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Modulation of the inflammatory response to LPS by the recruitment and activation of brown and brite adipocytes in mice

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Abstract.

Objectives. Numerous studies have shown that the recruitment and activation of thermogenic adipocytes, which are brown and beige/brite, reduces the mass of adipose tissue and normalizes abnormal glycaemia and lipidaemia. However, the impact of these adipocytes on the inflammatory state of adipose tissue is still not well understood, especially in response to endotoxaemia, which is a major aspect of obesity and metabolic diseases.

Methods. First, we analysed the phenotype and metabolic function of white and brite primary adipocytes in response to lipopolysaccharide (LPS) treatment *in vitro*. Then, 8-week-old male BALB/c mice were treated for one week with a β 3-adrenergic receptor agonist (CL316,243, 1 mg/kg/day) to induce recruitment and activation of brown and brite adipocytes and were subsequently injected with LPS (*E. coli* lipopolysaccharide, i.p., 100 μ g/mouse) to generate acute endotoxaemia. The metabolic and inflammatory parameters of the mice were analysed 6 hours later.

Results. Our results showed that in response to LPS, thermogenic activity promoted a local anti-inflammatory environment with high secretion of IL-1RA without affecting other anti- or pro-inflammatory cytokines. Interestingly, activation of brite adipocytes reduced the LPS-induced secretion of leptin. However, thermogenic activity and adipocyte function were not altered by LPS treatment *in vitro* or by acute endotoxaemia *in vivo*.

Conclusion. In conclusion, these results suggest an IL-1RA-mediated immunomodulatory activity of thermogenic adipocytes specifically in response to endotoxaemia. This encourages potential therapy involving brown and brite adipocytes for the treatment of obesity and associated metabolic diseases.

Keywords. brown adipose tissue; white adipose tissue; cytokines; inflammation; catecholamines.

1. Introduction

White adipocytes are specialized for the storage and release of energy (carbohydrates and lipids), while brown adipocytes dissipate this energy in the form of heat (thermogenesis) through the activity of uncoupling protein-1 (UCP1) (10). Brown adipocytes constitute brown adipose tissue (BAT) but can also be found within white adipose tissue (WAT). They are then called beige or brite adipocytes ("brown in white") and have a high thermogenesis capacity in response to conditions such as prolonged cold exposure (11).

Overweight (body mass index, BMI ≥ 25 kg/m²) and obesity (BMI ≥ 30 kg/m²) are the consequences of a positive energy balance (energetic substrate storage > expenditure) that leads to an increase in the mass of subcutaneous and visceral white adipose tissue. Obesity is a major risk factor for type 2 diabetes development, as 90% of patients suffering from type 2 diabetes are overweight or obese (16). It has also been shown that metabolic organs of obese subjects, especially WAT, are characterized by low-grade inflammation that can lead to metabolic disorders such as insulin resistance (20). Inflammation is characterized in WAT by an increase in inflammatory cytokines such as tumour necrosis factor α (TNF α), plasminogen activator inhibitor-1 (PAI-1) or interleukin-1 β and -6 (IL-1 β and IL-6). This promotes immune cell infiltration of adipose tissue, particularly infiltration of inflammatory macrophages (29, 30). Immune cell accumulation and secretion of inflammatory cytokines affect adipose tissue homeostasis and, more specifically, the recruitment and function of adipocytes in WAT and BAT (32). Previous studies have shown that TNF α inhibits adipocyte differentiation (21) and that IL-1 β blocks insulin signalling, thus favouring insulin resistance (22). Recently, it has also been shown that IL-1 β and TNF α affect the thermogenic function of brown adipocytes (18, 34, 39). Altogether, these studies showed that inflammatory cytokines participate in the development of an inflammatory environment, leading to the deregulation of adipose tissue homeostasis. However, the origin of low-grade metabolic inflammation is still under debate. Growing evidence

indicates that the gut microbiota is a major player in metabolic inflammation. The gut of obese patients is characterized by an alteration in the microbiota (dysbiosis) and a disruption of the intestinal barrier, increasing its permeability to microbiota metabolites and degradation products. Consequently, systemic endotoxin levels increase (endotoxaemia), including the levels of lipopolysaccharide (LPS) (46). It has been demonstrated using germ-free rodent models that this endotoxaemia is directly linked to adipose tissue inflammation (6, 7, 49). In this pathophysiological condition, LPS directly activates Toll-like receptor 4 (TLR-4), which is displayed by tissue macrophages and adipocytes (42). In response, white and brown/beige adipocytes exhibit an altered function (33, 34) and secrete inflammatory cytokines (TNF α and IL-1 β), which alter tissue homeostasis. Recently, it has been shown that brown and white adipocytes respond differently to *in vitro* LPS stimulation and mediate different inflammatory responses (13).

One of the strategies for treating type 2 diabetes associated with obesity is to increase energy expenditure by stimulating the recruitment and activity of brown and/or beige adipocytes (27). While little is known about the impact of this strategy on the balance and functionality of the immune system, it is a key element in the pathology of metabolic syndrome, especially in low-grade inflammation and inherent insulin resistance. In this work, we investigated the response of white and brown/beige adipocytes to LPS treatment with regard to their impact on the inflammatory environment induced by LPS. Using *ex vivo* and *in vivo* approaches in mice, we demonstrated that recruitment/activation of brown/beige adipocytes by β -adrenergic receptor agonists was not affected by LPS treatment and led to a reduced inflammatory response to LPS, especially by the overexpression of an IL-1 receptor antagonist (IL-1RA), coded by Il-1rn gene, that is known to inhibit the action of IL-1 β .

2. Materials and Methods

2.1. Reagents

Culture media and buffer solutions were purchased from Lonza (Ozyme, St-Quentin en Yvelines, France), foetal bovine serum (FBS) was purchased from Eurobio (Courtaboeuf, France), and insulin was purchased from Invitrogen (Cergy Pontoise, France). LPS (LPS-EK Ultrapure isolated from *E. coli* K12 strain) was obtained from InvivoGen (Toulouse, France). Other reagents were obtained from Sigma-Aldrich (Saint-Quentin Fallavier, France).

2.2. Animals

The experiments were conducted in accordance with the French and European regulations (2010/63/EU directive) for the care and use of research animals and were approved by national experimentation committees (MESR No.: APAFIS#18322-2018121809427035 v2). Eight-week-old male BALB/c mice from Janvier Laboratory (France) were maintained at housing temperature (22°C) on a 12:12-hour light-dark cycle, with *ad libitum* access to food and water.

The mice were treated daily with the β 3-adrenergic receptor agonist CL316,243 (1 mg/kg in saline solution, intraperitoneal injection, n=12) (Sigma-Aldrich) or with vehicle only (saline solution, n=12). To induce endotoxaemia, 6 mice from each group were injected at day 7 with LPS (100 μ g/mouse in PBS, intraperitoneal) or vehicle only and were sacrificed 6 hours later alternating one mouse from each group. At the end of the experiment, blood, interscapular brown adipose tissue (iBAT), epididymal white adipose tissue (eWAT) and inguinal subcutaneous white adipose tissue (scWAT) were sampled and used for different analyses. No mice have been excluded.

2.3. Cytokine and metabolic parameter quantification

For blood analysis, freshly prepared plasma was diluted twice before analysis. For tissue analysis, freshly sampled WAT and BAT were washed in PBS, weighed and incubated in free Dulbecco's modified Eagle's medium for 2 hours at 37°C. The media were preserved for analysis of various secreted proteins.

Leptin was assayed using a mouse leptin kit (Meso Scale Discovery, # K152BYC), and cytokines were measured using a mouse V-PLEX pro-inflammatory panel 1 kit (# K15048D) according to the manufacturer's instructions using a QuickPlex SQ 120 apparatus (Meso Scale Discovery, Rockville, Maryland, USA). IL-1RA levels were assayed using a mouse IL-1RA ELISA kit (#EMIL1RN) from Thermo Fisher Scientific (Courtaboeuf, France). Glycerol and triglyceride determinations were performed using a dedicated kit (free glycerol reagent and triglyceride reagent, Sigma Aldrich).

2.4. Histology

Freshly sampled tissues were fixed in 4% paraformaldehyde overnight at room temperature (RT) and then paraffin-embedded. Embedded tissues were cut into 5 µm sections and dried overnight at 37°C.

For histological analysis, the sections were stained with haematoxylin-eosin and mounted in vectamount (Vecto laboratories).

For immunohistochemical analysis, antigen retrieval was performed in buffer low pH in a de-cloaking chamber (Dako, S2367). The sections were then permeabilized in PBS with 0.2% Triton X-100 at room temperature for 10 min and blocked in the same buffer containing 3% BSA for 1 hour. The sections were incubated with rat anti-F4/80 antibody (Biorad, clone Cl:A3-1, dilution 1:100) overnight at 4°C. Following a 1 hour incubation with A568-coupled anti-rabbit secondary antibodies, nuclear staining was performed with DAPI, and the sections were mounted in PermaFluor mounting Media (Thermofisher).

Visualization was performed with an Axiovert microscope. Images were captured using AxioVision software (Carl Zeiss, Jena, Germany).

2.5. *Stromal vascular fraction cell isolation and culture*

Subcutaneous WAT (scWAT, inguinal) and interscapular BAT (iBAT) deposits were sampled from 8-week-old BALB/c male mice, washed in PBS, and minced. Adipose tissue samples were digested for 45 min at 37°C in DMEM containing 2 mg/ml collagenase A (Roche Diagnostics, Meylan, France) and 20 mg/ml bovine serum albumin (Sigma-Aldrich Chimie, Saint-Quentin Fallavier, France). The sample was successively filtered through 250, 100 and 27 µm nylon sheets and centrifuged for 5 min at 500 g. The pellet containing stromal vascular fraction (SVF) cells was subjected to red blood cell lysis.

SVF cells were plated and maintained in DMEM containing 10% Foetal Calf Serum until confluence. Differentiation was induced in the same medium supplemented with 1 µM dexamethasone, 0.5 mM isobutylmethylxanthine and 860 nM insulin for two days. Then, the cells were maintained for 7 days in the presence of 860 nM insulin for white adipogenesis and 860 nM insulin, 1 µM rosiglitazone and 2 nM triiodothyronine for brite and brown adipogenesis as previously published (36). Media were changed every other day. On the final day, the differentiated preadipocytes were stimulated with or without 1 µM isoproterenol for 6 hours.

2.6. *Isolation and analysis of RNA*

Procedures were performed according to MIQE recommendations (5). Total RNA was extracted using a TRI-Reagent kit (Euromedex, Souffelweyersheim, France) according to the manufacturer's instructions. For RNA isolation from organs, tissues were homogenized in TRI-Reagent using a dispersing instrument (ULTRA TURRAX T25). Reverse transcription-polymerase chain reaction (RT-PCR) was performed using M-MLV-RT (Promega). SYBR qPCR premix Ex Taq II from Takara (Ozyme, France) was used for quantitative PCR (qPCR), and

assays were run on a StepOne Plus ABI real-time PCR instrument (PerkinElmer Life and Analytical Sciences, Boston). The expression of selected genes was normalized to that of the TATA-box binding protein (TBP) and 36B4 housekeeping genes and then quantified using the comparative- Δ Ct method. Primer sequences are available upon request.

2.7. *Oxygen consumption analysis*

The oxygen consumption rate (OCR) and extra-cellular acidification rate (ECAR) of 10-day-old differentiated SVF cells was determined using an XF24 Extracellular Flux Analyser (Seahorse Bioscience, Agilent Technologies France, Courtaboeuf, France). Isoproterenol (1 μ M) and/or LPS (100 ng/mL) were used to characterize inducible respiration. Uncoupled and maximal OCR were determined using oligomycin (1.2 μ M) and carbonyl-cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP, 1 μ M). Rotenone and antimycin-A (2 μ M each) were used to inhibit mitochondrial respiration. All parameters were calculated as described previously (4).

2.8. *Statistical analysis*

Animal cohort size was determined using G*Power (14), and animals were allocated to experimental groups by randomization. The data were analysed using GraphPad Prism 6 software and evaluated by ordinary one-way ANOVA followed by Tukey's multiple comparisons post-test to assess significant differences between experimental groups. Differences were considered statistically significant with $p < 0.05$. The data are displayed as scatter plots of independent values and group mean values $\pm$ SD.

3. Results

3.1. *In vitro* phenotypic and functional metabolic response of white, brown and brite differentiated preadipocytes to LPS treatment

White and brite adipocytes were obtained after differentiation of preadipocytes that were isolated from the stromal vascular fraction of mouse subcutaneous white adipose tissue, and brown adipocytes from stromal vascular fraction of mouse brown adipose tissue, as previously published (36). On the last day of differentiation, the differentiated pre-adipocytes were treated for 6 hours with 1 μ M isoproterenol (a nonspecific β -adrenergic receptor agonist that activates lipolysis and/or UCP1 activity) and/or 100 ng/ml LPS and were then used for mRNA expression (Figure 1), or treated acutely with the same compounds for functional metabolism modification analysis (Figure 2).

LPS treatment did not alter perilipin 1, adiponectin and Glut4 mRNA expressions in all kind of adipocytes. Leptin mRNA was scarcely detected in all adipocytes limiting our interpretation about the strong inhibition found in white adipocytes under LPS and isoproterenol treatments (Figure 1). White adipocytes barely expressed Ucp1 and perilipin 5 mRNA, and LPS and isoproterenol did not alter these expression levels. In brite adipocytes, perilipin 5 which was slightly inhibited by LPS treatment independent of isoproterenol. Interestingly, LPS alone did not alter Ucp1 expression in brite adipocytes but blunted the increase in Ucp1 mRNA expression due to acute isoproterenol treatment (Figure 1). In brown adipocytes, perilipin 5 and Ucp1 mRNA levels were increased by isoproterenol but unaffected by LPS. Interestingly, in all kinds of adipocytes Ppar γ (peroxisome proliferator-activated receptor γ) mRNA levels were inhibited by LPS and by isoproterenol treatment (Figure 1).

Analysis of Tnf α and Il-6 mRNA levels showed that they were overexpressed after acute LPS treatment. Interestingly, isoproterenol completely inhibited Tnf α overexpression in the three kinds of adipocytes. A more complex situation was found for Il-6 mRNA, which was positively

affected by the combination of isoproterenol and LPS treatment in white adipocytes but inhibited by the same cotreatment in brite and brown adipocytes (Figure 1). Analysis of IL-1 β mRNA expression showed an increase after LPS treatment of adipocytes. Isoproterenol did not alter this expression except when white adipocytes were treated with both isoproterenol and LPS. As IL-1 β mRNA encodes the pro-form of the protein before its maturation and secretion, these results need to be analysed with caution. mRNA expression of IL-1rn, which codes for the IL-1RA protein that counteracts the effect of IL-1 β , was increased after LPS treatment, similar to that of IL-1 β mRNA. Interestingly, brite adipocytes expressed more IL-1rn than white and brown adipocytes. This expression in brite adipocytes was increased by LPS treatment and, more importantly, by isoproterenol with or without LPS (Figure 1).

At the metabolic level, isoproterenol treatment induced glycerol release (a reflection of lipolysis) in the three types of adipocytes, but cotreatment with LPS had no effect (Figure 2A). Interestingly, LPS slightly but significantly increased glycerol release in white, brite and brown adipocytes. The extracellular acidification rate (ECAR) (Figure 2B) and oxygen consumption rate (OCR) (Figure 2C) were analysed concomitantly and were increased by acute isoproterenol treatment in brite and brown adipocytes, while only the OCR was increased in white adipocytes. Uncoupling mitochondrial oxygen consumption (the OCR due to proton leakage) was increased in brite and brown adipocytes after isoproterenol stimulation, which is a reflection of Ucp1 activity (Figure 2C). Neither ECAR nor OCR was affected by LPS treatment (Figure 2B-C).

Taken together, these *in vitro* adipocyte experiments suggest that activation of adipocyte β -adrenergic receptors decreased the inflammatory phenotype and that LPS treatment did not clearly affect the thermogenic phenotype.

3.2. *In vivo effects of acute LPS treatment on browning and BAT activation induced by the β 3-adrenergic receptor agonist (CL316,243)*

Eight-week-old male BALB/c mice were treated daily with a β 3-adrenergic receptor agonist (CL316,243, 1 mg/kg) that activates brown and brite adipocyte thermogenesis and adipocyte lipolysis and were finally treated with 100 μ g of LPS for 6 hours. Body weight was unaffected by treatment with CL316,243 or LPS (Figure 3A). Different weights of epididymal WAT showed that fat mass was decreased after CL316,243 treatment but was unaffected by LPS treatment (Figure 3A). While the plasma glycerol level did not change, we found a decrease in plasma triglyceride levels, probably due to the high use of this substrate by activated brown and brite adipocytes (Figure 3B). Interestingly, LPS treatment also decreased triglyceride levels, but no additive effect was found in the cotreated mouse group (Figure 2B). Leptin plasma levels increased in LPS-treated mice, and the effect was blunted when the mice were pre-treated with CL316,243 (Figure 3C). The same kind of results were found for secreted leptin using scWAT explants sampled from these mice (Figure 3C). Thus, in our experiment, leptin levels did not follow the fat mass of the animals and corresponded to an adipose tissue inflammatory response to LPS.

To assess the effects of acute LPS treatment on brown and brite adipocyte recruitment and activation, we performed histological and molecular analyses of WAT and BAT from mice in each group. As shown in Figure 3D, CL316,243 decreased quantity of lipid droplets in brown adipose tissue and slightly increased Ucp1 and Cpt1m (Carnitine O-Palmitoyltransferase 1M) mRNA expression, which is characteristic of activated BAT, without affecting Perilipin 1 and 5 expressions as well as adiponectin, Glut4 (glucose transporter 4) and Ppar γ mRNA expressions (Supplementary Figure 1A). scWAT histological sections from CL316,243-treated groups displayed massive recruitment of multiloculated adipocytes (Figure 3D), which is a characteristic morphology of brite adipocytes. This was confirmed by the overexpression of Ucp1 and Perilipin 5 mRNA (Supplementary Figure 1B).

Perilipin 1, adiponectin and Leptin mRNA expression in scWAT did not change with LPS and/or CL316,243 treatment. This discrepancy between leptin secretory levels and mRNA

expression supports an acute response independent of fat mass change (Supplementary Figure 1B). Differently to Glut4 mRNA which was increased after isoproterenol treatment and less affected by LPS treatment, Ppar γ mRNA levels were strongly inhibited by LPS independently to isoproterenol exposition (Supplementary Figure 1B). Interestingly, we did not detect cell infiltration or crown structure after LPS treatment by histological analysis (Figure 3D), which was confirmed by negative staining for the macrophagic marker F4/80 (data not shown). Certainly, the time was too short between LPS treatment and analysis to allow infiltration of adipose tissue by immune cells.

3.3. *Acute LPS treatment induces an inflammatory response and secretion of anti-inflammatory cytokines.*

Inflammation is mainly triggered by inflammatory cytokine secretion, followed by a resolution step that is characterized by secretion of anti-inflammatory cytokines. This effect was observed in mice that were treated with LPS. Terminal LPS treatment induced an increase in the plasma level of IL-1 β , as well as IL-1 β secretion in BAT and scWAT explants, and CL316,243 did not alter this effect (Figure 4A). IL-1RA plasma levels equivalently increased after administration of LPS to untreated and CL316,243-treated mice (Figure 4B). In contrast, in scWAT and BAT, while LPS treatment increased IL-1RA secretion, CL316,243 also increased IL-1RA secretion, and this effect was additive in the presence of LPS (Figure 4B).

In addition to IL-1 β /IL-1RA, other cytokine levels were affected. TNF α and IL-12 plasma levels were increased after LPS treatment independent of CL316,243 treatment, and the same trend was found for IL-6 (Supplementary Figure 2). KC/GRO (CXCL-1) was detected in the plasma of mice but was not affected by the different treatments. Interestingly, LPS-induced IFN γ and IL-2 plasma levels increases were abrogated when the mice were treated with CL316,243 (Supplementary Figure 2).

A different profile of secreted cytokines was found in the adipose tissue explant media. IFN γ was undetected, and IL-2 and IL-12 were barely detected (Figure 5), with a slight increase only in the iBAT of LPS-treated mice (Figure 5A). Secreted IL-6 increased in the iBAT of LPS-treated mice independent of CL316,243 treatment and only in LPS plus CL316,243 in scWAT (Figure 5). In contrast, KC/GRO and TNF α increased in all groups of LPS-treated mice independent of CL316,243 treatment and had the same trend (Figure 5).

Although the anti-inflammatory cytokine IL-4 was barely detected in plasma and undetected in explants of adipose tissues, the plasma level of IL-10 was increased in response to LPS (Supplementary Figure 3). None of these were altered by CL316,243. In contrast, local secretion of IL-10 by adipose tissue was not stimulated by LPS alone, but an increasing trend was observed after CL316,243 treatment, especially in BAT that was cotreated with LPS (Supplementary Figure 3).

4. Discussion

Metabolic endotoxaemia, found for example in obese patients and sepsis, appears after gut epithelium disturbance which allows LPS from gram-negative gut bacteria to enter the systemic circulation (8, 24). This leads to systemic and local inflammation, which is among others a central feature of metabolic syndrome development (6). Several studies have shown the action of LPS on white and brown adipocyte differentiation and function. LPS modulates adipocyte functions directly via TLR-4 activation or indirectly, especially through macrophage activation which in turn secrete cytokines that modulate adipocyte functions. In WAT, LPS directly induces lipolysis (51) and cytokine secretion by adipocytes and indirectly (mainly by macrophage-derived cytokines) inhibits differentiation and promotes insulin resistance (41, 50). In BAT, LPS has been suspected to be an activator of brown adipocyte thermogenesis (9), but recent studies demonstrated that acute (*in vitro*) and chronic (*in vivo*) exposure to LPS inhibits

UCP1 expression and function in these adipocytes (1, 33). However, little is known about the influence of white and brite or brown adipocytes on local and systemic inflammation due to LPS exposure, especially the secretion of inflammatory mediators such as cytokines and adipokines. Herein, we analysed the impact of brown and brite adipocyte recruitment and activation on systemic and local (adipose tissue) responses to acute LPS treatment, which mimics endotoxaemia.

Leptin is a well-known modulator of pyrexia in response to LPS (15, 37, 38). We found an expected increase in leptin secretion in plasma that correlated with scWAT leptin level, a chief origin of this adipokine with others adipose tissues (25), in mice that were stimulated with acute LPS treatment. This positive effect was not found at the mRNA level in the scWAT of these mice or in primary white adipocytes that were exposed to LPS. These results suggested that the modulation in leptin levels were not due to change in adipose tissue mass, but that LPS stimulated adipocyte leptin secretion without affecting its mRNA expression. Interestingly, these increased leptin levels were completely abolished when the mice were pre-treated with CL316,243, which induces brown/brite adipocyte recruitment and activation and thus thermogenesis. As leptin induces fever independently of thermogenesis (15), we suspect negative feedback that prevents hyperthermia due to the addition of thermogenesis and pyrexia. Unfortunately, leptin levels cannot be related to fever, as body temperature was not available in our work. Inconsistently, numerous studies have shown an inhibitory effect of chronic LPS treatment on Ucp1 expression and function *in vivo* and thus on thermogenesis (33). In our work, we showed that LPS did not modify Ucp1 expression or the histology of BAT and scWAT after CL316,243 treatment, strongly suggesting that LPS does not affect thermogenesis. The difference between our results and those of Okla and colleagues is probably due to the duration of LPS treatment (6 hours vs. 2 weeks) (33). This was confirmed by an *in vitro* experiment on brite adipocytes showing that LPS slightly inhibited Ucp1 mRNA expression due to isoproterenol treatment but did not modify Ucp1 function, as demonstrated by oxygen consumption analysis. It

is possible that a difference exists between brite and brown adipocytes, as LPS inhibits Ucp1 function in brown adipocytes *in vitro* (1).

As mentioned previously, LPS is a known inducer of lipolysis which is confirmed in our *in vitro* experiment (51). In the same way, thermogenic activity induced by CL316,243 treatment involved a sustained lipolysis supplying fatty acids as energy substrate to brown and brite adipocytes and releasing glycerol in circulation (47). *In vivo*, we did not detect any increase in glycerol plasma level both after LPS and CL316,243 treatment. While the short treatment time with LPS can explain this, it is not the case for chronic CL316,243 treatment. We hypothesized that, in this mouse strain, a higher uptake of glycerol by liver or an active glycerol recycling by adipocytes, could limit level of circulating glycerol in the blood (3, 35).

Dowal and colleagues used coculture of human adipocytes and macrophage cell lines to show that brown adipocytes reduced macrophage IL-6 secretion in response to LPS compared to that of white adipocytes (13). Under similar conditions, mRNA expression of other inflammatory markers, including IL-10, in macrophages was inhibited by brown adipocytes. These results suggest that brown adipocytes and perhaps brite adipocytes display general immunomodulatory properties of interest. Our *ex vivo* analysis showed the same results, with decreased mRNA expression of IL-6 in brite adipocytes compared to that in white adipocytes and to a lesser extent for TNF α . We hypothesised that the inhibition of inflammatory cytokine expression in brown adipocytes participates in its immunomodulatory activity on macrophages. Unfortunately, this inhibition of pro-inflammatory and anti-inflammatory cytokine levels was not confirmed *in vivo*. Control mice and mice that were pre-treated with CL316,243 displayed an equivalent increase in the secretion of TNF α and IL-6 after LPS treatment. The same results were obtained for other pro- and anti-inflammatory cytokines in plasma and adipose tissue, except for IFN γ and IL-2 plasma levels, which were strongly decreased in the CL316,243 group after LPS treatment. As no IFN γ and very low quantities of IL-2 were found in BAT and scWAT, we can exclude the involvement of these tissues in this immunomodulatory effect. Moreover, the

discrepancies between plasma and adipose tissue cytokine levels demonstrated that the local production of cytokines found in the adipose tissue had a limited impact on systemic inflammatory response to LPS. Nevertheless, in addition to cytokines, oxygenated derivatives of ω 6 polyunsaturated fatty acids (n-2 series prostaglandins and n-4 series leukotrienes) and of ω 3 polyunsaturated fatty acids (n-3 series prostaglandins, n-5 series leukotrienes, resolvins (Rv) and protectins) are highly synthesized and secreted by adipocytes, and participate in the inflammatory and resolutive state of immune response (19, 31). As we have demonstrated previously that recruitment and activation of brown and brite adipocytes modulate these syntheses (12, 17), we can hypothesize that these metabolites could participate to local, and even systemic, inflammatory response to LPS of the adipose tissue.

Along pro-inflammatory cytokines, IL-1 β has a central role in the immune response to LPS and in adipose tissue homeostasis (45). More specifically, IL-1 β secretion induced by LPS treatment disrupts insulin signalling in WAT (2, 22, 26) and inhibits UCP1 function in BAT both *in vitro* and *in vivo* (9, 18). As expected, we found that LPS treatment induced IL-1 β production in all conditions assayed, as shown by mRNA expression in white and brite adipocytes *in vitro* and secretion of the mature form of IL-1 β in BAT and scWAT *in vivo*. In addition, we showed that recruitment and activation of brown and brite adipocytes did not modulate IL-1 β levels and thus did not seem to prevent inflammatory action of this cytokine. IL-1 β is mainly regulated at the expression, maturation and secretion levels, especially by modulation of the NF-kB transcription factor and activation of inflammasomes. Another pathway exists to modulate IL-1 activity, which is the production and secretion of IL-1RA. This cytokine antagonizes the biological effects of IL-1 β by competing for binding to the IL-1 receptor without inducing a cellular response (43). Moreover, IL-1RA is highly produced by adipose tissue (23). Interestingly, we demonstrated that IL-1rn mRNA was more highly expressed in brite adipocytes *in vitro* than in white adipocytes and that this difference was amplified when the cells were treated with a β -adrenergic receptor agonist. Importantly, these observations were confirmed *in vivo*, where CL316,243 treatment

increased IL-1RA levels in WAT and BAT, with or without LPS treatment. In contrast, plasma levels of IL-1RA increased only in the presence of LPS and independently of CL316,243 treatment. Taken together, these results clearly demonstrate that brite and brown adipocytes express and/or secrete more IL-1RA than white adipocytes, especially in response to acute LPS treatment and certainly *via* direct activation of TLR4 pathway. In addition to a potential anti-inflammatory role of IL-1RA, we hypothesise that the simultaneous secretion of IL-1RA and IL-1 β corresponds to a protective mechanism to preserve adipocyte function in an inflammatory context.

Increased IL-1 β or decreased IL-1RA levels are linked to the development of obesity and diabetes, and among therapeutic strategies developed to normalize these levels, some studies have focused on the use of exogenous IL-1RA (44, 45). In rodents, chronic IL-1RA treatment of mice that were fed a high-fat/high-sucrose diet prevented glucose metabolism alteration and normalized the metabolic parameters linked to obesity without affecting fat mass (40). In addition, using IL-1RA-overexpressing mice, we detected a normalization of inflammatory marker mRNA in the WAT of mice fed a high-fat diet (40). In humans, several clinical trials using anakinra (recombinant human IL-1RA) have been developed with obese and diabetic patients and have shown, among others, better pancreatic function and decreased systemic inflammation after treatment (28, 48). As we demonstrated that IL-1RA adipose levels are increased after β -adrenergic receptor agonist treatment, we suggest that increasing brown/brite adipocyte recruitment and activity in humans, in addition to decreasing fat mass, protects adipose tissue from the adverse effects of IL-1 β .

Our study was performed in lean BALB/c mice at ambient temperature. Thus, i) we cannot exclude that the results displayed herein was inherent to the model chosen; ii) we have a basal thermogenic activity due to ambient temperature which could limit the difference between CL316,243 treated and untreated mice and it will be interesting to reproduce this work at thermoneutrality to match more the human situation; iii) as we have decided to develop our

431 study on lean mice in order to exclude any additional effect of obesogenic environment, it will be
432 of interest to transpose now this study in an obese mouse model mimicking human situation
433 where endotoxaemia is mainly found.

434 Finally, we have demonstrated that recruitment and activation of brown and brite
435 adipocytes in the adipose tissue of mice led to a local anti-inflammatory phenotype characterized
436 by an increased IL-1RA level and decreased leptin secretion in response to endotoxaemia
437 without modulation of systemic inflammation.

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439

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Competing Interests. The authors declare no conflict of interest.

Legends.

Figure 1. Effect of LPS on white, brite and brown differentiated preadipocytes *in vitro*.

White and brite adipocytes were obtained after differentiation of stromal vascular fraction cells isolated from mouse scWAT, and brown adipocytes from stromal vascular fraction of iBAT. mRNA expression analysis by qPCR of white (leptin, perilipin 1, Pparg, Glut4 and Adiponectin), brite/brown (Ucp1 and perilipin 5) and inflammatory markers (Tnf α , Il-6, Il-1 β , and Il-1rn) after 6 hours of treatment with isoproterenol (1 μ M) and/or LPS (100 ng/ml). The results are displayed as the mean $\pm$ SEM. n = 6 (2 replicates of 3 independent cultures). * p <0.05 vs. control; \$ p <0.05 vs. LPS; § p <0.05 vs. isoproterenol; £ p <0.05 vs. white adipocytes.

Figure 2. Effect of LPS on white, brite and brown differentiated preadipocytes *in vitro*.

White and brite adipocytes were obtained after differentiation of stromal vascular fraction cells isolated from mouse scWAT, and brown adipocytes from stromal vascular fraction of iBAT. (A) Glycerol levels were assayed in the supernatants of white and brite adipocytes that were treated for 2 hours with LPS and/or isoproterenol. (B-C) Adipocytes were analysed for oxygen consumption and extracellular acidification following sequential injection of isoproterenol and/or LPS, oligomycin A (1.2 μ M) and rotenone/antimycin A (2+2 μ M). (B) ECAR in response to LPS and/or isoproterenol is displayed as % of basal ECAR. (C) Plots show mitochondrial OCR and the indicated injections. Histograms display mitochondrial OCR in response to LPS and/or isoproterenol (induced/basal mitochondrial OCR) and uncoupling mitochondrial respiration. The results are displayed as the mean $\pm$ SEM. n = 6 (2 replicates of 3 independent cultures). * p <0.05 vs. control; \$ p <0.05 vs. LPS; § p <0.05 vs. isoproterenol; £ p <0.05 vs. white adipocytes.

Figure 3. Effect of CL316,243 and LPS on general mouse metabolic parameters.

Mice were analysed after 1 week of treatment with CL316,243 daily (1 mg/kg/day) or vehicle only (NaCl), and with or without LPS treatment (1 mg/kg) during the final 6 hours. (A) Mouse body weights,

epididymal white adipose tissue (eWAT) weights, (B) triglycerides and glycerol plasma levels and (C) secreted leptin levels in plasma and subcutaneous white adipose tissue (scWAT). (D) Haematoxylin-eosin staining of interscapular BAT (iBAT), scWAT and eWAT sections. The results are displayed as independent values (dots) and the mean $\pm$ SE. n = 6 (mice and plasma) or 8 (explant). * $p < 0.05$.

Figure 4. Effect of CL316,243 and LPS on IL-1 β /IL-1RA. IL-1 β (A) and IL-1RA (B) protein levels were assessed in plasma (upper panel) and in the media of iBAT (middle panel) and scWAT (lower panel) explants from mice that were treated for 1 week with CL316,243 daily (1 mg/kg/day) or vehicle only (NaCl), and with or without LPS treatment (1 mg/kg) for the final 6 hours. The results are displayed as independent values (dots) and the mean $\pm$ SE. n = 6 (plasma) or 8 (explant). * $p < 0.05$.

Figure 5. Local inflammatory response to LPS. IFN γ , TNF α , IL-6, IL-12-p70, KC/GRO (CXCL-1) and IL-2 levels were assessed in the media of iBAT (A) and scWAT (B) explants from mice that were treated for 1 week with CL316,243 daily (1 mg/kg/day) or vehicle only (NaCl), and with or without LPS treatment (1 mg/kg) for the final 6 hours. The results are displayed as explant values (dots) and the mean $\pm$ SE. n = 8. * $p < 0.05$.

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Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5.

A iBAT

B scWAT