used a negative selection protocol on autoMACS to deplete contaminating cell types (CD14+ macrophages, CD66b+ granulocytes, HLA-G+ trophoblast and HAI-1+ trophoblasts), from lymphocyte isolates. By FACS sort the highest lymphocyte purity was obtained. Thereby the FACS sort enables the use of multicolour immune fluorescence labelling whereby multiple marker can be used for purification of particular cell subsets. However logistics to use the FACS sort facility is limited and therefore not all freshly delivered placenta's can be purified using FACS sort. In contrast, autoMACS separation contains more limited separation possibilities, but the use of autoMACS separation is easily accessible and provides the flexibility that is required to purify freshly delivered placentas at any time. Positive autoMACS selection for CD45+ cells will not only select CD45+ lymphocytes, but also CD45+ macrophages and CD45+ granulocytes whereas positive selection for CD3 may inhibit T cell function. Therefore we used a negative selection protocol to deplete CD14+ macrophages, CD66b+ granulocytes, HLA-G+ trophoblast and HAI-1+ trophoblasts from the lymphocyte fractions (17). However this selection did not result in high lymphocyte purity and non lymphocyte cell types remain present after separation. Additional MoAbs to deplete more contaminating cell types, like endothelial cells (CD31) trophoblast subsets (CD9) and fibroblasts (CD9, FSA) can be included in the depletion cocktail to further enhance lymphocyte purity (18).

Decidual leukocyte isolation protocols are crucial to investigate the dynamics and functional capacities of human decidual leukocytes subsets. Thereby, in vitro experiments using purified human decidual leukocytes subsets will improve our understanding of the role of these cells in acceptance of the allogeneic fetus and their possible role in development of pathology during pregnancy. However, we show that decidual macrophage isolation and purification procedures can alter macrophage phenotype and functional activity. In contrast the isolation and purification procedure has minimal effect on lymphocyte phenotype and proliferative capacity whereas changes in cytokine production by lymphocytes are also observed. For this reason the isolation protocols require constant improvement and appropriate controls must be included in all studies. For phenotypic analysis, flowcytometry can be used next to IHC controls. In addition, controls assessing the influence of enzymatic treatment and percoll gradient selection on the leukocyte subsets of interest should be included in all future studies assessing phenotypic and functional characteristics of decidual leukocyte subsets.

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