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# Metabolic endotoxemia directly increases the proliferation of adipocyte precursors at the onset of metabolic diseases through a CD14-dependent mechanism<sup>\*</sup>



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## ABSTRACT

Metabolic endotoxemia triggers inflammation, targets cells from the stroma-vascular fraction of adipose depots, and metabolic disease. To identify these cells we here infused mice with lipopolysaccharides and showed by FACS analyses and BrdU staining that the number of small subcutaneous adipocytes, preadipocytes and macrophages increased in wild type but not in CD14-knockout (KO) mice. This mechanism was direct since in CD14KO mice grafted subcutaneously and simultaneously with fat pads from CD14KO and wild-type mice the concentration of cytokine mRNA was increased in the wild-type fat pad only. Conversely, the mRNA concentration of genes involved in glucose and lipid metabolism and the number of large adipocytes was reduced. Eventually, a pretreatment with LPS enhanced HFD-induced metabolic diseases. Altogether, these results show that metabolic endotoxemia increases the proliferation of preadipocytes through a CD14-dependent mechanism directly, without recruiting CD14-positive cells from non-adipose depot origin. This mechanism could precede the onset of metabolic diseases.

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**Keywords** Metabolic endotoxemia; Inflammation; Gut microbiota; LPS; Adipose tissue

## 1. INTRODUCTION

Intestinal microbiota is now considered as a veritable organ that regulates energy metabolism [1–3] and is causally involved in metabolic diseases [3–5]. Recent data have revealed a close association between intestinal microbiota, adipose tissue biology [6] and obesity [7,8]. We, and further confirmed by others, showed that the blood concentration of lipopolysaccharides (LPS), components from the outer membrane of gram negative bacteria, is increased by feeding a fat-enriched diet in humans [9–11] and in mice [12,13] thus defining a state of metabolic endotoxemia. Elevated LPS levels are associated with increased adipose tissue mass in the mouse [13] and in human [14] that could be related to the observed chronic low grade inflammation [15–17] involving both macrophages [16–23] and lymphocytes [18,24–26]. Recent data provide further evidence for the importance of LPS and its receptor system CD14/TLR4 on HFD-induced obesity [27]. However, the *in vivo* mechanisms through which

metabolic endotoxemia directly triggers adipose depot inflammation, development and metabolic disease are unknown. Previous data led to the hypothesis that metabolic inflammation originates from bone marrow infiltrating cells [21,28]. Inflammatory factors, including free fatty acids [29] were initially proposed to activate TLR4-expressing macrophages and trigger inflammation in adipose depot. However, we demonstrated, using functional analyses and microarray technology, that adipocyte progenitors and macrophages were characterized by a closed genome and phenotypome [30,31] suggesting that adipose-resident cells are sensitive to endotoxemia and could be involved in the changes observed in adipose tissue. Hence, we here suggest that both infiltrating and resident cells are involved in the processes characterizing metabolic inflammation in adipose tissue. This process would be tightly dependent on changes in intestinal microbiota and consequently on the production of bacterial fragments such as LPS. Therefore, we undertook to determine whether LPS could directly target CD14 expressed by adipose tissue resident cells as a first step in the

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generation of inflammation, which cells were targeted, and whether this process enhanced high-fat diet-induced metabolic diseases. This process could directly control the proliferation and biology of adipose precursors.

## 2. MATERIALS AND METHODS

### 2.1. Animals and treatments

Twelve-week-old C57bl6/J male mice (Charles River, France) and CD14 mutant male mice (Jackson laboratory, Bar Harbor, ME) bred in a C57bl6/J background were housed in a controlled environment (inverted 12-h daylight cycle, lights off at 10:00 am) with free access to food and water. In a first set of experiments mice were fed with either a normal chow diet (NC, energy content: 12% fat, 28% protein, and 60% carbohydrate, A04, Villemoisson sur Orge, France) or a high-fat carbohydrate-free diet which specifically induces metabolic endotoxemia (HFD, energy content: 72% fat, 28% protein and < 1% carbohydrate) for 4 weeks, as previously described [12,32,33]. In some mice metabolic endotoxemia was mimicked by infusing low rates of LPS through implanted osmotic mini-pumps, as described [13] (Alzet Model 2004; Alza, Palo Alto, Ca). The pumps were filled either with NaCl (0.9%) or LPS (from *Escherichia Coli* 055:B55; Sigma, St. Louis, MO) to infuse  $300 \mu\text{g kg}^{-1} \text{ day}^{-1}$  for 4 weeks. A subset of these treated mice was used to study the influence of LPS *in vivo* (osmotic pumps) on adipose precursor cell proliferation (Figure 1E and F). These mice were treated with BrdU (100 mg/kg i.p. Sigma, St Louis, MO) every 48 h and 2 weeks later were fed a HFD (Figure 1G).

In a second set of experiments, the direct role of LPS/CD14 was studied in CD14KO mice that were grafted with approximately 100 mg of 2 subcutaneous fat pads, one from a 12-week old wild-type (WT) donor and the other from a CD14KO donor (Supplementary Data 1). A 1 cm incision was made in the middle of the back skin and the two fat pads were symmetrically grafted towards each side of the animal and the incision was then sutured. Osmotic mini-pumps delivering NaCl or LPS were implanted for 4 weeks. At completion of the treatment the mice were sacrificed, the stroma vascular fraction of the adipose depot isolated and studied by FACS analysis or by immunohistochemistry.

In a subset of mice we grafted fat pads from wild type (genotype CD45.1) mice under the skin of CD14KO (genotype CD45.2) and fed a HFD for 1 month. FACS analyses have been performed on the grafted fat pads to identify the colonization by the host cells i.e. % of CD45.1 and CD45.2 over the total number of cells from the stroma vascular fraction (Supplementary data 2-H).

In a third group of experiments to analyze the influence of LPS pretreatment on the onset of metabolic diseases mice were fed a diet which induces both hyperglycemia and body weight gain. This high-fat and carbohydrate diet (HFCD) containing 45% fat, 20% proteins, and 35% carbohydrates (Research diets laboratory), was given for 8 weeks after the LPS-pretreatment. This diet induces obesity and glucose intolerance over the course of 3–4 months. At completion of the treatment the mice were sacrificed and FACS analysis or immunohistochemistry was performed on the adipose depots.

All the animal experimental procedures were approved by the local ethical committee of the Ranguel Hospital.

### 2.2. Quantitative real-time PCR

The concentrations of TNF $\alpha$ , IL-1 $\beta$ , PAI-1, MCP-1, MIF, OSN, GLUT4, HKII, FAS, ChREBP, PPAR $\gamma$  and aP2 mRNA were evaluated by quantitative RT-PCR analysis. Total mRNA was extracted using TriPure

reagent (Roche, Basel, Switzerland). PCRs were performed using an AbiPrism 7900 Sequence Detection System instrument and software (Applied Biosystems, Foster City, CA, USA), as previously described [13]. The concentration of each mRNA was normalized for RNA loading for each sample using RPL19 rRNA as an internal standard. Primer sequences for the targeted mouse genes are available upon request Supplementary Table 1.

### 2.3. Adipose tissue morphometry, F4/80 and BrdU staining

Paraffin-embedded hematoxylin and eosin counterstained sections were performed. The size of the adipocytes was assessed using image analyzer software (Image J). The number of adipocytes was then classified according to their size ( $\mu\text{m}^2$ ), which was estimated by a point-counting, and expressed as the percentage of the total population counted, as described previously [13]. The total count ranged from 1936 to 5270 cells per condition. F4/80 staining was carried out as follows. Ethanol-fixed, paraffin-embedded adipose tissue sections were de-paraffinized and rehydrated. Sections were blocked in normal serum and incubated overnight with primary rat anti-mouse F4/80 monoclonal antibody (1/1000; Serotec, Oxford, U.K.). Endogenous peroxidase activity was quenched by incubation with 3% hydrogen peroxide for 20 min. Secondary antibody staining was done using goat anti-rat biotinylated Ig Ab (1/500, 30 min, room temperature) and streptavidin horseradish peroxidase conjugated (1/500, 30 min, room temperature) and detected with 3,3'-diaminobenzidine. Sections were counterstained with hematoxylin eosin before dehydration and cover-slip placement. The number of F4/80 positive cells per microscope field was counted and divided by the total number of adipocytes in the sections. Five to 12 fields were counted per sample. BrdU staining was performed on ethanol-fixed, paraffin-embedded adipose tissue sections that were de-paraffinized, rehydrated and permeabilized to allow the binding of anti-BrdU biotinylated antibody. A horseradish peroxidase coupled streptavidin was used to detect the bound antibodies as described (BD Pharmingen, Palo Alto, CA, USA).

### 2.4. Measurement of body composition by nuclear magnetic resonance

The body composition of the mice, including the fat and lean masses, was analyzed by NMR using EchoMRI-100TM equipment (Echo Medical Systems, Houston, Texas).

### 2.5. Isolation of the stroma vascular fraction (SVF)

Cells were isolated according to Björntorp et al. with minor modifications [34]. Briefly, the grafted fat pads were digested at 37 °C in phosphate buffered saline containing 0.2% bovine serum albumin and 2 mg/ml collagenase for 30 min (collagenase A, Roche Diagnostics, Meylan, France). After elimination of undigested fragments by filtration through 25  $\mu\text{m}$  filters, adipocyte fractions were separated from the pellets of the stroma-vascular fraction (SVF) by centrifugation ( $600 \times g$ , 10 min). SVF cells were incubated for 5 min in a buffer to hemolyse red blood cells (140 nmol/l NH $_4$ Cl and 20 mmol/l Tris, pH 7.6), washed, and centrifuged in PBS. The number of isolated SVF cells was then counted with cell counter (Coulter Z2). SVF cells were either used for flow cytometry analyses or plated *in vitro*.

### 2.6. Cell phenotyping

Cells isolated from the adipose depots were analyzed by flow cytometry (FACS). Freshly isolated SVF cells were stained in staining buffer consisting of phosphate-buffered saline containing 0.5% fetal calf serum and FcR Blocking reagent (BD Biosciences). Cells were incubated with anti-mouse monoclonal antibodies (mAb) or rat immunoglobulins

(isotypes) conjugated with FITC, phycoerythrin, PercP or allophycocyanin for 30 min at 4 °C. Triple staining was performed by incubating cells with antibodies directed against cell surface antigens labeled with dyes such as FITC, phycoerythrin, PercP in one step to characterize the different cell populations. Cells were washed in staining buffer and then analyzed by FACS (FACS Canto II, Becton Dickinson, Mountain View, CA). Data acquisition and analysis were then carried out using DIVA software (Becton Dickinson). For BrdU detection, SVF cells were stained according to the manufacturer's protocol (BrdU Flow Kit, BD Pharmingen, Palo Alto, CA) with minor modifications. Briefly, cells were fixed and permeabilized with BD Cytofix/Cytoperm buffer. The cells were then treated with DNase (300 µg/ml) to expose incorporated BrdU and finally immunofluorescent staining of cell surface antigens was carried out as described above.

## 2.7. *In vitro* proliferation and differentiation assay

### 2.7.1. Cell proliferation

SVF cells were plated at a density of 5500 cells/cm<sup>2</sup> in DMEM:F12 supplemented with 10% fetal calf serum, biotin (16 µmol/l), panthothenic acid (18 µmol/l), ascorbic acid (100 µmol/l), and amphotericin (25 µg/ml), streptomycin (10 mg/ml), and penicillin (10,000 U/ml). The medium was changed every 2 days. The cells were counted each day with a cell counter (Coulter Z2) over 6 days.

### 2.7.2. Cell differentiation

Cells from the SVF were plated similarly. When they reached confluence the adipogenic differentiation process was induced with dexamethasone (33 nmol/l), insulin (2 nmol/l), 3,3,5-tri-iodo-L-thyronine (T3; 2 nmol/l) and transferrin (10 µg/ml) for 10 days. The medium was changed every 2 days. At the end of the culture period (after 14 days), the cells were lysed with 0.1 N NaOH, neutralized and the triglyceride (TG) content measured using a commercially available kit (Triglycerides enzymatiques PAP 150, Biomerieux, Marcy l'Etoile, France). The total protein content was determined using the DC Protein Assay Kit (BioRad, Marne la Coquette, France).

### 2.8. Glucose tolerance test

Intraperitoneal glucose tolerance tests were performed in 6-h fasted mice injected with glucose into the peritoneal cavity (1 g/kg glucose, 20% glucose solution). Blood glucose was determined with a glucose meter (Roche Diagnostics) on 3.5 µl blood collected from the tip of the tail vein. The glycemic index was calculated by dividing the sum of the glycemia values determined at all of the time points by the time period following glucose administration.

### 2.9. Statistical analyses

Results are presented as means ± SE. Statistical significance of differences was analyzed by *t*-test or two-way ANOVA followed by post hoc (Bonferroni's multiple comparison test) using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA). Data labeled with \* are statistically different with *p* < 0.05.

## 3. RESULTS

### 3.1. Metabolic endotoxemia increases subcutaneous adipose tissue precursor proliferation

Four weeks of low rate LPS infusion increased the number of small adipocytes (< 500 µm<sup>2</sup>) in the subcutaneous fat pads from wild type (WT) mice and reduced concomitantly the number of larger adipocytes (Figure 1A). These changes were not observed in CD14KO mice

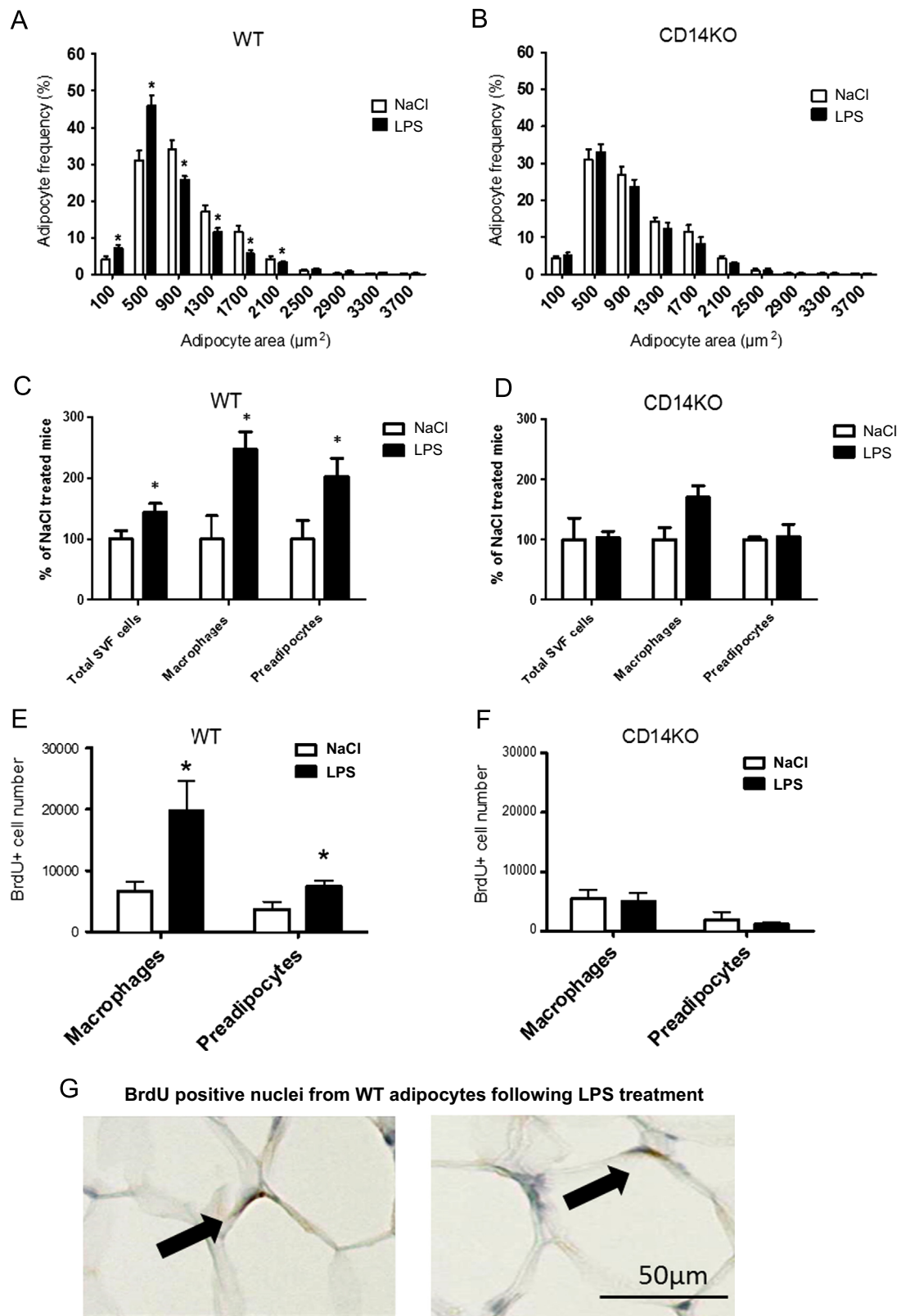
(Figure 1B). Importantly, no difference was observed in the lipolysis rates of WT and CD14KO adipocytes (not shown). We then analyzed by FACS the impact of LPS infusion on the cell types present in the stroma vascular fraction (SVF). LPS treatment increased the total cell number in WT mice (Figure 1C) as well as the number of macrophages (CD45<sup>+</sup>/CD11b<sup>+</sup>/F4/80<sup>+</sup>) and preadipocytes (CD45<sup>+</sup>/CD34<sup>+</sup>/CD31<sup>+</sup>) (Figure 1C). No significant change was observed following LPS infusion in CD14KO mice (Figure 1D). It was then shown by BrdU incorporation that LPS infusion increased the number of BrdU-positive adipocyte precursors and macrophages in the subcutaneous depot of wild type but not of CD14KO mice (Figure 1E and F). No change was observed for the other fat pads (data not shown). When these mice were subsequently fed on a HFD for 2 weeks to promote adipogenesis, we observed adipocyte nuclei stained with BrdU, suggesting that, after proliferation the preadipocytes differentiated *in vivo* into adipocytes (Figure 1G).

### 3.2. *In vitro* LPS directly regulates adipose precursor proliferation and differentiation

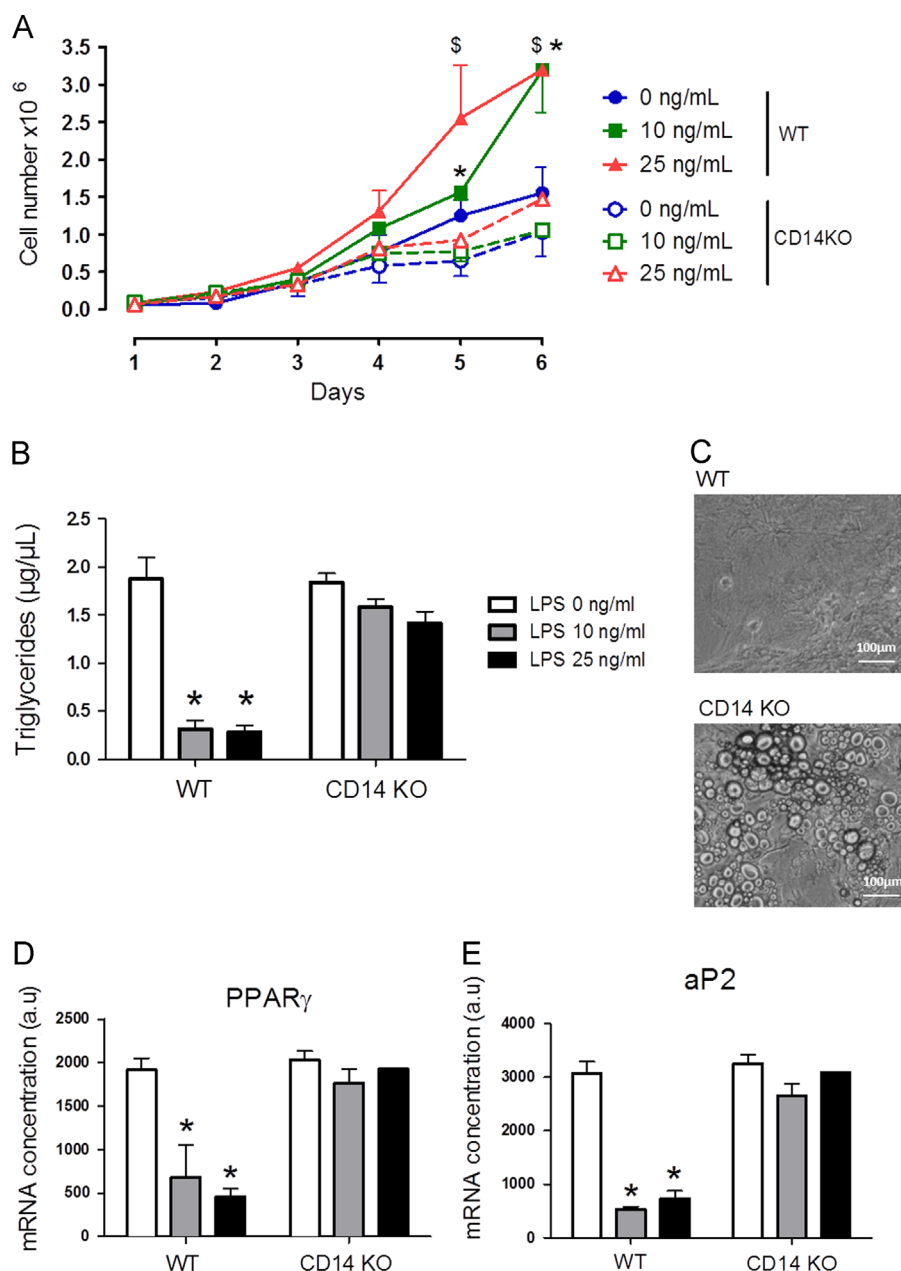
To further consolidate our *in vivo* observation that LPS directly increases the number of precursors in the SVF of adipose tissue, we incubated preadipocytes *in vitro* with different concentrations of LPS. The data showed that LPS treatment increased the number of adipocyte precursor cells (Figure 2A). Furthermore, triglyceride accumulation during the differentiation of the precursor cells into adipocytes was lower in the presence of LPS (Figure 2B and C). This effect was not observed with cells from CD14KO mice (Figure 2B and C). The expression of key genes of adipocyte differentiation such as PPARγ (Figure 2D) and aP2 (Figure 2E) was reduced by the LPS treatment in a CD14-dependent manner (Figure 2D and E).

### 3.3. *In vivo* metabolic endotoxemia directly targets and influences the inflammatory status of CD14-expressing cells in adipose tissue

We then set up a procedure to determine the direct role of LPS on cells from the adipose depot without targeting CD14 positive cells outside the fat pads (Supplementary Figure 1). To this aim we grafted fat pads simultaneously from wild type and CD14KO mice under the skin of CD14KO mice. After 1 month of LPS infusion FACS analyses showed that metabolic endotoxemia increased significantly the number of macrophages in the grafted wild type fat pads only (Figure 3A). No significant increase was observed in fat pads from CD14KO mice suggesting that the increase in macrophage number induced by metabolic endotoxemia was CD14 mediated (Figure 3A). Immunohistochemistry analyses confirmed this conclusion (Figure 3B and C). Furthermore, after LPS infusion the mRNA concentrations of plasminogen activator inhibitor 1 (PAI-1; Figure 3D), tumor necrosis factor alpha (TNFα; Figure 3E), interleukin 1b (IL1b; Figure 3F), macrophage chemo-attractant peptide 1 (MCP1; Figure 3G) and macrophage inhibitory factor (MIF; Figure 3H), were increased in the grafted fat pad from wild type mice but not from CD14KO when compared to saline-infused mice. Similar results were obtained in subsets of mice fed a high-fat diet (Supplementary Figure 2). In a subset of mice we aimed to identify the percentage of hematopoietic (CD45 positive) cells from the host infiltrating the grafted fat pads and hence to determine the role of CD14 on that process. FACS analyses showed that when the wild type fat pad (genotype CD45.1) was grafted under the back skin of CD14 KO mice (genotype CD45.2) the frequency of hematopoietic cells from the host (genotype CD45.2) within the grafted fat pad (genotype CD45.1) was over 90% of the total number of cells from the stroma vascular fraction (Supplementary data 2H). A similar frequency was observed when the fat pad was grafted under the back skin of a wild type mouse



**Figure 1:** Metabolic endotoxemia increases subcutaneous adipose tissue precursor proliferation rate. Wild type (WT) (A, C, and E) and CD14KO (B, D, and F) mice were infused with Saline (NaCl;  $n=6-8$ ) or LPS ( $n=8-9$ ) for 1 month. (A and B) The quantification of the adipocyte frequency is reported according to their size ( $\mu\text{m}^2$ ). (C and D) The total stroma vascular fraction cell, macrophages (CD45+, CD11b+, F4/80+), and preadipocyte (CD45-, CD34+, CD31-) number (E and F) and the number of macrophages and preadipocytes labeled with BrdU is shown ( $n=6$ ). (G) BrdU WT adipocytes labeled (both panels) are shown after 4 weeks of LPS infusion followed with 2 weeks of HFCD treatment. Data are means  $\pm$  SE. \* $P < 0.05$  vs NaCl control by  $t$ -test.



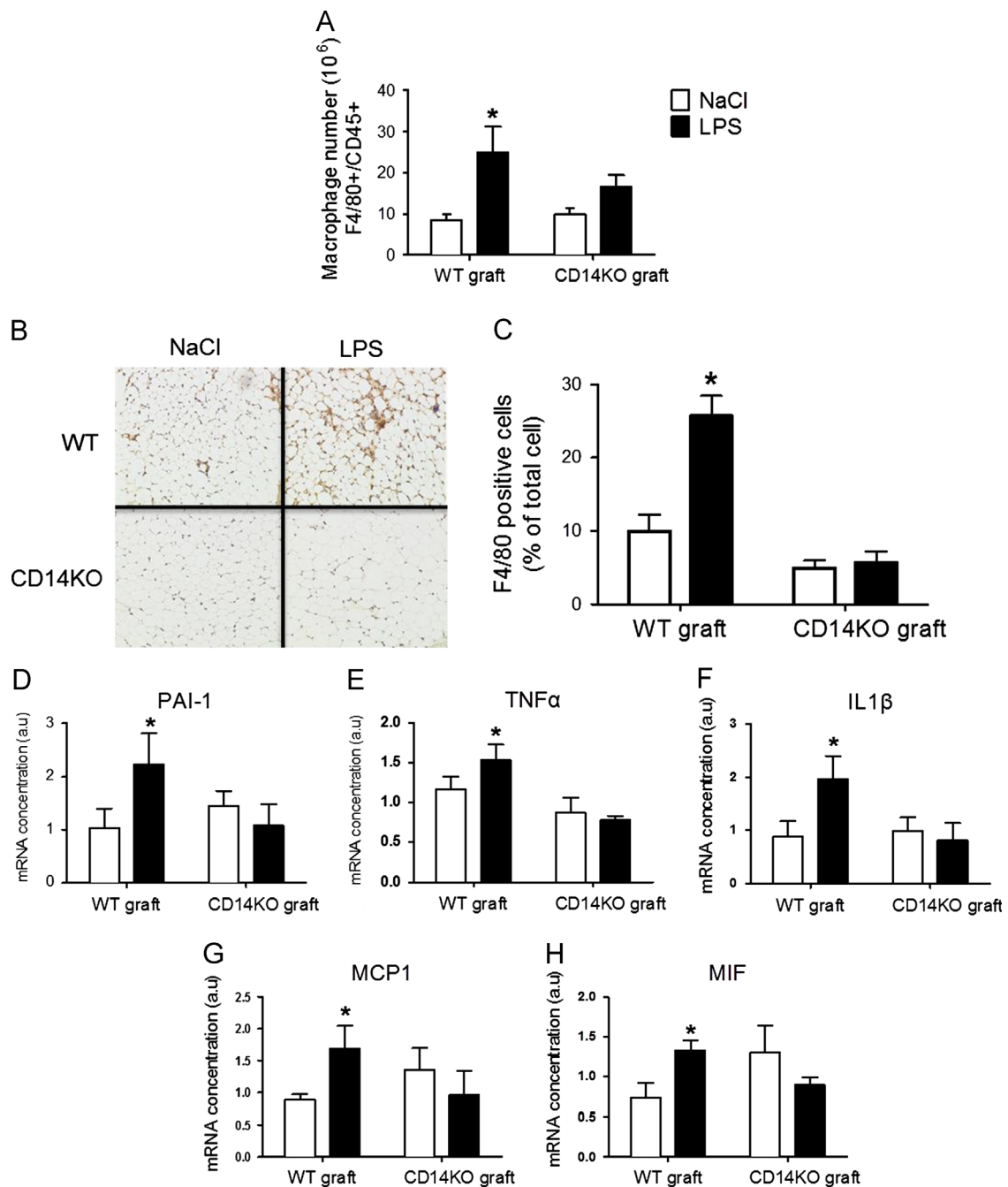
**Figure 2:** LPS increases the proliferation and reduce triglyceride accumulation in vitro on cells from the stroma vascular fraction from adipose tissue. The cells from stroma vascular fraction from WT and CD14KO mice were incubated for 6 days in the presence of different concentrations of LPS (A; ng/ml). \* $P < 0.05$  vs CD14KO 10 ng/ml condition and \$ $P < 0.05$  vs CD14KO 25 ng/ml condition control by a *Post-hoc* ANOVA analyses. Triglyceride content (B; μg/μl) and representation (C) of the corresponding cells when incubated for 6 days with different concentrations of LPS. The scale (μm) is represented. The mRNA concentrations of genes involved in adipocytes differentiation such as PPAR (D) and aP2 (E) are represented. Data show mean  $\pm$  SE of duplicates and are representative of at least two independent experiments. \* $P < 0.05$  vs 0 ng/ml condition control by a *post-hoc* ANOVA analyses.

suggesting that the infiltrating process was independent from CD14 expression of the host (Supplementary data 2H).

### 3.4. Metabolic endotoxemia directly impacts genes from adipose tissue metabolism

The mRNA concentrations of glucose transporter type 4 (GLUT4; Figure 4A), hexokinase II (HKII; Figure 4B), fatty acid synthase (FAS; Figure 4C), and carbohydrate responsive transcription factor (ChREBP; Figure 4D) were reduced in wild-type grafted fat pads from mice infused with LPS when compared with saline-infused mice (Figure 4A–D). No effect was observed in fat pads from CD14KO mice (Figure 4A–D) except for ChREBP (Figure 4D) which was still significantly increased by

LPS. A non-significant trend was observed for GLUT4 suggesting that non-CD14 dependent LPS receptor effect, although modest, could still be observed, which could be attributed to TLR4. Similar results were obtained in subsets of mice fed a high-fat diet (Supplementary Figure 3). Furthermore, in wild type fat pads, metabolic endotoxemia induced an increase in the number of small adipocytes whereas the number of large adipocytes was decreased (Figure 4E). The size distribution of adipocytes was different in grafted fat pads from CD14KO compared to control mice (Figure 4F). We also showed that the mean size of the adipocyte fat pads was reduced in response to LPS in wild type depots but increased in the fat pad from CD14KO mice (Figure 4G). In addition, in response to metabolic endotoxemia the number of preadipocytes was



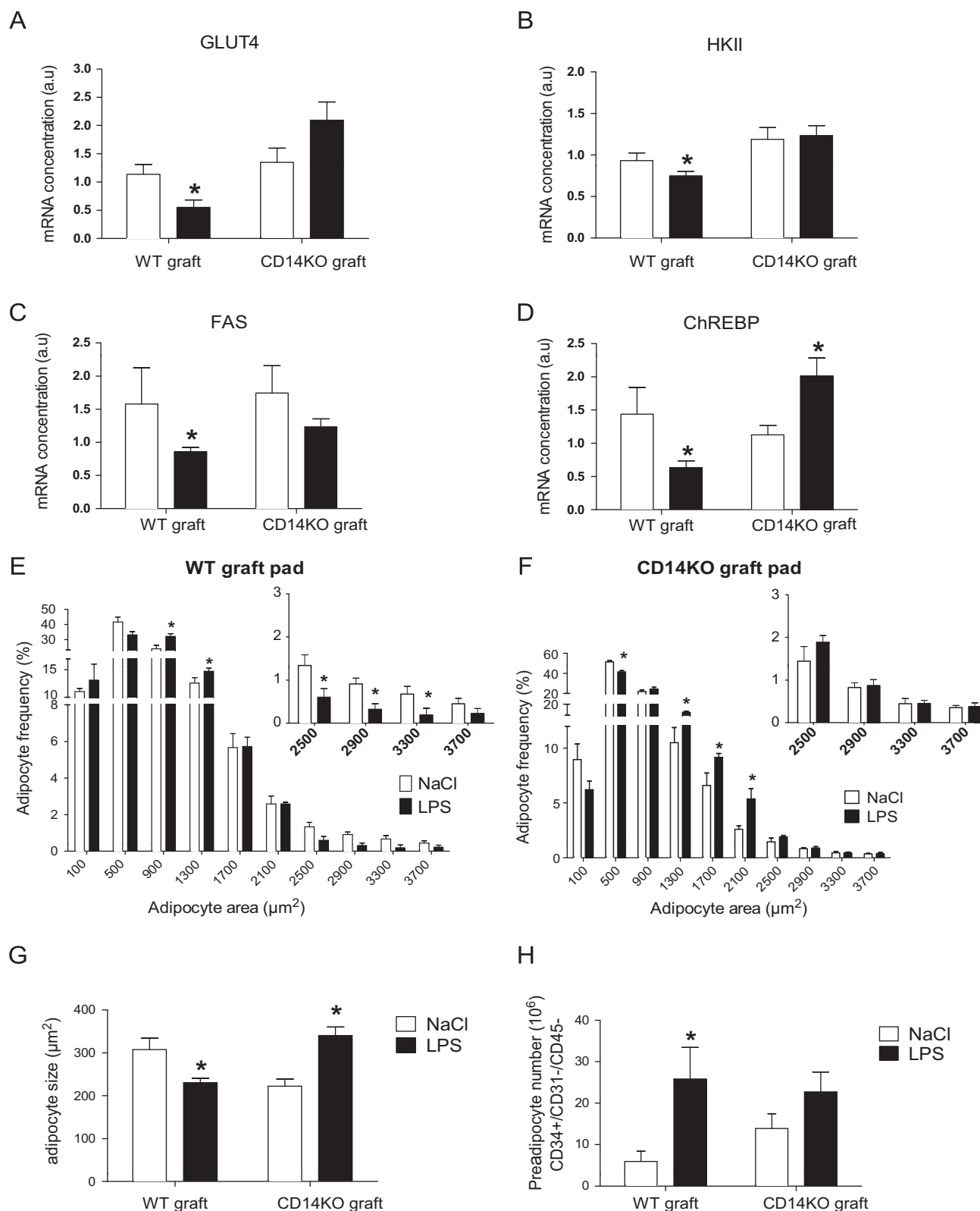
**Figure 3:** Metabolic endotoxemia directly targets adipose tissue and modifies the inflammatory status of resident CD14 depots. CD14KO mice were grafted with adipose fat pads from wild type and CD14KO mice and were infused with saline or LPS for 1 month. At the end of 4 weeks the number of macrophages (F4/80+ CD45+) contained within the stroma vascular fraction of the grafted fat pads was determined within by FACS analysis (A) and by immunohistochemistry (F4/80) (B and C) ( $n=13$  NaCl and  $n=10$  LPS). The mRNA concentrations of genes coding for inflammatory markers as PAI-1 (D), TNF (E), IL1 $\beta$  (F), MCP1 (G) and MIF (H) are evaluated. Data are means  $\pm$  SE. \* $P < 0.05$  vs NaCl as assessed by  $t$ -test.

dramatically increased in the grafted fat pads from wild type mice (Figure 4H). A small, non-significant increase was also noticed in the grafted fat pads from CD14KO mice (Figure 4H). The number of preadipocytes was slightly increased in the CD14 KO fat pad when compared to WT. We have no definitive explanation for this trend which might be due to the adaptation of the CD14KO mice to the genetic deletion in order to maintain some level of preadipocytes. Since LPS induces preadipocyte proliferation in wild type mice, it might suggest that in CD14KO mice other adaptive mechanisms occurred to overcome

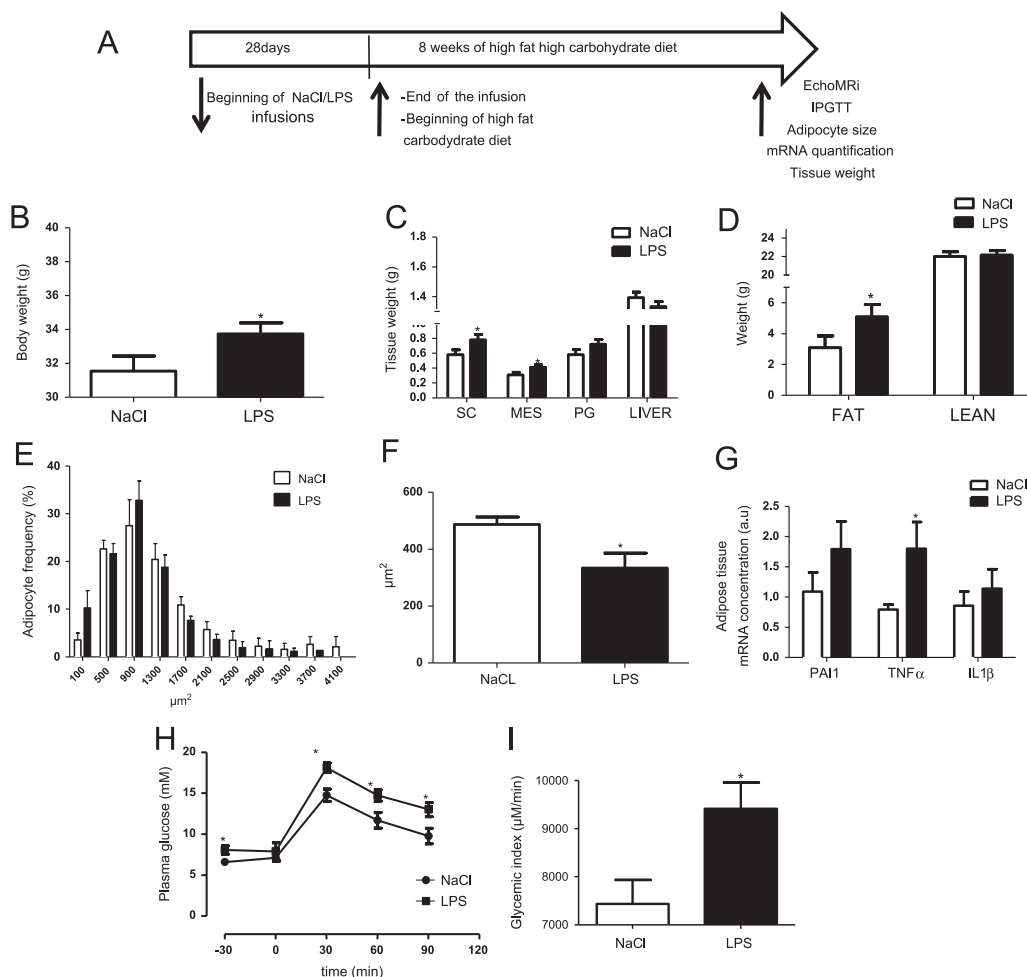
the deletion. Although, this putative mechanism is not, or very mildly, responding to LPS infusion.

### 3.5. Metabolic endotoxemia enhances high-energy diet-induced total fat mass, body weight gain and glucose intolerance

We then studied the effect of metabolic endotoxemia on the control of preadipocyte proliferation in relation to the onset of high-energy diet-induced metabolic diseases. Metabolic endotoxemia was first induced for 1 month by LPS infusion in order to prime adipose tissue and



**Figure 4:** Metabolic endotoxemia directly impacts adipose tissue metabolism. CD14KO grafted mice were infused with saline or LPS for 1 month. At the end of 4 weeks the mRNA concentrations of genes coding for metabolism markers as GLUT4 (A) HKII (B) FAS (C) ChREBP (D) is represented ( $n=13$  NaCl and  $n=10$  LPS). The quantification of the adipocyte frequency is reported according to their size ( $\mu\text{m}^2$ ) (E and F) and the mean size of grafted fat pad adipocytes is represented (G). Preadipocytes (CD34+/CD31-/CD45-) number was determined by FACS experiments (H,  $n=6$  NaCl and  $n=5$  LPS). Data are means  $\pm$  SE. \* $P < 0.05$  vs NaCl control by a  $t$ -test analyses.



**Figure 5:** LPS is a prerequisite to high-fat diet-induced obesity and glucose intolerance. (A) LPS ( $n=8$ ) or NaCl ( $n=9$ ) was infused in mice for 28 days using osmotic implantable minipumps. Mice were then fed a high-fat carbohydrate diet (HFCD) for 8 weeks before the final phenotyping assays. (B) Body weight (g), (C) subcutaneous (SC), mesenteric (MES), perigonadal (PG) adipose tissue and liver weights (g), (D) echo MRI analyses showing grams of fat or lean masses. (E) The frequency (% of total number of adipocytes counted) of subcutaneous adipocytes is represented according to the size of the adipocytes ( $\mu\text{m}^2$ ) in WT mice at the end of a 8 weeks HFCD treatment which followed the LPS or Saline (NaCl) pretreatment period. (F) represents the mean size of adipocytes counted in (E). (G) represents mRNA concentrations for adipose tissue cytokines (in arbitrary units). (H) represents intraperitoneal glucose tolerance and (I) the corresponding glycemic index calculated from  $-30$  to  $90$  min ( $\mu\text{M}/\text{min}$ ). Data are means  $\pm$  SE. \* $P < 0.05$  vs NaCl pretreatment calculated by a  $t$ -test and a post-hoc ANOVA analyses.

subsequently the mice were fed a high-energy diet (Figure 5A) for 8 weeks to trigger energy storage. Compared to non-LPS-treated controls, this treatment led to an increase in body weight (Figure 5B) as well as the weight of different adipose depots, whereas that of the liver remained unchanged (Figure 5C). This was confirmed by echo magnetic resonance imaging analysis since metabolic endotoxemia increased the fat but not the lean mass (Figure 5D). Importantly, although the size distribution of adipocytes was not significantly different between groups (Figure 5E) the mean size was lower in LPS-infused mice. The treatment was associated with an increase in inflammatory markers (Figure 5G). It is noticeable that the increase was significant for TNF $\alpha$  only whereas there was only a strong trend that did not quite reach significance for PAI1 and IL1- $\beta$ . We also observed an increase in the intolerance of the mice to glucose (Figure 5H and I).

#### 4. DISCUSSION

We here demonstrate *in vivo* that metabolic endotoxemia directly increases the proliferation of adipocyte precursors i.e. hyperplasia and

the macrophage number within the fat pad itself, through a CD14-dependent mechanism. LPS also directly reduced adipocyte differentiation. However, in the presence of an excess in energy available, such as a high-fat high-carbohydrate diet the LPS-induced adipocyte precursor proliferation mechanism favours the development of metabolic diseases. This novel finding suggests that bacterial components of intestinal origin are risk factors that trigger mechanisms which reshape adipose depots in response to changes in nutritional status and which directly involved CD14. The molecular control of this mechanism could be of great importance in protecting against obesity and diabetes.

A substantial number of publications have demonstrated the important role of intestinal microbiota in the control of adipose tissue development [6,8,35,36] and metabolic inflammation [13,27,35,37,38]. The metabolic inflammation is attributed to the increased number of macrophages within the stroma vascular fraction of the adipose depots at the onset of metabolic disease [39]. These cells could be resident, as recently suggested [30,31,40], or originate from bone marrow [21,28]. Our recent data show that the origin of tissue LPS could be linked to phagocyte-mediated translocation of bacteria from the intestine towards the adipose tissue [41]. Inflammatory bacterial components such as LPS

and peptidoglycan may then trigger proliferation of preadipocytes and macrophages in the SVF of adipose tissue. This hypothesis is further supported by recent data which show the importance of bacterial components and their receptors, such as NOD1, in relation to the control of insulin resistance and inflammation [41,42]. It has been suggested that non-adipose tissue resident cells, such as bone marrow-derived cells contribute to gut microbiota-induced metabolic inflammation [43]. Wild type mice grafted with CD14 KO bone marrow stem cells do not develop HFD-induced inflammation [43]. However, these observations could be influenced by the experimental conditions used since the irradiation process employed in the bone marrow grafting model could have impaired adipose tissue function by eliminating proinflammatory cells. The data presented here show that CD14-positive cells resident in the fat pad are sufficient to mediate metabolic inflammation. Fat pads from wild type mice grafted on to a CD14 KO background and infused with LPS are still characterized by a rise in cytokine mRNAs and the presence of F4/80 positive cells. The LPS infusion procedure leads to results that are very similar to those obtained with mice fed a high-fat diet. In addition, we have here shown that metabolic endotoxemia stimulates the proliferation of preadipocytes and macrophages leading to an increase in the total number of cells in the stroma vascular fraction. This mechanism suggests that LPS could contribute to obesity by triggering hyperplasia. The observed increase in preadipocytes is supported by a study which showed that inflammatory stimulation induces preadipocyte proliferation through a mechanism could be due to the production of Activine A by preadipocytes which activates the proliferation of the precursor cells [44]. We are tempted to speculate that the increased proliferation of preadipocytes induced by metabolic endotoxemia could lead to an increased adipocyte number. This conclusion is supported by our data which show that labeling of preadipocytes with BrdU is observed in adipocytes once adipogenesis is induced by a high-fat high-carbohydrate diet as recently suggested since adipogenesis is reduced when 3T3L1 preadipocytes are incubated with LPS [45]. The induction of hyperplasia is associated with conversely a reduction of hypertrophy since the expression of PPAR $\gamma$ , FAS, ChREBP, HKII and GLUT4 and consequently, the adipocytes is reduced. This last result, which could seem paradoxical to the development of obesity, could be considered as a risk factor of metabolic diseases, since the treatment of mice with LPS prior to a high-fat high-carbohydrate diet enhances body weight gain and glucose intolerance. Therefore, we can speculate that LPS induce preadipocyte proliferation and hence adipose tissue hyperplasia while inhibiting adipocyte differentiation. This was observed in human [46], in 3T3L1 adipocytes [45], and even brown adipocyte differentiation [47]. The impact of LPS on the triggering of body weight gain suggests that in response to an increase in nutrient, lipids/carbohydrate availability the anti-differentiation impact of LPS is overridden by the nutrients leading to hypertrophy. This is further supported by our data since after a short period of time of 1 month of HFD the number of small adipocytes was increased by the LPS treatment suggesting that the cells could be on the way to develop and enlarge further overtime. We could not here distinguish between the respective impact of LPS on macrophages and on preadipocytes on the induction of metabolic diseases. We do believe that LPS triggers adipocyte hyperplasia by inducing preadipocyte proliferation while triggering cytokine production by adipose tissue macrophages. We cannot disregard the fact that the cytokines would have also contributed to the impact on adipose tissue biology and certainly on insulin resistance. Hence, the preconditioning of adipose fat pads by LPS favours hyperplasia first to increase the risk of developing metabolic diseases. Hypertrophy would secondarily develop in response to the

increased energy available. In the light of the present results on LPS-mediated adipose tissue inflammation the ADAM17 (A Disintegrin and A Metalloproteinase 17) pathway could also be implied since this molecule is involved in the regulation of TNF $\alpha$  production [48]. This hypothesis is supported by the fact that heterozygotes knockout mice for ADAM17 partly resisted HFD-induced obesity and insulin resistance linked to a reduced MCP-1 expression, as we reported here. The molecular link between adipose tissue inflammation, LPS-induced TNF $\alpha$  and adipose tissue biology was also observed in mice where tissue inhibitor of metalloproteinase 3, the regulator of ADAM17, was manipulated [49,50]. A change in metabolic endotoxemia that could be due to changes in intestinal microbiota, as described [13,14,51,52], could be considered as an early risk factor of metabolic diseases. The direct action of LPS on adipose tissue cells was confirmed *in vitro* in primary cultures of SVF cells since LPS directly and dose-dependently induced cell proliferation through a CD14-dependent mechanism. Our results cannot rule out that CD14 negative cells outside the adipose fat pad could have infiltrated the tissue in response to LPS infusion. We could identified an increase in F4/80+/CD14- cells in the CD14 WT grafted fat pad only and in LPS infused mice when compared with saline infused mice. These cells could originate from the host. However, they are not CD14 positive therefore, demonstrating a direct action of LPS outside the fat pad does not require CD14. No increase in the cell number was observed in the CD14KO grafted fat pad in response to LPS further demonstrating that CD14 was required in the fat pad itself.

Associated with this phenotype, LPS slowed down triglyceride accumulation, most likely due to the reduction of the expression of genes involved in lipogenesis such as C/EBP $\beta$  [44]. Altogether, this mechanism could explain the reduced size of adipocytes in response to LPS and may be implicated at the onset of disease since we showed that following a prolonged high-fat feeding period the fat mass increased. LPS could increase the number of adipocyte precursors which would subsequently differentiate and favor body weight gain. This conclusion is in line with our previous results which showed that genetic deletion of CD14 in a leptin KO background reduced mesenteric adipose tissue weight and inflammatory tone [12]. The data presented here showed that LPS can precondition cells of the adipose depot, notably preadipocytes, which in turn could initiate the secondary development of the adipose depot under HFD.

The mechanisms through which LPS reach the adipose depots are still unclear but could be related to their association with lipoproteins. Indeed, lipoproteins have been shown to buffer plasma LPS [53]. Recent data suggest that dietary fat promotes the intestinal absorption of LPS from the gut microbiota [54–56]. LPS could be transported by lipoproteins [53] towards target tissues such as the liver, muscle and adipose depots and consequently contribute to various inflammatory disorders [54]. This hypothesis is supported by our data since chemokine mRNA content is increased in response to LPS or HFD in wild-type fat pads only, which could, in a second step, increase the number of proliferating and infiltrating host macrophages, further enhancing inflammation. We show here that the LPS pre-treatment is sufficient for the engagement of the adipose depot towards metabolic diseases. The increased proliferation of adipocyte progenitor cells induced by LPS preceded adipose tissue development when energy is available.

In summary, our data show that an increased plasma LPS concentration could directly trigger adipose tissue inflammation and stimulate adipocyte progenitor cell and macrophages proliferation. This CD14-dependent mechanism further demonstrates a crosstalk between gut microbiota, the innate immune system and tissues and would be one mechanism that could explain body weight gain and insulin resistance.

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## CONFLICT OF INTEREST

The authors declare having no conflict of interest.

## APPENDIX A. SUPPLEMENTARY MATERIALS

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.molmet.2013.06.005>.

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