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PEDIATRIC ORIGINAL ARTICLE

Effects of pregnancy on obesity-induced inflammation in a mouse model of fetal programming

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OBJECTIVE: Maternal obesity is associated with increased risk of metabolic dysfunction in the offspring. It is not clear whether it is the metabolic changes or chronic low-grade inflammation in the obese state that causes this metabolic programming. We therefore investigated whether low-grade inflammation was present in obese dams compared with controls dams at gestation day 18 (GD18).
METHODS: Female mice were fed either a standard chow diet or a highly palatable obesogenic diet for 6 weeks before conception. Mice were either kileed before mating ($n = 12$ in each group) or on GD18 ($n = 8$ in each group). Blood and tissues were collected for analysis.
RESULTS: The obesogenic diet increased body weight and decreased insulin sensitivity before conception, while there was no difference between the groups at GD18. Local inflammation was assayed by macrophage count in adipose tissue (AT) and liver. Macrophage count in the AT was increased significantly by the obesogenic diet, and the hepatic count also showed a tendency to increased macrophage infiltration before gestation. This was further supported by a decreased population of monocytes in the blood of the obese animals, which suggested that monocytes are being recruited from the blood to the liver and AT in the obese animals. Gestation reversed macrophage infiltration, such that obese dams showed a lower AT macrophage count at the end of gestation compared with pre-pregnancy obese mice, and there were no longer a tendency toward increased hepatic macrophage count. Placental macrophage count was also similar in the two groups.
CONCLUSION: At GD18, obese dams were found to have similar macrophage infiltration in placenta, AT and liver as lean dams, despite an incipient infiltration before gestation. Thus, the obesity-induced inflammation was reversed during gestation.

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Keywords: fetal programming; inflammation; macrophage; pregnancy

INTRODUCTION

Maternal obesity before and during pregnancy increases the risk of metabolic disorders, such as obesity, hyperphagia, hypertension, non-alcoholic fatty liver disease and insulin resistance in the offspring.[1,2] The maternal cues that programme metabolic changes in the offspring are still unknown, but inflammatory mediators produced in the mother due to the obesity-induced low-grade chronic inflammation could be causative agents. This inflammatory state is characterized by increased infiltration of immune cells into the white adipose tissue (AT) and the liver, as well as enhanced levels of pro-inflammatory cytokines.[3] Prenatal exposure to tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6), at levels reflecting infections, results in increased bodyweight and adiposity in the offspring, and exposure to IL-6 furthermore resulted in decreased glucose uptake.[4–7] There is as yet no data on the effects of fetal exposure to the sub-acute levels seen in obesity-induced low-grade inflammation. Although it is well established that excessive adiposity induces low-grade inflammation, pregnancy is characterized by local anti-inflammatory skewing of the immune response to avoid rejection of the fetus.[8,9] We therefore hypothesize that pregnancy might attenuate the effects of obesity-induced inflammation, and hence might have a less pronounced role in fetal metabolic imprinting than otherwise anticipated.

Various hypotheses have been raised to explain why excess adiposity is associated with sub-clinical inflammation; two recent models are the endotoxemia hypothesis and the lipotoxicity hypothesis. According to the endotoxemia hypothesis, recruitment of monocytes to AT is initiated through lipopolysaccharide (LPS)-mediated activation of circulating monocytes, via a Toll-like receptor 4 (TLR4)- and CD14-dependent mechanism, that propagates the inflammatory phenotype.[10–12] This hypothesis is supported by observations that obese subjects have increased levels of circulating LPS.[10,13] However, neutrophils,[14,15] mast cells[16] and also T cells[17] infiltrate the AT, coincident with a depletion of eosinophils, before monocyte infiltration and the endotoxemia hypothesis is unlikely to explain all these changes. The lipotoxicity hypothesis alternatively suggests that the triglyceride storage capacity of adipocytes becomes limiting, causing ectopic lipid accumulation and formation of lipotoxic intermediates, such as ceramide. Ceramide impairs metabolic function through inhibition of insulin receptor signalling[18] and might participate in inducing an inflammatory phenotype in the tissue.[19] Ceramide accumulation in skeletal muscles, liver and heart have been linked to the development of type 2 diabetes and increased risk for cardiac failure.[20–22] Furthermore, AT expansion alters the adipokine expression profile, which, among other effects, results in increased leptin and monocyte chemotactic

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protein-1 (MCP-1) production, which initiate migration of monocytes and neutrophils concurrent with cytokine release.[23,24]

Regardless of the aetiology of obesity-induced inflammation, maternal obesity-induced inflammation during gestation might cause programming effects in the offspring, leading to metabolic impairment, but it requires that the low-grade inflammation is not counter-balanced by the gestation-induced anti-inflammatory response. To test our hypothesis that gestation attenuate obesity-driven tissue inflammation, we have determined the level of obesity-associated inflammation in a commonly used mouse model of obesity-induced fetal programming of metabolic dysfunction,[25,26] before mating and at day 18 of gestation (GD18). Furthermore, we also tested whether maternal obesity affected ectopic lipid deposition in the offspring at GD18.

MATERIALS AND METHODS

Animal experiment

All studies were approved by the local Ethics Committee and conducted according to the Home Office Animals (Scientific Procedures, UK) Act 1986. The animal model used has been described in detail by Samuelsson *et al*.[1,26] In previous studies, offspring of second pregnancies were studied, where in the current study it is the first pregnancy that is being characterized. In brief, female C57BL/6 mice at weaning were fed a regular chow diet (3% fat (w/w), RM1, Special Diets Services, Witham, UK) ($n=20$) or a highly palatable obesogenic diet consisting of a high-fat pellet diet (22% fat (w/w), 824053, Special Diets Services, Witham, UK) supplemented with sweetened condensed milk (Nestle, Croydon, UK) ($n=21$) (Supplementary Figure 1). The composition of the diets was described by Samuelsson *et al*.[1,26] After 6 weeks on the diets, 12 mice in each group were killed by rising CO_2 concentrations, followed by immediate cardiac puncture; plasma was isolated and stored at − 80 °C until analysed, tissues were collected and treated as described below. The remaining mice were mated and killed on GD18, along with the unborn fetuses, followed by tissue and blood plasma sampling (lean $n=8$, obese $n=9$). Before and during gestation the dams were weighed weekly and body fat was evaluated by time-domain nuclear magnetic resonance (Bruker minispec LF series, Bruker Optik GmbG, Ettlingen, Germany).

Blood stimulations and identification of monocytes in blood by flow cytometry

Blood from the saphenous vein was collected in heparin tubes 2 days before the animals were killed for profiling of monocyte numbers, TLR4 and CD14 expression levels and for stimulation with LPS. Blood was stained with CD45-PE, CD11b-FITC, CD14-PerCP-Cy5.5 and TLR4-APC (eBiosciences, Hatfield, UK) for 30 min at 4 °C followed by lysis of red blood cells with FACS lysing solution (BD Biosciences, Oxford, UK). Samples were subsequently analysed by flow cytometry on a FACS Calibur (BD Biosciences). The remaining blood was diluted tenfold in complete media (RPMI-1640 with 1% penicillin and streptomycin and 1% L-glutamine) and stimulated for 24 h with 500 ng ml^{-1} LPS (*Escherichia coli* O111:B4, InvivoGen, Tououse, France) or complete media. After stimulation, supernatants were collected for TNF-α analysis (Meso Scale Discovery TNF-α kits and Meso Scale Selector Imager 6000, Meso Scale Discovery, Rockville, MD, USA) according to the manufacturer's instructions. The cells were collected for flow cytometry analysis and analysed as described for the whole blood. Monocytes were gated as shown in Supplementary Figure 2.

Blood glucose and plasma parameters

Blood glucose was analysed by a glucometer (AlphaTRAK, Abbott Animal Health, IL, USA) on blood collected from the tail vein after 4 h of fasting. Plasma triglycerides were analysed on a Cobas Mira Plus with a Horiba ABX Pentra kit (Montpellier, France). Plasma MCP-1, TNF-α, IL-6, IL-10, CXCL1, insulin and leptin were analysed on a Meso Scale Selector Imager 6000 with Meso Scale Discovery kits according to the manufacturer's instructions. Due to lack of plasma, TNF-α and IL-6 could, however, only be analysed in four control and nine obese animals at week 6, and seven control and seven obese animals on GD18.

Adipocyte size

Parametrial AT was fixed in 4% paraformaldehyde and embedded in paraffin, before sectioned (5 μm) and stained with haematoxylin and eosin (Pioneer Research Chemicals, Colchester, UK). Slides were evaluated on a light microscope using Cell^P to calculate adipocyte area from approximately 300 adipocytes per animal.

Lipid analysis

Livers were snap-frozen in liquid N_2 and stored at − 80 °C until analysed. Total lipids were extracted from tissues, as earlier described in Pedersen *et al*.[27] with the exception that the internal standards ^{2}H- ceramide (N-palmitoyl(d31)-D-*erythro*-sphingosine (Avanti Polar Lipids, Alabaster, AL, USA), pentadecanoic acid, tri-pentadecanoylglycerol (all Larodan Fine Chemicals, Malmo, Sweden) and β-sitostanol (Sigma-Aldrich, St Louis, MO, USA) were added before extraction. The total lipid extract was fractionated on Strata NH2 cartridges (Phenomenex, Torrance, CA, USA). The cartridges were rinsed and activated with 2 × 1000 μl heptane, samples were applied in 200 μl $CHCl_3$ and consecutively eluted with 2 × 2.0 ml $CHCl_3$:2-propanol (2:1, v-v) (elutes neutral lipids) and 5.0 ml 2% acetic acid in diethylether (elutes free fatty acid). The neutral-lipid fraction was dried down, re-dissolved in 200 μl $CHCl_3$ and applied to a new NH2 cartridge and further fractionated into cholesterol esters, triacylglycerol (TAG) and a fraction containing cholesterol, diacylglycerol and ceramide through elution with 2 × 2.0 ml hexane (cholesterolesters), 2 × 1.0 ml hexane: $CHCl_3$:ethylacetate (100:5:5 (TAG) and 2 × 2.0 ml $CHCl_3$:2-propanol (2:1). The fractionation method has been validated in-house through spiking of authentic samples with synthetic lipids. TAGs were analysed as earlier described,[27] using gas chromatography–flame ionisation detector. The fraction containing cholesterol and ceramide was analysed using liquid chromatography–mass spectrometry as described in Supplementary Materials.

Histology

Placenta and liver from female mice and fetuses were fixed in 4% paraformaldehyde and incubated in 30% sucrose overnight before sectioned (7 μm). Slides were washed in 60% isopropanol, stained with Oil Red O (Sigma, St Louis, MO, USA) and counterstained with haematoxylin to detect lipid accumulation within the various tissues.

Immunohistochemistry

Liver, parametrial AT, placenta and fetal livers were fixed in 4% paraformaldehyde and embedded in paraffin before sectioned (5 μm). Antigens were retrieved by proteinase K (Qiagen, Hilden, DE, Germany), followed by blocking of endogenous peroxidases with 0.3% hydrogen peroxide for 30 min. Slides were then incubated with 10% rabbit serum with avidin (Vector laboratories, Peterborough, UK) to block endogenous biotin for 40 min, followed by incubation with a primary rat anti-mouse F4/80 antibody (liver, placenta and fetal liver: BM8, Abcam (Cambridge, UK), 1:200 dilution; AT: Cl:A3, AbD Serotec (Kidlington, UK), 1:150 dilution) with added biotin (Vector laboratories) for 2 h. Hereafter, slides were incubated with a biotinylated rabbit anti-rat IgG antibody (1:200 dilution) for 1 h (Vector laboratories), treated with ABC reagent (Vector laboratories) for 30 min and developed with a 3,3'-diaminobenzidine tetrahydrochloride reagent (Vector laboratories). Slides were finally counterstained with haematoxylin. Liver macrophage infiltration was measured by counting all F4/80-positive cells in six images obtained at × 10 magnification. Macrophage infiltration in AT was evaluated by counting adipocytes and F4/80-positive cells in six images taken at × 10 magnifications and a macrophage-to-adipocyte ratio was calculated. Placenta samples were analysed by counting all F4/80-positive cells in a placental cross-section. The liver is involved in formation and development of blood cells during the perinatal period, where resident macrophages form erythroblastic islets with other blood cells.[28] This complicates macrophage count in the fetal liver, so instead macrophage accumulation was quantified by estimating the area with F4/80-positive stain. Images were obtained at × 20 magnification and four images from each fetal liver were quantified by the 'color segmentation' plug-in in ImageJ (http://imagej.en.softonic.com/).

Statistical analysis

Data are presented in box-plots with whiskers that represent 5–95 percentiles. Not all data followed a Gaussian distribution or had equal

variance among groups, therefore the gestation and diet effects were analysed by a permutated two-way analysis of variance ANOVA in the lmperm package in R (http://www.r-project.org/), as described by Anderson, 2001.[29] *Post hoc* tests were performed as a pair-wise comparison between groups by a two-sample permutation test in the coin package in R and adjusted for multiple testing by the Holm method.[30] Effects were considered significant when $P < 0.05$.

RESULTS

We characterized the metabolic status of non-pregnant female mice after 6 weeks intake of the obesogenic diet, as well as on GD18.

Weight gain and adiposity upon 6 weeks of obesogenic diet and effects of gestation

Female mice on the obesogenic diet gained more weight and had higher adiposity than mice on the control diet from 2 weeks of feeding (Figures 1a and c). After 6 weeks, the obese mice had higher body weights (26.7 ± 3.5 g vs 22.9 ± 1.6 g, $P < 0.001$), increased adiposity (Figure 1c, $P < 0.001$), larger parametrial AT depots (Figure 1e, $P < 0.01$) and larger adipocytes compared with the lean mice (Figures 1f–g, $P < 0.001$). The two groups had similar weight gain from conception (Figure 1b). On GD18, the obese dams still had increased total adiposity ($P < 0.05$) and larger parametrial AT depots ($P < 0.05$) (Figures 1d and e). Gestation did not affect adipocyte size. Liver weights were identical between the groups both after 6 weeks and at GD18. However, the liver almost doubled in weight in both diet groups during gestation (control: from 0.81 ± 0.11 g to 1.73 ± 0.19 g, obese: from 0.94 ± 0.35 g to 1.58 ± 0.17 g) (Figure 1e). We observed no effect of maternal obesity on placental weight, fetal body weights, fetal liver weights or the liver-to-body weight ratios (Figures 2a–d).

Maternal metabolic parameters in blood

Fasting blood glucose levels were not different between diet groups after 6 weeks or at GD18 (Figure 3a). However, the obesogenic diet resulted in increased fasting insulin levels after 6 weeks on the diet ($P < 0.05$). Fasting insulin levels were increased as an effect of gestation only in lean dams ($P < 0.01$), and hence there was no significant difference between the two groups at GD18 (Figure 3b, $P = 0.34$). Leptin was elevated in the obese animals both at week 6 and on GD18, although leptin levels also increased in the control mice with gestation (Figure 3c, $P < 0.05$). The levels of plasma triglycerides were unaffected by diet, but gestation had a lowering effect (Figure 3d, $P < 0.05$).

Lipid accumulation in maternal liver, placenta and fetal liver

The obesogenic diet had no effect on liver weight or hepatic lipids after 6 weeks of feeding. However, at GD18 the diet significantly increased hepatic TAG accumulation compared with lean dams ($P < 0.01$) (Figures 4a and c accumulation). Hepatic ceramide level was also unaffected by the obesogenic diet at week 6, while gestation increased the levels in both groups ($P < 0.0001$ for 6 week vs GD18). At GD18 this increase was attenuated in obese dams, which had lower hepatic ceramide than lean dams (Figure 4b, $P < 0.05$). We found no differences in triglyceride or ceramide concentrations in the placenta (Figures 4d–f); however, fetuses from obese dams had higher hepatic triglyceride ($P < 0.05$) and tended to have increased ceramide levels (Figures 4g–i, $P = 0.06$).

Based on all metabolic parameters, it was apparent that the obesogenic diet used in this model of fetal programming induced significant metabolic changes not only in the dams, but also in the fetuses.

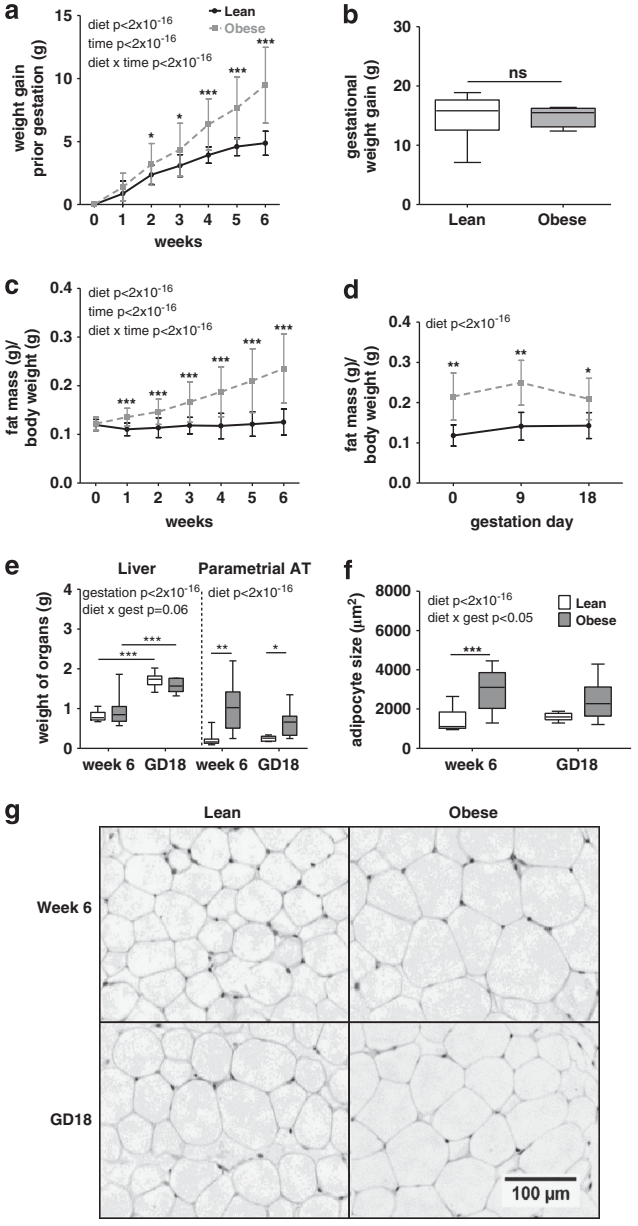

Figure 1. Maternal characteristics. Maternal weight gain before gestation (**a**) and at GD18 (**b**) in lean (solid line and open box) and obese (broken line and gray box) female mice. Their percentage fat mass was determined by TD-NMR before (**c**) and during gestation (**d**), and parametrial fat pads and livers were weighed at post-mortem (**e**). Finally the parametrial AT was sectioned and H&E stained (**g**) to evaluate the adipocyte size (**f**). Open boxes/broken lines: lean dams, shaded boxes/solid lines: dams fed obesogenic diet. Boxes show the 25–75 percentile and whiskers the 5–95 percentile. In (**a**, **c** and **d**), data are given as mean ± s.d. At week 6 $n = 12$, and on GD18 $n = 8$ in lean and $n = 9$ in the obese group. Only significant values from the two-way analysis of variance (ANOVA) and the *post hoc* test are reported on the figures. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, NS, non significant.

Levels of tissue macrophage infiltration

AT macrophages were enhanced in obese mice before gestation ($P < 0.05$, Figures 5a and b), while liver macrophage counts tended to be higher upon 6 weeks on the obesogenic diet ($P = 0.07$) (Figures 5c and d). Interestingly, macrophage counts were similar in obese and lean dams at GD18 in AT (Figures 5a and b),

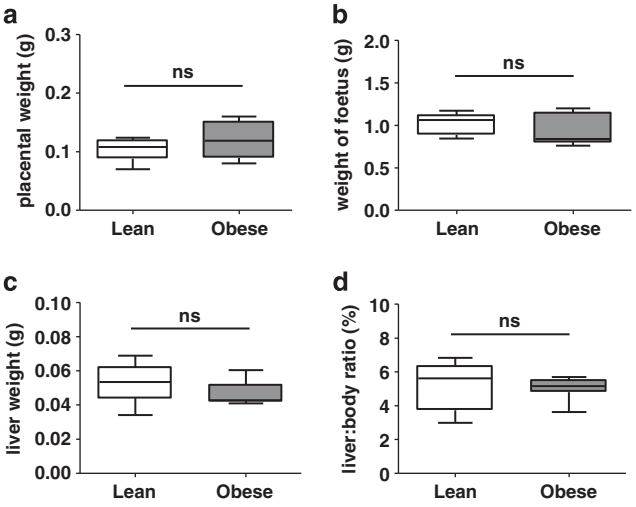

Figure 2. Fetal characteristics. On GD18, dams were killed, and placenta and fetuses were removed and weighed (**a** and **b**, respectively). Livers from fetuses were also removed and weighed (**c**) and a liver-to-body ratio was calculated (**d**). Open boxes lean, gray boxes obese. All pups from a single dam were considered as a single natural replicates, so the average weight of placenta, fetuses, fetal livers and the average fetal liver-to-body ratio from each dam are presented (lean $n=8$, obese $n=9$). NS, non significant.

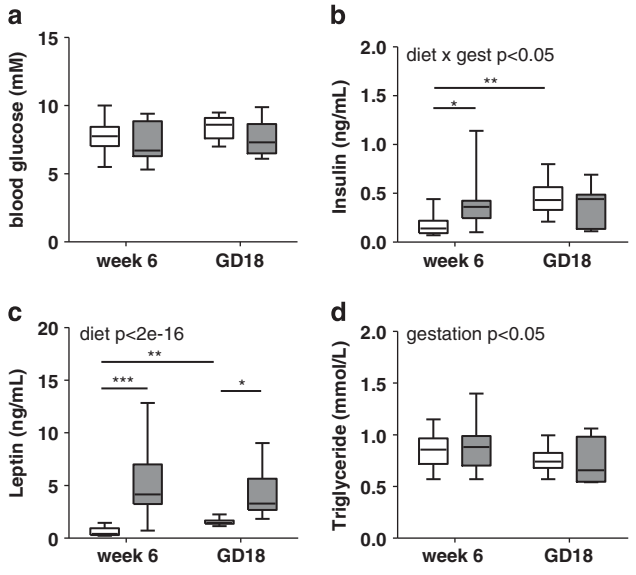

Figure 3. Metabolic markers in plasma. At week 6 and on GD18, blood was collected after a 4-h fast from animals fed chow (white boxes) or an obesogenic diet (gray boxes). Blood glucose levels (**a**) were measured in whole blood from the tail vein, and insulin (**b**), leptin (**c**) and triglycerides (**d**) were analysed in plasma obtained from blood collected by cardiac puncture. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

liver (Figures 5c and d) and placenta (Figures 5e and f) as well as in fetal livers (Figures 5g and h).

Gestational effects on monocyte recruitment and activation propensity

Since we found increased macrophage infiltration in AT following 6 weeks intake of the obesogenic diet, we determined the general recruitment of blood monocytes by tissues via MCP-1. MCP-1 was on an average higher in the obese mice at week 6, although this effect did not reach significance ($P=0.07$). At GD18, MCP-1 levels in the pregnant obese dams were identical to the lean (Figure 6a). However, the levels of plasma MCP-1 correlated significantly with both parametrial AT mass ($P<0.05$) and macrophage count in AT ($P<0.01$) (Figure 6b and Supplementary Figure 3) implying a link between circulating MCP-1 levels, adiposity and macrophage infiltration in AT. Concomitant with the enhanced macrophage infiltration, there was a decrease in the number of blood monocytes in obese mice at week 6 (Figure 6c, $P<0.05$) supporting that monocytes were recruited to tissues at this time point (Figures 5a and c). At GD18, there were no differences in blood monocyte counts (Figure 6c) or AT macrophage counts (Figure 5a) between obese and lean dams. However, blood monocyte numbers increased twofold during gestation independent of diet (Figure 6c).

Since MCP-1 tended to increase in the obese dams at week 6, but appeared normal at GD18, we also analysed the pro-inflammatory cytokines IL-6 and TNF-α, the anti-inflammatory cytokine IL-10 and the neutrophil-attractant chemokine CXCL1 (Figures 6d–g). For IL-6, there was a significant effect of diet ($P<0.01$, Figure 6d), and at GD18, the plasma concentration was significantly higher in the obese dams, than in the lean ($P<0.05$). This is in contrast to IL-10 and CXCL-1 (Figures 6f and g), which showed a similar pattern as MCP-1; IL-10 and CXCL-1 levels were increased at 6 weeks ($P<0.01$ and $P<0.05$, respectively) in the obese group, while plasma concentrations were normalized at GD18. Thus, among analysed cytokines and chemokines, only IL-6 showed a unique response pattern, with significantly increased levels in the obese dams at GD18. Noteworthy, MCP-1, CXCL-1 and IL-10 were correlated to adipose macrophage count ($P<0.01$, $P<0.05$ and $P<0.05$, respectively), while there was no such correlation for IL-6 and TNF-α (Supplementary Figure 3).

As an indication of pre-activation by endotoxemia, we measured the expression of CD14 and TLR4 on monocytes from freshly drawn blood, as well as TLR4 expression level after stimulation with LPS for 24 h. The obesogenic diet resulted in a highly significant reduction in the expression of TLR4 (Figure 6h; $P<2\times10^{-16}$) and tended to decrease CD14 (Figure 6i, $P=0.06$) when analysed by two-way analysis of variance. These effects were most pronounced at GD18, where there were significant differences ($P<0.05$) between control and obese dams. This could indicate a systemic adaptation to chronic endotoxemia. To test whether the lower TLR4 expression level was indicative of LPS tolerance, we stimulated the blood with LPS for 24 h. If LPS tolerance is induced, we would expect TLR4 expression levels and also secreted TNF-α to be less upregulated upon *ex vivo* LPS stimulation.[31] Notably, the LPS stimulation resulted in an equal upregulation of TLR4 and CD14 in both the diet groups (Figures 6j and k, $P=0.27$ and $P=0.80$, respectively), and TNF-α secretion was also similar (Figure 6l), suggesting no apparent LPS pre-activation of blood monocytes *in vivo*.

DISCUSSION

Maternal obesity is a risk factor for metabolic dysfunction in the offspring.[1,2] Obesity and AT expansion induces many metabolic changes, but also a low-grade inflammatory phenotype. Both metabolic and inflammatory components might have a key role in impaired metabolic programming of the offspring. However, since gestation induces an anti-inflammatory state in order to avoid rejection of the fetus, both pro- and anti-inflammatory cues are in play in the obese mother. In order to understand the potential role of inflammatory signals in this metabolic programming, it is important to determine to what extent metabolic inflammation is attenuated by gestation in models of offspring metabolic programming by maternal obesity. Therefore, we determined both the inflammatory and metabolic state in the mothers before pregnancy and at the end of gestation in a mouse model of

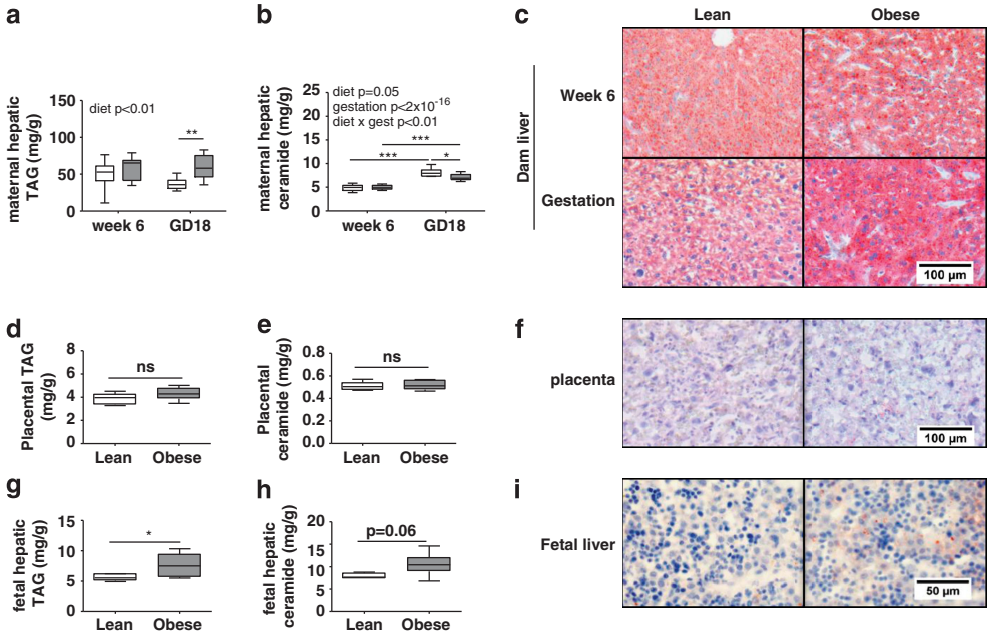

Figure 4. Hepatic and placental lipid accumulation. Lipids were extracted from maternal and fetal liver, as well as the placenta, and TAG content was analysed. (**a**) TAG content in maternal liver; (**b**) ceramide content in maternal liver; (**c**) lipid accumulation in maternal liver determined Oil-Red O staining; (**d**) placental TAG content; (**e**) placental ceramide content; (**f**) lipid accumulation in placenta determined with Oil Red O staining (**g**) TAG content in fetal livers; (**h**) hepatic ceramide levels in fetuses; (**i**) lipid accumulation in fetal liver stained with OilRed O. $P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, NS, non significant.

maternal obesity with well-described negative metabolic implications for the offspring.[26,32]

Obesity-associated inflammation and metabolic impairment is induced after 6 weeks of obesogenic feeding

In this model, 6 weeks on a high-fat diet supplemented with condensed milk before mating induced an obese phenotype in the dams apparent by increased body weight, increased adiposity and decreased insulin sensitivity, as reflected by an elevated insulin level to maintain a normal glycemia. The obesity development was associated with increased macrophage infiltration in the parametrial AT and a tendency to increased macrophage infiltration in the liver. Hepatic macrophage infiltration has previously been reported to be associated with hepatic lipid accumulation. Ceramide is known to induce an inflammatory phenotype;[33] however, our data indicate that obesity-induced hepatic inflammation might occur before changes in the lipid content, since no significant increases in liver triglycerides or ceramide were observed at this time point. The data from the macrophage counts were supported by a tendency to increased levels of MCP-1 in the blood. This cytokine is produced as a result of an early tissue response to high fat intake,[34] and it activates circulating monocytes and mobilizes them to a site of inflammation.[35] Furthermore, the number of circulating monocytes was also reduced in the obese dams before gestation, indicating that the increased concentration of MCP-1 provoked recruitment of circulating monocytes from the blood into tissues. Furthermore, the inflammatory chemokine CXCL-1, as well as IL-10, were significantly increased at 6 weeks, while IL-6 tended to increase. Altogether, our data indicate that obesity-induced tissue inflammation is initiated upon 6 weeks on obesogenic diet, before to mating, although a regulatory response, as seen by the increased IL-10, also is apparent.

Since the metabolic-endotoxemia hypothesis proposes that obesity leads to a chronic LPS exposure and thereby induces relative tolerance in target cells,[31] we investigated whether the LPS-driven activation of monocytes differed between the two groups. Although our data showed a strongly significant reduction in TLR4 expression and a tendency ($P = 0.06$) to reduced CD14 expression on the blood monocytes of obese dams, their response to stimulation with LPS for 24 h was similar to that of the lean dams, suggesting there was no endotoxemia/LPS tolerance.[36] Thus, although the reduced TLR4 and CD 14 expression could be interpreted as an adaptation to chronic LPS exposure, this did not result in functional LPS tolerance.

Effect of gestation on obesity-induced inflammation and metabolic impairment

The main objective of our study was to investigate if obesity-induced inflammation is attenuated by gestation in a well-described model of metabolic programming.[1] Dams fed the obesogenic diets had higher fat mass and circulating leptin levels at GD18, but despite this, our data showed that tendencies toward obesity-induced inflammation, to a large extent, were normalized during gestation. Among the inflammatory markers, only IL-6 was significantly increased at GD18. In contrast to the other inflammatory cytokines, IL-6 level was, however, not correlated with either AT mass (not shown) or AT macrophage count (Supplementary Figure 3). Hence, the IL-6 response did not seem to be related to AT inflammation in this model. The placenta is a major site for IL-6 secretion into both maternal and fetal circulation during pregnancy.[37] IL-6 stimulate fatty acid transfer from maternal circulation to the placenta,[38] suggesting that the elevated IL-6 on GD18 might explain the increased hepatic TAG accumulation observed in the fetuses. Pregnancy doubled monocyte abundance in the blood, independent of the diets, which is in accordance with previous studies.[39,40] We found no change in placental macrophage counts at GD18. This is in contrast to previous findings by Challier *et al.*,[41] who reported increased macrophage accumulation in human placenta from obese mothers.[41] However, our data are consistent with other studies, which have reported that pro-inflammatory-related

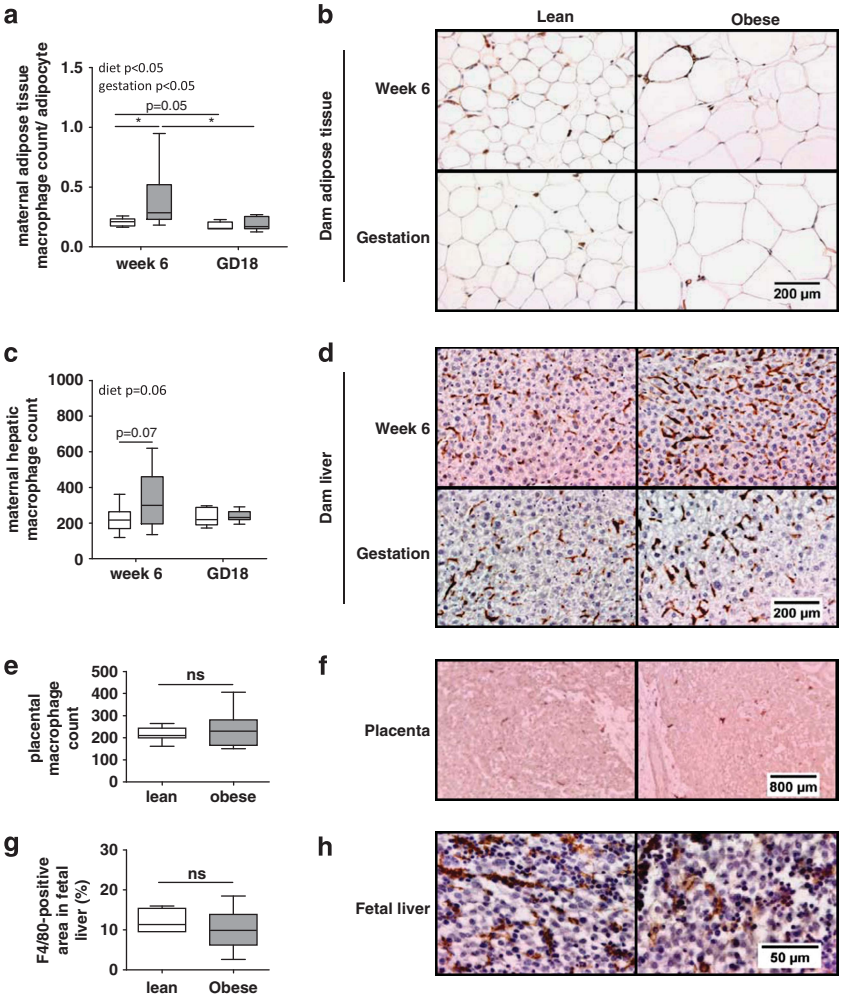

Figure 5. Macrophage infiltration in AT, liver and placenta. Mice were killed and AT (**a** and **b**), liver (**c** and **d**), placenta (**e** and **f**) and fetal liver (**h**) were collected, fixed and stained with an antibody targeting a macrophage surface marker (F4/80). Macrophages were counted in AT (**a**), liver (**c**) and placenta (**e**), but during the perinatal period macrophages form erythroblastic islets with other blood cells in the liver, which obstruct a macrophage count. It was therefore only possible to estimate the F4/80-positive stained area in the fetal livers (**g**), which were done by the 'color segmentation' plugin in ImageJ. $P < 0.05$, NS, non significant.

diseases such as multiple sclerosis, rheumatoid arthritis and psoriasis are improved during pregnancy. In addition, pregnant women have been shown to be more susceptible to infections such as influenza, due to the suppression of the pro-inflammatory immune system. Moreover, persistent infections, like malaria, during pregnancy increase the risk of abortion and preterm delivery, because the body is incapable of sustaining the anti-inflammatory state (reviewed in Robinson and Klein[42]). Despite the anti-inflammatory properties of gestation, this study cannot rule out that the inflammatory state at the time of embryonic implantation potentially could have programming effects on offspring.

However, while gestation skews the systemic immune response toward an anti-inflammatory direction, it has been shown that maternal obesity dramatically increases fetal exposure to lipids. Zhu *et al.*[43] reported increased lipid transport to the fetus in obese ewes and McCurdy *et al.*[44] have reported that a maternal high-fat diet results in lipid accumulation in fetal liver of non-human primates. Although we found no lipid accumulation or any sign of increased inflammation in the placenta after the relatively short pre-gestational time on the obesogenic diet, we did observe increased accumulation of triglycerides and ceramides in fetal livers. Gestation increased ceramide levels in maternal livers in both groups, but unexpectedly this increase was less pronounced in livers from obese mothers, resulting in a significantly higher hepatic ceramide level in lean dams compared with obese dams. It is well established that maternal insulin sensitivity is reduced during pregnancy[45] in order to optimize nutrient flow to the fetus. It is therefore noteworthy that ceramide accumulation in the livers of the lean dams occurred concomitant with reduced systemic insulin sensitivity during pregnancy, manifested as increased fasting insulin. To find whether the observed hepatic ceramide accumulation is part of this normal metabolic adaption to pregnancy requires further study.

The model of developmental programming used in this study is more susceptible to metabolic diseases and cardiac dysfunctions later in life.[26,32,46] If the higher tissue ceramide content in the fetuses reflects persistent alterations in ceramide metabolism, this could explain the observed increased incidence and earlier onset of metabolic diseases in pups from the obese mothers.

In conclusion, in this study we found no evidence that fetal programming of metabolic dysfunction in mice born by obese dams is driven by sub-clinical inflammation during gestation. On the contrary, the data support a model in which gestation induces an anti-inflammatory skewing of the immune system and an attenuation of the metabolic inflammation induced by the obesogenic diet. Since the mice in our study were in an early phase of the inflammatory development before mating, this

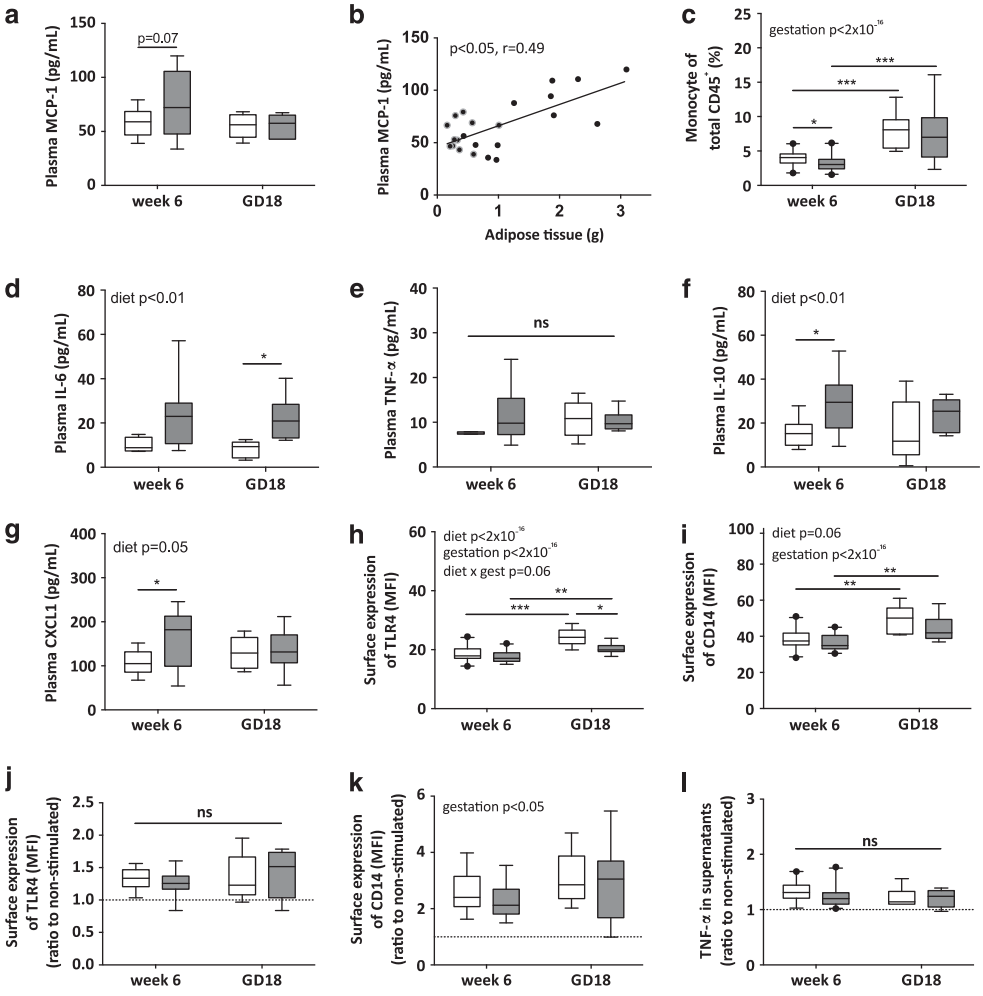

Figure 6. Monocyte recruitment and activation in blood. Blood was collected and monocyte recruitment investigated by analyses of plasma MCP-1 levels (**a**). Plasma MCP-1 levels were correlated with adiposity among the obese animals at week 6 (**b**), $P < 0.05$, which could indicate increased monocyte recruitment to the tissues. Monocytes in the blood were identified by flow cytometry ($CD45^{+}$ $CD11b^{high}$) (**c**). Plasma levels of the inflammatory cytokines IL-6 (**d**) and TNF-α (**e**) were analysed, along with the anti-inflammatory cytokine IL-10 (**f**) and the neutrophil-attractant chemokine CXCL1 (**g**). Surface expression of TLR4 (**h**) and CD14 (**i**) was also analyzed in monocytes in non-stimulated conditions. In addition, blood was also stimulated with 500 ng ml^{-1} LPS for 24 h to investigate the change in surface expression of CD14 (**j**) and TLR4 (**k**) and secretion of TNF-α into the supernatants (**l**). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. NS,non significant. Lean, week 6 $n = 21$; obese, week 6 $n = 22$; lean, GD18 $n = 8$; obese, GD18 $n = 9$. For IL-6 and TNF- α, 6 weeks lean $n = 4$; obese = 9, GD 18 lean = 7; obese = 7 (see Materials and methods).

model will not answer if gestational anti-inflammatory effects are able to reverse more pronounced pro-inflammatory states. However, the obesogenic diet caused increased ectopic deposition of TAG and ceramide in the fetal liver before birth. Hence, the results indicate that ectopic lipid deposition, rather than maternal inflammation, may provide a mechanism for the increased susceptibility to metabolic diseases later in life in this model of developmental programming.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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