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Obesity in pregnancy stimulates macrophage accumulation and inflammation in the placenta

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Abstract

Obesity and pregnancy are associated with a combination of insulin resistance and inflammatory changes which exacerbate in combination. Based on the similarity between the inflammatory transcriptomes of adipose tissue and placenta, we hypothesized that the placenta develops exaggerated inflammation in response to obesity. The aim of this study was to characterize placental inflammatory mediators and macrophage accumulation in relation to peripheral inflammation in obesity.

Placental macrophages and maternal peripheral mononuclear cells from 20 obese and 15 lean women were functionally and phenotypically characterized using immunohistochemistry, flow cytometry and expression for macrophage markers and inflammatory cytokines. The number of resident CD68+ and CD14+ cells was increased 2-3 fold in placenta of obese as compared to lean women. The enhanced macrophage population was characterized by a marked phenotypic heterogeneity with complex subsets of CD14+, CD68+ and CD11b+ cells and by an increased expression of the pro-inflammatory mediators IL-1, TNF-alpha, IL-6. Placental inflammation was associated with an activation of peripheral blood mononuclear cells with high amount of CD14, TNF-alpha, IL-6 and the chemokine receptors CCR2 and IL8-R in maternal but not fetal circulation. Additionally, plasma CRP and IL-6 concentrations were higher in obese compared to lean women.

In conclusion, the chronic inflammation state of pre-gravid obesity is extending to *in utero* life with accumulation of an heterogeneous macrophage population and proinflammatory mediators in the placenta. The resulting inflammatory milieu in which the fetus develops may have critical consequences for short and long term programming of obesity.

Keywords

Obesity; pregnancy; placenta; inflammation; cytokines; fetus; programming

The association between excess neonatal adiposity and high pre-gravid BMI and the alarming rise in the number of overweight women of reproductive age, call for elucidating

the adverse consequences of obesity in pregnancy (1,2). In non gravid individuals, obesity is described as a low grade inflammatory condition associated with increased production of pro-inflammatory factors which originate from the macrophages infiltrating the adipose tissue (3,4). During pregnancy, however, the placenta becomes a significant source of a variety of cytokines and adipocytokines whose expression is dysregulated by maternal diabetes and obesity (5-7).

Pregnancy is considered as a natural inflammatory state evident in activation of maternal leucocytes and increased systemic concentration of acute phase reactants and cytokines (8). The physiological inflammatory state of pregnancy becomes exaggerated when pregnancy is complicated by pre-eclampsia and gestational diabetes and returns to baseline levels after delivery (9-11). Products of inflammatory stress secreted by the placenta and microparticles shedding from the syncytial surface of the placenta into the systemic circulation have been proposed as mechanisms driving adaptive immunity (12-14). Given its ability to regulate both innate and adaptive immune responses, the placenta is the primary candidate to mediate pregnancy-specific inflammatory changes. Macrophages originating from fetal hematopoietic cells are detected in the placenta at very early developmental stages (15,16). They display a large secretory repertoire of cytokines and immunoregulatory molecules however, their role is not well understood. We and others have identified TNF-alpha activated pathways as potential links between increased placenta inflammation and altered maternal metabolic homeostasis (17,19) suggesting a link between macrophage secretory activity and the metabolic changes. Activation of TNF-alpha through IKK and PPAR gamma are potential pathways connecting inflammation, insulin resistance and obesity in peripheral tissues (20, 21).

Based on these observations, we hypothesized that the stimuli generated by the low grade inflammation of obese women will challenge the placenta to develop an inflammatory response. Our studies have identified an accumulation of macrophages with enhanced expression of pro-inflammatory cytokines in placenta of obese women analogous to chronic villitis. The association of maternal systemic and placental inflammation suggests that the chronic inflammation of obesity extends from pre-gravid to *in utero* life with potential adverse consequences for the fetus i.e developmental programming.

1. Subjects and Methods

The monoclonal antibody against human CD68, clone PGM-1 and the FITCconjugated anti-CD68 were from Dako Cytomaton (Glostrup, Denmark). The monoclonal mouse antibody against human CD14, clone 7 was from Novobiosystems Novocastra (Newcastle, UK). The PE and FITC-conjugated CD14 and CD11b were from Abcam (Cambridge, MA). The FITC-conjugated CD133 was from Miltenyi-Biotech (Auburn, CA). Anti-activated caspase 3 was from R&D Systems (Minneapolis, MN). The secondary antibodies anti-Rb Biotin and Streptavidin-HRP were from Vector (Burlingame, CA).

1.1. Study subjects

Women with a singleton pregnancy were recruited at the time of admission for elective cesarean delivery at term (38-40 weeks). Obesity was defined as pre-gravid BMI >30.

Anthropometrics and metabolic parameters for 15 lean and 20 obese women are given in Table 1. The protocol was approved by the MetroHealth Medical Center Institutional Review Board and GCRC Scientific Review Committee.

Volunteers gave their written informed consent in accordance with the MetroHealth Medical Center guidelines for the protection of human subjects. Gestational diabetes was ruled out based on a normal one hour 50 g glucose screen or 100 g oral glucose tolerance test according to the criteria defined by Carpenter and Coustan (22). Neonatal anthropometric measurements were obtained within 24 hours from delivery by the same examiner experienced in technique. Body composition measurements were evaluated using skinfolds (23). Maternal blood was drawn on admission to labor and delivery, prior to placement of an intravenous line for hydration. Umbilical venous blood was drawn via syringe at the time of delivery of the placenta. Plasma was separated by centrifugation and kept frozen at -20°C for glucose, insulin and cytokine assays.

1.2. Analysis of RNA expression

Placental biopsies were performed within 10 min of delivery and snap-frozen in liquid nitrogen or immediately processed for trophoblast isolation. Total RNA was prepared from frozen whole villous tissue samples using CsCl gradient (24) and from peripheral mononuclear cells (PMNC) using an RNeasy kit (Qiagen, Valencia, CA). RNA samples were reversed transcribed using standard method (Stratagene, La Jolla, CA). cDNA were analyzed by real-time PCR using SYBR green (Lightcycler, Roche Molecular Diagnosis, IN). Specific primers were designed within the 3' coding region of the genes. Specific primers were designed within the 30 coding region of the genes. CD14 (BC-010507) forward: 50-tctctgtccccacaagtcc-30 reverse: 50-cccgctccaggtgcaggttacc-30; CD68 (NM_001040059) forward: 50-gaaccccaacaaaaccaag-30 reverse: 50-gatgagaggcagcaagatg-30; EMR-1 (NM_001974) forward: 50-ccaaggggataagatgaag-30 reverse: 50-caccaaggagatgattaatgcc-30; IL-6 (NM_000600) forward: 50-tacccccaggagaagattcc-30 reverse: 50-ttttct gccagtgccctttt-30; TNF-alpha (NM-000594) forward: 50-tccttcagacaccctcaacc-30 reverse: 50-agggcccagttgaattctt-30. Quantification of relative gene expression normalized for actin was performed by the comparative C_T method and expressed as the fold difference between groups.

1.3. Isolation of primary placental cells

Placental cells were isolated according to Kliman et al (25). Approximately 30 to 45 g of villous tissue was dissected from placenta, blotted and washed with sterile saline to remove excess blood. The finely minced tissue was washed and digested three times at 37°C in a shaking water bath for 30 min each with 0.25 per cent trypsin and 300 U/ml DNase I in Hanks' balanced salt solution (HBBS), Ph 7.4. Cells were centrifuged from digestion supernatants, washed with culture medium and filtered through 100µm nylon mesh (Becton Dickinson, NJ, USA) and purified by Percoll (Amersham Pharmacia Biotech, NJ, USA) gradient centrifugation. The resultant purified cell fraction composed of villous stromal cells, trophoblast and endothelial cells was used to isolate CD14 positive resident macrophages. Briefly, CD14-coupled magnetic beads (Dynalab, Invitrogen) were incubated with the purified cells (50 µl beads/10⁷ cells) at 4°C for 30 min under constant shaking. The

magnetic bead coupled fraction was separated by placing the cells in front of a magnet. The CD14+ enriched fraction was washed twice in PBS with 2% FCS, counted and pelleted by centrifugation (200 g, 5 min).

1.4. Immunohistochemistry

For placenta sections, specimen were cut off longitudinally from the amnion to the basal plate, fixed in 10 % formalin and included in paraffin. Endogenous peroxidase of deparaffinized sections was neutralized by hydrogen peroxide 3% for 10 min. Antigen unmasking was performed by incubating the slides in a pH 9 buffer at 98°C for 45 min. The non specific reactions were blocked by incubation for 30 min with normal swine serum (CD68) or 0.4% casein solution (CD14). Primary antibodies were added for 30 min (CD68) or 60 min (CD14) at room temperature. For CD68 staining the biotinylated secondary antibody was applied for 30 min followed by the streptavidin-horseradish peroxidase conjugate for 15 min. Peroxidase was developed using the DAB working solution (LSAB +system, DAKO) and washed in deionised water. For CD14, a penetration enhancer was applied for 30 min followed by the anti-mouse/rabbit-HRP polymer (Novolink Polymer-detection-system RE7 290K). The DAB-chromogen (DAKO) was added to develop the enzyme activity. Sections were counterstained using hematoxylin Gill II (Sigma). Digitized images were obtained using a Nikon E600 microscope equipped with DXM200 camera. Six to 12 images of a slide were made at a low magnification (x10) to obtain a wide section with significant labelling. Image analysis was performed using the ImageJ software from NIH 1.35q (Bethesda, MD). The number of CD68+ macrophages was counted after surrounding each villous with a freehand line tool. The villous size was comprised between 1,700 μm^2 and 150,000 μm^2 , corresponding to mature intermediate villi (30-80 μm diameter) up to ramii chorii (300-600 μm diameter).

1.5. Fluorescence-activated cell sorter (FACS) analysis

FACS analysis was performed simultaneously on maternal blood and isolated placental cells. 100 μl maternal venous blood collected on EDTA or freshly isolated placental cells re-suspended at a concentration of $10^7/\text{ml}$ in HBSS buffer PH7.4 were incubated (30 min at 4°C) with fluorescent primary antibodies (CD14-PE, CD68-FITC, CD11b-PE, CD133-FITC) or control IgG. Cells were washed, fixed in 0.5% paraformaldehyde and analyzed using a FACScan (Becton Dickinson). Propidium iodide (50 mg/ml) was used to determine the absolute number of live cells and control for cell viability in placental cell analysis. Multicolor population analysis was performed with paint-a-gate software 3.0.0 PPC (Becton Dickinson). Blood samples were lysed with FACS lysis solution pelleted and fixed with 0.5% paraformaldehyde. The proportion of monocytes and granulocytes in the maternal blood was determined by using light scatter characteristics and expressed as percentage of white blood cells. All gating and data analysis were performed with CellQuest software 3.2.1f1 (Becton Dickinson).

1.6. Plasma Assays

All plasma samples were run in duplicate in a single assay. Plasma insulin was measured using radioimmunoassay kits (Linco, St Charles, MO) with intra-assay coefficient of variations (CVs) of 2.9 - 6.0%. Leptin was measured by a commercially available double

antibody radioimmunoassay kit (Linco) with intra-assay CVs of 3.0 - 6.2%. Plasma TNF-alpha and interleukin-6 were assayed by ELISA (Quantiglo R&D Systems, Minneapolis, MN) with the following CVs: 5.3 - 7.8 and 2.6 - 3.4%. Plasma CRP was measured by ELISA (Alpha Diagnostics International, San Antonio, TX) with a CV of 3.0 - 4.7%.

1.7. Statistical analysis

All data are presented as mean $\pm$ SE. Significance for statistical differences was calculated using ANOVA and unpaired Student's t-test.

2. Results

2.1. Obesity in pregnancy is associated with peripheral inflammation

Increased circulating concentrations of IL-6 (4.2 ± 0.3 vs 2.7 ± 0.2 ng/ml) and CRP ($10,450 \pm 602$ vs 8162 ± 926 ng/ml) were detected in the plasma of obese women compared to lean women. By contrast there was no change in circulating TNF-alpha (Table 2). The doubling in insulin concentrations was associated with a 4 fold increase in insulin resistance measured by the HOMA index (Table 2). The higher adiposity of the obese women was supported by their higher circulating leptin concentration. Obese women displayed only mild leucocytosis with no change in the amount of monocytes and a higher proportion of circulating granulocytes (69.6 ± 2.7 vs $65.5 \pm 2.0\%$), which did not quite reach statistical significance compared to lean women ($p = 0.09$). These changes were associated with an increased gene expression of several inflammatory markers in maternal peripheral blood mononuclear cells (PBMC) of obese women (Fig. 1A) including the CD14 and CD68 monocyte maturation and differentiation antigens (1.4 ± 0.1 and 1.5 ± 0.1 fold vs lean, $p < 0.0001$) and the pro-inflammatory cytokines TNF-alpha and IL-6 (1.6 ± 0.1 and 1.5 ± 1.2 fold vs lean, $p < 0.0001$). None of these inflammatory markers were significantly modified in PBMC from the umbilical cord in neonates of obese compared to lean mothers (Fig. 1B).

2.2. Accumulation of resident macrophages in placenta of obese women

Macrophages were identified by immunohistochemistry of placental sections using CD68 antibody against macrosialin and CD14 against the LPS receptor which label myelomonocytic lineage.

A small number of CD68 and CD14 positive cells were identified in the placenta of lean subjects (Figure 2 A-B) at contrast with the large number observed in tissue sections from obese women (Figure 2C-D). The number of macrophages was doubled in placenta of obese compared to lean women (Figure 2 right panel). A higher magnification (Figure 3) showed that the CD14+ and CD68+ cells accumulating in the stromal core of the villi were large cells (average diameter: 20 ± 3 μ m) with the characteristic granular structure of Hofbauer cells, the resident placental macrophages. The labeled macrophages were clearly distinguished from adjacent non labeled fibroblasts based on their structural characteristics. There was no evidence of CD14+ or CD68+ cells in the direct vicinity of syncytial knots however, the localization of the CD14+ cells appeared to be more peripheral i.e closer to the trophoblast layer.

Given the huge diversity of currently used macrophage markers as relate to their species and tissue specificity, we sought to further identify the placental macrophages by flow cytometry. We used freshly harvested placental cells consisting of trophoblast, stromal and endothelial cells to preclude artifactual monocyte activation through direct macrophage isolation. Two color FACS analysis was performed using antibodies directed against the trophoblast protein CD133 (26) and CD14 or CD68 as macrophage markers (Figure 4 A-B). The double staining showed a distinct population of CD68 (blue) and CD14 (red) labeled cells which amounted to 5.8 ± 1.4 and 11.3 ± 2 % of the total cells harvested from placenta of obese women reflected the higher abundance of CD14⁺ cells found by immunohistochemistry (Figure 2). Three color FACS analysis of the same cell population adding the CD11b (green) as marker of activated monocytes revealed three distinct subsets of cells (Figure 4C-D). Two double positive cell populations expressing either CD68 (turquoise) or CD14 (yellow) in conjunction with CD11b were identified. Color merging indicated that 75.7 ± 6.8 % of CD68 (turquoise) and 71.7 ± 5.5 % of CD14 (yellow) cells also expressed the CD11b marker, whereas there were only 10.5 % overlapping between the CD14 and CD68 populations. The third subset of CD11b positive cells (green) which did not stained for the other macrophage markers accounted for 22.1 ± 0.04 % of the phenotyped cells. Altogether these data revealed the marked heterogeneity of the macrophage population accumulating in the placenta of obese women.

2.3. Placental macrophages of obese women have increased expression of cytokines and macrophage specific factors

The functional implication of macrophage accumulation was assessed by measuring macrophage specific antigens and cytokine gene expression in whole placental villous tissue and CD14⁺ macrophages separated from the other placental cell types by immunoselection. Macrophage enrichment was confirmed by a higher expression of genes for the macrophage markers CD14, CD68 and EMR-1 in placental villous tissue (Fig. 4, left). The activated state of the macrophages was supported by a 2-4 fold increase ($p < 0.001$) in the expression of the inflammatory cytokines IL-6, TNF-alpha and IL-1 in CD14⁺ cells (Fig. 4, right). Additionally, an increased expression of the gene encoding for the chemoattractant protein 1 (MCP-1) facilitating monocyte adhesion and infiltration of inflamed tissues was observed in isolated CD14⁺ macrophages.

3. Discussion

Accumulation of macrophages with production of proinflammatory molecules, an immunological response of inflamed tissues, has emerged as a common feature of the chronic metabolic disturbances associated with insulin resistance. For example, macrophage infiltration in expanding adipose tissue is a mechanism contributing to the development of obesity and its stream of associated co-morbidities [27]. Pregnancy is another situation characterized by profound metabolic modifications i.e. increased fat mass, insulin resistance, dyslipidemia, which all become exacerbated with pre-gravid obesity [28,29]. We have examined the inflammatory status exacerbates the basal inflammatory state of pregnancy. Increased insulin resistance and higher leptin concentrations reflecting the increase in fat mass of obese women were associated with higher circulating concentrations of CRP and

IL-6 supporting findings from our group and others that obesity in pregnancy worsens the pre-gravid inflammatory state [30,31]. In contrast, plasma TNF-alpha concentrations remained unchanged suggesting that production from placental cells and PBMC does not contribute significantly to the circulating concentration similar to TNF-alpha from white adipose tissue [32]. An increased expression of the CD14 and CD68 monocyte maturation and differentiation antigens on maternal PBMC also indicated systemic inflammation in obese mothers. This enrichment in CD14 and CD68 expression was suggestive of monocyte activation although the gene expression was not directly measured in isolated monocytes. The activated state of the PBMC was further supported by an increase in the pro-inflammatory cytokines IL-6 and TNF-alpha in maternal PBMC. The absence of the modification of markers of monocyte activation in the umbilical PBMC from fetuses of obese mothers suggested that the inflammatory condition was confined to the maternal and placental compartments.

In contrast to pre-eclampsia, where placental dysfunction results in a pro-inflammatory response in the mother [33], our data suggest that the pre-existing systemic inflammation of obese women is the *primum movens* to trigger placenta inflammatory signals. Based on our previous studies showing an over-expression of the inflammatory secretome in the placenta of diabetic obese women [5] we hypothesized that the activated circulating monocytes of obese women contribute to the accumulation of macrophages in their placenta. We found that the abundance of CD68⁺ and CD14⁺ cells was doubled in the placenta of obese compared to lean women indicating an enrichment in resident macrophages. Resident macrophages which initially colonize the placental villi differentiate from primitive fetal macrophages without passing through the monocyte lineage [14,34]. They represent the primitive macrophages known as Hofbauer cells which can be identified by CD68 staining [35]. Their high intrinsic proliferative and mitogenic capacities suggest that *in situ* mitogenesis may contribute to the enrichment we observed in the present study [36,37]. Flow cytometry analysis using discriminative macrophage markers demonstrated that besides CD68⁺ cells, additional subsets of macrophages expressing CD14 and CD11b (mac-1) also co-exist in the stromal core of the placental villi. There was little overlapping between CD68 and CD14 positive cells, suggesting that the two antigen markers recognize distinct macrophage populations. However, we also identified subsets of cells expressing both CD14 and CD11b or CD68 and CD11b and a subset of cells exclusively labeled with CD11b. This phenotypic heterogeneity strongly suggested that macrophages located in the placenta of obese women may derive from both endogenous and exogenous sources as recently demonstrated in the adipose tissue of mice fed a high fat diet [20]. Because placental cells are of fetal origin, the genotypic determination of the placental macrophages would help to further understand the mechanisms for their accumulation. Infiltration of vascularized tissues or organs by circulating monocytes is considered a general mechanism to cope with systemic inflammation [38,39]. The increased placental gene expression of the chemoattractant protein MCP-1 suggested that the maternal monocytes have the potential to be recruited by placental cells. In support of the hypothesis of an increased chemotaxis are previous studies showing that monocytes can adhere to syncytiotrophoblast cells and damage the trophoblast barrier opening a way to cellular infiltration and villitis [40,41]. The pattern of macrophage accumulation enrichment in resident macrophages. Resident

macrophages which initially colonize the placental villi differentiate from primitive fetal macrophages without passing through the monocyte lineage [14,34]. They represent the primitive macrophages known as Hofbauer cells which can be identified by CD68 staining [35]. Their high intrinsic proliferative and mitogenic capacities suggest that in situ mitogenesis may contribute to the enrichment we observed in the present study [36,37]. Flow cytometry analysis using discriminative macrophage markers demonstrated that besides CD68⁺ cells, additional subsets of macrophages expressing CD14 and CD11b (mac-1) also co-exist in the stromal core of the placental villi. There was little overlapping between CD68 and CD14 positive cells, suggesting that the two antigen markers recognize distinct macrophage populations. However, we also identified subsets of cells expressing both CD14 and CD11b or CD68 and CD11b and a subset of cells exclusively labeled with CD11b. This phenotypic heterogeneity strongly suggested that macrophages located in the placenta of obese women may derive from both endogenous and exogenous sources as recently demonstrated in the adipose tissue of mice fed a high fat diet [20]. Because placental cells are of fetal origin, the genotypic determination of the placental macrophages would help to further understand the mechanisms for their accumulation. Infiltration of vascularized tissues or organs by circulating monocytes is considered a general mechanism to cope with systemic inflammation [38,39]. The increased placental gene expression of the chemoattractant protein MCP-1 suggested that the maternal monocytes have the potential to be recruited by placental cells. In support of the hypothesis of an increased chemotaxis are previous studies showing that monocytes can adhere to syncytiotrophoblast cells and damage the trophoblast barrier opening a way to cellular infiltration and villitis [40,41]. The pattern of macrophage accumulation a pathology of unknown origin where the placental villous is invaded by maternal lymphocytes [42]. Our data provide experimental support to epidemiological studies showing a positive association between the incidence of chronic villitis and high maternal BMI [43].

Accumulation of the macrophages within the stromal core of the placenta is associated with an increased expression of TNF-alpha, IL-1 and IL-6 indicating that they are in an activated state. We and others have shown that the quantity in which a given cytokine is released from the placenta into the blood stream depends upon the cell type in which it is produced. For example, leptin produced by trophoblast cells is preferentially released into the maternal circulation whereas TNF-alpha produced by macrophages is not [16,44]. This suggests that the cytokines secreted by the macrophages into the villous stroma are unlikely to be released into the systemic circulation (either fetal or maternal) across the placental barrier. Consequently, the high cytokine content in the placenta of obese women is more likely to promote intra-placental inflammatory cascades through specific gene transcription rather than directly enhance systemic inflammation as shown for TNF-alpha in human adipose tissue [32].

In conclusion, obesity in pregnancy results in an exaggerated inflammatory response in the placenta with accumulation of multiple subsets of macrophages and production of proinflammatory mediators, a signature of chronic villitis. The chronic inflammation of obese women prior to pregnancy initiates a cascade of events which translate into an inflammatory in utero environment. We speculate that the inflammatory milieu in which the

fetus develops may result in detrimental consequences with metabolic programming of obesity and the insulin resistance syndrome.

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Figure 1. Systemic inflammatory status in obese pregnant women

Peripheral blood mononuclear cells (PBMC) were separated from maternal and umbilical venous plasma samples obtained at the time of elective cesarean delivery. Aliquots were processed for total RNA purification. Expression of inflammatory markers was assessed by real-time RT-PCR. Results normalized for actin are expressed as fold change in obese vs lean women. Results are given as mean $\pm$ SE of duplicate determinations with 19 obese and 15 lean women. *: $p < 0.001$. White bars: lean, grey bars: obese.

Figure 2. Accumulation of CD68+ and CD14+ macrophages in placenta of obese women

Upper left panel: histochemical analysis of whole placental villous sections from a representative lean (pre-gravid BMI:22.5) woman shows rare CD68⁺ (A) and CD14⁺ (B) staining. By contrast, the sections from a representative obese (pre-gravid BMI: 31.7) woman show an increased number of dark brown CD68⁺ (C) and CD14⁺ (D) stained cells. Original magnification A-D: x20. Right panel: quantification of CD68⁺ and CD14⁺ macrophage number in placental sections. Mean ± SE of N = 10 placental sections. *: p < 0.001 obese vs lean. Lower panel: higher magnification showing localization of macrophages (thick arrows) in the villous stroma with no staining in the syncytiotrophoblast outlayer (maternal side) or vascular endothelium (fetal side). Original magnification (x60). Thin arrow: syncytiotrophoblast nuclei, #: fetal blood space, *: maternal blood space. Scale bar indicates 20 μm.

Figure 3. Identification of placental macrophages based on flow cytometry analysis

(A,B) Representative double staining side scatter analysis of isolated cells from placenta of an obese woman (BMI: 31.6) performed with CD14 (red) or CD68 (blue) FITC antibodies and CD133-PE (grey), a marker of trophoblast cells. (C,D) Representative three color analysis of the same cell population performed with CD11b-PE antibody (green) in addition to the three antibodies described in A-B. The turquoise color in panel C indicates that positive cells express both the CD11b and CD68 markers. The yellow color in panel D indicates that positive cells express both the CD11b and CD14 markers. Similar results were obtained from independent analysis of six placenta of obese women. PE: phycoerythrin, FITC: Fluorescein isothiocyanate.

Figure 4. Expression of macrophage markers and inflammatory cytokines in CD14⁺ macrophages from the placenta of obese women

mRNA expression of inflammatory markers was measured in whole placenta villous tissue (white bars) and in isolated placental CD14⁺ macrophages (black bars). Results of duplicate real-time PCR analysis were normalized for actin and expressed as fold change in obese vs lean women. Mean $\pm$ SE of duplicate determinations processed from 15 placenta of obese and 15 placentas of lean women. *: $p < 0.001$ in obese compared to lean women. White bars: lean, grey and black bars: obese.

Table 1Anthropometric parameters of study subjects (mean $\pm$ SEM)

	Obese group (n = 20)	Lean group (n = 15)
Maternal age (years)	26.9 $\pm$ 1.2	27.9 $\pm$ 1.7
Gestational age at delivery (weeks)	39.1 $\pm$ 0.5	38.9 $\pm$ 0.1
Pre-gravid BMI (kg/m ²)	40.6 $\pm$ 1.4 **	22.3 $\pm$ 0.4
BMI at delivery (kg/m ²)	44.9 $\pm$ 1.3 **	28.5 $\pm$ 0.62
Birth weight (g)	3379 $\pm$ 95 *	3028 $\pm$ 62
Neonatal % body fat at birth	14.0 $\pm$ 0.5 *	12.1 $\pm$ 2.5
Placental weight (g)	673 $\pm$ 23 *	599 $\pm$ 29.4

*
p < 0.05,**
p < 0.001.

Table 2Metabolic and inflammatory parameters in maternal plasma (mean $\pm$ SEM)

	Obese group (n $\pm$ 20)	Lean group (n $\pm$ 15)
Insulin (uU/ml)	26.9 $\pm$ 3.5**	10.6 $\pm$ 1.7
Insulin sensitivity at delivery (HOMA index)	4.3 $\pm$ 0.5**	1.2 $\pm$ 0.3
Leptin (ng/ml)	63.4 $\pm$ 6.2**	27.6 $\pm$ 2.6
TNF-alpha (pg/ml)	1.1 $\pm$ 0.2	1.1 $\pm$ 0.1
IL-6 (ng/ml)	4.2 $\pm$ 0.3**	2.8 $\pm$ 0.2
CRP (ng/ml)	10,453 $\pm$ 602*	8162 $\pm$ 926
% Monocytes	5.6 $\pm$ 0.5	5.3 $\pm$ 0.4
% Granulocytes	69.2 $\pm$ 1.2	65.5 $\pm$ 2.0

*
p < 0.05.**
p < 0.001.