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Profiling of the Three Circulating Monocyte Subpopulations in Human Obesity

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Three subpopulations of circulating monocytes have been described: CD14+CD16− (classical monocytes [CM]), CD14+CD16+ (intermediate monocytes [IM]), and CD14−CD16+ (nonclassical monocytes [NCM]). We previously showed that obesity is associated with an increased proportion of IM and NCM. Our objective is to decipher the migratory and inflammatory functions of each monocyte subset in obesity-related low-grade inflammation. Twenty-six healthy, normal-weight and nonobese volunteers (C) and 40 obese nondiabetic (Ob) individuals were included in this study. We explored the gene expression profile of 18 inflammatory genes in each subset of C and Ob subjects and measured protein expression of the upregulated genes. We then tested their functional response to TLR signaling in both groups. We showed an increased expression of CX3CR1 in all monocyte subpopulations and of CCR2 and CCR5 in CM and IM in the Ob group. We found negative correlation between CCR2 and CX3CR1 expressions and high-density lipoprotein-cholesterol, whereas CCR5 expression was positively linked to obesity-related metabolic traits. Production of inflammatory proteins upon bacterial LPS and viral ssRNA stimulation was higher in CM and NCM of the Ob group compared with the C group. Our work highlights an enhanced inflammatory phenotype of monocytes with a higher response to TLR4 and TLR8 stimulations in obesity. Moreover, it suggests an increased migration capacity of CM and IM subpopulations.

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Obesity is an ongoing worldwide epidemic now recognized as a low-grade inflammatory disease characterized by increased concentrations of a large panel of cytokines, chemokines, and acute-phase proteins in the circulation (1, 2). Obesity-related inflammation favors the development of metabolic and cardiovascular complications (3, 4). This systemic inflammation results, at least in part, from activation of circulating immune cells (5), vascular endothelium (6), and excessive accumulation of inflammatory cells in adipose tissue (7). Monocytes, acting critically in several inflammatory diseases, have been recently categorized in three subsets according to the expression of LPS (CD14) and the FcγRIII (CD16) receptors (8, 9). The

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The online version of this article contains supplemental material.

Abbreviations used in this article: BMI, body mass index; C, normal-weight and nonobese volunteer; CM, classical monocyte; FMO, fluorescence minus one; HDL-c, high-density lipoprotein-cholesterol; HS, human serum; IM, intermediate monocyte; MFI, mean fluorescence intensity; NCM, nonclassical monocyte; Ob, obese nondiabetic.

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Materials and Methods

Subjects

Two groups of subjects were included in this study (Table I). The first group included 40 obese nondiabetic (Ob) individuals involved in a bariatric surgery program and recruited to the nutrition division of Pitié-Salpêtrière Hospital (Reference Center for the Medical and Surgery Care of Obesity, Institute of Cardiometabolism and Nutrition, ICan La Pitie-Salpêtrière Hospital, Paris, France). Patients met the criteria for bariatric surgery as described earlier (12). The second group comprised 26 healthy normal-weight and nondiabetic volunteers (C), living in the same region as the Ob subjects. The Ethics Committee (CpP Ile de France 1) approved the clinical investigations for both Ob and C individuals. All subjects gave written informed consent. The study was conducted in accordance with the Declaration of Helsinki and was registered in a public trial registry (NCT00476658 and NCT01655017).

For each of the experiments, we used fasting blood samples from only part of the two groups. For ethical reasons, blood withdrawal for monocyte isolation was restricted to a maximum of 10 ml per patient. This resulted in isolation of 1 × 10⁷ to 4 × 10⁸ monocytes per patient, which limited the number of IM and NCM after FACs sorting, and experimental design was adapted accordingly (Supplemental Fig. 1).

Mononuclear cell isolation and FACs sorting of monocyte subpopulations

PBMCs were isolated from blood by centrifugation on a Ficoll-Hypaque gradient (PAO Laboratories). Monocytes were then enriched by a centrifugation on a Hyper-osmotic Percoll® Gradient (GE Healthcare) (18). Monocyte-enriched cell fraction was further subdivided by FACs. For the gene expression experiment, cells were stained with allolymphocytin-CD14 and FITC-CD16. Doubledts and dead cells were excluded, monocytes were selected based on their light scatters and CD14 and CD16 expres- sions, and sorted into CD14⁺CD16⁻, CD14⁻CD16⁺, and CD14⁺CD16⁺ cells directly in RNeasy Lysis Buffer (Qiagen) using a FACs Vantage cell sorter (BD Biosciences). Sorted cells were kept at -80°C before RNA isolation. For cytokine production, we added a PE-elimination channel (RANTES), CXCL10 (IP10), and IL-8 (CXCL8) by the human 9-flex bacterium, adapted accordingly (Supplemental Fig. 1). To analyze cytokine production, we cultivated 1 × 10⁶ monocytes per patient, which limited the number of IM and NCM after FACs sorting, and experimental design was adapted accordingly (Supplemental Table I). Describes Abs used for these studies.

RNA extraction, reverse transcription, and cDNA preamplification

Total RNA was isolated from 9000 to 90,000 sorted monocytes. RNeasy Mini Kit (Qiagen) was used according to manufacturer’s instructions including DNase treatment. RNA quality and quantity were analyzed by capillary electrophoresis (BioAnalyzer; Agilent). Equal amounts of cDNA were preamplified using the TaqMan PreAmp Master Mix (Applied Bio- systems).

Real-time PCR

Preamplified cDNA samples were amplified using Made-to-Order Low Density Array System according to manufacturers’ instructions. The arrays were carried out on the Applied Biosystems 7900HT. TaqMan Low Den- sity Array was designed to screen for changes in gene expression of CX3CR1, CSF-1R, SELPLG, TNF, CCR2, CCR3, CD36, ICAM1, IL-10, IL-8, IL-1β, CCL2, CCL3, TLR2, TLR4, TLR7, TLR8, peroxisome proliferator-activated receptor γ genes, and actin as endogenous controls. A detailed list of probes is given in Supplemental Table 1. Each sample was run in duplicate, and the results were analyzed using the RQ Manager software (Applied Biosystems) and expressed as relative gene expression (2⁻ΔΔCt method) using β-actin expression as endogenous control.

Cytokine production

To analyze cytokine production, we cultivated 1 × 10⁸ FACs-sorted CM, IM, or NCM in 100 μl RPMI 1640 and 10% FBS. Each subset was stimulated with 10 ng/ml LPS, 10 μg/ml viral ssRNAs (ssRNA40/LyoVec; Invivogen), or media alone. After 18 h, supernatants were harvested and kept at -80°C for cytokine quantification. Monocyte supernatants were quantified for TNF, IL-1β, IL-6, IL-10, IL-12p70, CCL2 (MCP-1), CCL5 (RANTES), CXCL10 (IP10), and IL-8 (CXCL8) by the human 9-flex cytokine bead array. Each set of probes was analyzed on the manufacturer’s instruction. acquisition was performed on an LSRII and quantification calculated with FCAP array v3 software (all from Becton Dickinson).

Protein expression

Flow cytometry using 8-fluorophores was performed on monocyte-enriched PBMCs. To prevent nonspecific binding, we blocked FcRs using 2% human serum PBS (2% HS) for 10 min. Abs were diluted in the same buffer and incubated 30 min in the dark at 4°C. Monocyte subsets were selected as described earlier. Fluorescence minus one (FMO) was used to determine the cutoff between cells expressing FITC-CSF-1R, PE/CF594-CXCR5, PerC/Pc CX5-C5-CR2, or PE/Cy7-CX3CR1 and negative cells (Supplemental Fig. 2). Expression of TLR4 and TLR8 was determined separately on whole blood. Whole blood was FcR blocked in 2% HS for 15 min. Anti-TLR4 Ab or matching isotype control was added to cells before a mix of allolymphocytin-CD14, allolymphocytin/CD16, V50-Cd3, V50-Cd19, V50-Cd55, and a fixable dead cell marker. After 30 min of surface staining, RBC lysis and fixation were performed simultaneously using VersaFix (Beckman Coulter). TLR8 intracellular staining was performed after surface staining; cells were permeabilized with 0.1% saponin 2% HS for 10 min at 4°C. Anti-TLR4 or matching isotype was diluted in this buffer and incubated 30 min in the dark at 4°C. Monocyte subsets were selected as described earlier, and FMO, including PE-isotype, was used to determine the cutoff between cells expressing TLR4 or TLR8 and negative cells. Labeled cells were washed and analyzed using FACSIAria III (8-fluorophore flow cytometry) or LSRII (TLR4 and TLR8) (both instruments from BD Biosciences). Analyses were standardized over time by the use of both BD Cytoometer Setup and Tracking software and the application setting functions available in BD FACS Diva software. We used the mean fluorescence intensity (MFI) to evaluate the expression of CSF1R, CCR5, CCR2, CX3CR1, TLR4, and TLR8. Abs de- scription and dead cell markers are given in Supplemental Table II.

Statistical analyses

Data are expressed as means ± SEM. Comparisons of mean for quanti- tative variables between groups were performed using the nonparametric Mann–Whitney U test. Relationship among the different quantitative var- iables was determined by calculating the Spearman correlation coefficient. For all analyses, a p value <0.05 was considered statistically significant. Statistical analyses were done with GraphPad Prism 5.0 (GraphPad Soft- ware).

Results

Specific gene expression profile in monocyte subsets in obesity

In accordance with previous findings (12), monocyte absolute numbers were increased in each subset of the obese subject group, as well as the proportion of IM and NCM (Fig. 1). To unravel the molecular signature of monocytes in obesity, we compared expression of 18 candidate genes related to monocyte functions in 3 monocyte subpopulations isolated from C and Ob subjects before and after gastric surgery–induced weight loss (Fig. 2). We found that each monocyte subset displayed a specific gene expression signature. Regarding molecules involved in chemotaxis, mRNA expression of CX3CR1 was significantly increased (2- to 2.5-fold) in the Ob group compared with the C group in the CM and IM, and with a trend in the NCM. After weight loss, the expression of CX3CR1 decreased in all monocyte subsets. In obese CM and IM, CCR2 and CCR5 were significantly 2- to 2.5-fold overexpressed. Decreased mRNA expression was observed after weight loss for CCR5 in the CM and for CCR2 in the IM. The expression of CCL2 chemokine was increased by 3.6-fold in the IM of Ob subjects and decreased to levels comparable with C group after weight loss. We found no difference in CCL3 ex- pression in Ob group. Peroxisome proliferator-activated receptor γ, which regulates fatty acid storage and glucose metabolism, was increased in the NCM of Ob individuals and in the CM after weight loss.

For genes involved with the pathogen recognition function, we found a 2- to 4-fold increased expression of TLR4 and TLR8 in all monocyte subsets of the Ob group. Conversely, the expres- sion of TLR2 and TLR7 was similar in both groups. After weight loss, the expression profiles of these TLRs was not significantly modified.
For genes involved in cytokine production, we found increased expression of IL-1β in NCM and of TNF in IM in Ob individuals. No significant difference in NCM expression level of IL-1β was observed after weight loss, whereas IM expression level of TNF tended to return to normal value.

Taken together, these results show modified mRNA expression of gene related to monocyte functions in obesity. We observed specific characteristics for each monocyte subset for genes involved in cytokines production, chemotaxis, fatty acid storage and glucose metabolism, and pathogen recognition. Indeed, TLR4 and TLR8 overexpression could be considered as a molecular signature of monocytes in obese patients.

Monocyte subsets are characterized by stronger responses upon TLR4 and TLR8 stimulation in obesity

While observing only a slight increase, however not statistically significant, in TLR4 and TLR8 expressions at the protein level in monocytes of obese patients compared with lean subjects (Fig. 3A, 3B), we demonstrated that monocyte subsets from obese patients compared with lean subjects have increased ability to secrete inflammatory molecules upon stimulation with LPS or viral ssRNA respective ligands of TLR4 and TLR8.

We found that some cytokines involved in the inflammatory response were increased in the monocyte subsets (Fig. 3C). CMs were characterized by an increased production of IL-1β in response to LPS and an increased production of TNF and IL-10 upon ssRNA stimulation in the Ob group compared with C. The IM subset did not differ in terms of cytokine production in response to LPS or ssRNA. Finally, the NCM subset displayed higher production of IL1-β upon ssRNA stimulation.

Chemokine production analysis (Fig. 3D) revealed an increased CM production of CCL5 when stimulated by LPS in the Ob group compared with C. The IM subset did not differ in terms of chemokine production in response to LPS or ssRNA. Finally, the NCM subset of Ob subjects displayed higher secretion of CCL5 upon ssRNA stimulation.

We present in this article convincing evidence that CM and NCM are characterized by a higher response upon TLR4 and TLR8 activation in obese patients compared with lean subjects.

Monocyte subsets are characterized by changes in chemokine receptor expression related to obesity-related bioclinical features

The analysis of gene expression signatures revealed specificities between monocyte subsets regarding chemotaxis. The heat-map representation of CCR2, CCR5, CX3CR1, and CSF-1R protein expression revealed a general overexpression of these receptors in
all subsets of the Ob group (Fig. 4). Thus, the overexpression is significant only for CCR2, CCR5, and CX3CR1 in the CM and IM subsets and for CX3CR1 at the surface of NCM subset (Fig. 4 and Supplemental Fig. 2). Taken together, these findings suggest higher migration ability and increased survival of monocytes in Ob compared with C subjects. Indeed, the highest increased

FIGURE 3. Comparison of inflammatory molecule secretion by monocyte subsets upon TLR4 and TLR8 stimulation. (A) Protein expression of TLR4 and TLR8 measured by flow cytometry. n = 12 normal-weight and nondiabetic volunteers (C), n = 10 obese nondiabetic subjects (Ob). (B) Representative examples of TLR4 surface staining and TLR8 intracellular staining of one C and one Ob. Black histogram represents obese patient, gray histogram represents lean subject, and clear histogram represents FMO, with matching isotype control for TLR8. (C) Cytokines TNF-α, IL-1β, IL-6, IL-10, and IL-12p70, and (D) chemokines MCP-1 (CCL2), RANTES (CCL5), IP-10 (CXCL10), and IL-8 (CXCL8) measured by cytometric bead array upon 18-h LPS (10 ng/ml) or ssRNA (10 μg/ml) stimulation. C is represented by clear bar and Ob by black bar. n = 5 C, n = 5 Ob. Statistical comparisons between each group were obtained by the nonparametric Mann–Whitney U test (* p < 0.05, ** p < 0.01).
expression of CCR5 and CCR2 in the CM and the IM imply that those two cell subpopulations could have higher migration capacity than the NCM (19).

To evaluate the clinical relevance of increased expression of chemokine receptors, we performed correlation analysis between protein expression of CCR2, CCR5, CX3CR1, and CSF-1R and phenotypes related to corpulence (body mass index [BMI]) and metabolic status (lipid and diabetes-related traits) listed in Table I. The complete linkage clustering of correlation coefficients of obese clinical outcomes and protein expression levels of CCR2, CCR5, CX3CR1, and CSF-1R showed that for each receptor the expression level of CM and IM segregated from the one of NCM (Fig. 5). We found that monocyte surface expression of CX3CR1 and CCR2 was negatively correlated with high-density lipoprotein–cholesterol (HDL-c), a protective marker of cardiovascular risk. The strength of the negative correlation was particularly strong for BMI and metabolic traits (glycemia and triglycerides) in the three subsets. Furthermore, expression of CCR5 and CSF-1R in NCM correlated with obesity-related low-grade inflammation. The overexpression of CCR5 and CSF-1R in NCM could therefore be considered a hallmark of metabolic alterations, especially insulin resistance, in Ob subjects.

Fig. 6 proposes a model of the presumed functions of monocyte subsets in obesity-related low-grade inflammation.

**Discussion**

Recent studies in healthy individuals have characterized gene expression profiles of the three monocyte subsets (11, 16, 17). However, little is known about these different subpopulations in inflammatory-related diseases. In this study, we show that the three subsets display specific modified gene expression and protein expression profiles of inflammatory molecules, as well as differential functional response to the TLR signaling in obesity, a well-known low-grade (sterile) inflammatory pathology.

It is well established that monocytes migrate into sites of inflammation in response to several chemokines and obesity is closely associated with an enhanced number of adipose tissue macrophages (7). We showed that a common characteristic of the three subsets of monocytes in obesity is the overexpression of CX3CR1, suggesting their increased potential to migrate in response to CX3CL1, the chemokine ligand for CX3CR1. This result is consistent with the demonstration by Shah et al. (20) that CX3CL1 is secreted by adipose tissue and mediates monocyte adhesion to human adipocytes. They concluded that CX3CL1 is a novel adipose tissue chemokine in humans and may modulate the recruitment of monocytes into adipose tissue. Consistently, Polyák et al. (21) demonstrated that the CX3CL1-CX3CR1 chemokine system is implicated in the development of metabolic visceral adipose tissue inflammation in obese mice. This is further supported by several studies performed in murine models (22, 23) and clinical investigations in humans (24–26) that suggest a role in obesity-related metabolic diseases.

### Table I. Bioclinical characteristics of obese and control subjects

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Ob</th>
<th>C</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subjects, n</td>
<td>40</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>Age (mean ± SEM), y</td>
<td>41 ± 2</td>
<td>37 ± 2</td>
<td>0.211</td>
</tr>
<tr>
<td>BMI (mean ± SEM), kg/m²</td>
<td>43.2 ± 0.7</td>
<td>21.0 ± 0.4</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Sex, female/male</td>
<td>36/4</td>
<td>24/2</td>
<td></td>
</tr>
<tr>
<td>Glycemia (mean ± SEM), mmol/L</td>
<td>5.4 ± 0.1</td>
<td>4.6 ± 0.1</td>
<td>0.002</td>
</tr>
<tr>
<td>Insulinemia (mean ± SEM), mU/L</td>
<td>15.6 ± 1.4</td>
<td>7.8 ± 1.1</td>
<td>0.001</td>
</tr>
<tr>
<td>Total cholesterol (mean ± SEM), mmol/L</td>
<td>5.1 ± 0.1</td>
<td>5.0 ± 0.3</td>
<td>0.883</td>
</tr>
<tr>
<td>HDL-c (mean ± SEM), mmol/L</td>
<td>1.2 ± 0.1</td>
<td>1.7 ± 0.1</td>
<td>0.002</td>
</tr>
<tr>
<td>LDL-c (mean ± SEM), mmol/L</td>
<td>3.2 ± 0.1</td>
<td>3.0 ± 0.3</td>
<td>0.197</td>
</tr>
<tr>
<td>Total triglycerides (mean ± SEM), mmol/L</td>
<td>1.3 ± 0.1</td>
<td>0.8 ± 0.1</td>
<td>0.001</td>
</tr>
<tr>
<td>CRP (mean ± SEM), mg/L</td>
<td>9.8 ± 1.2</td>
<td>5.5 ± 0.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Total WBCs (mean ± SEM), 10³/µL</td>
<td>7.4 ± 0.3</td>
<td>6.4 ± 0.6</td>
<td>0.054</td>
</tr>
<tr>
<td>Lymphocytes (mean ± SEM), %</td>
<td>32.7 ± 1.2</td>
<td>30.1 ± 2.2</td>
<td>0.323</td>
</tr>
<tr>
<td>Neutrophiles (mean ± SEM), %</td>
<td>57.4 ± 1.3</td>
<td>60.0 ± 2.2</td>
<td>0.440</td>
</tr>
<tr>
<td>Monocytes (mean ± SEM), %</td>
<td>7.1 ± 0.3</td>
<td>5.8 ± 0.4</td>
<td>0.022</td>
</tr>
<tr>
<td>Total monocytes (mean ± SEM), 10⁶/µL</td>
<td>0.5 ± 0.0</td>
<td>0.4 ± 0.0</td>
<td>0.003</td>
</tr>
</tbody>
</table>

Statistical comparisons between each group were obtained by the nonparametric Mann–Whitney U test.

CRP, C-reactive protein; LDL, low-density lipoprotein–cholesterol.
Moreover, our results suggest higher chemotactic ability specifically secreted by activated endothelium and adipose tissue. Furthermore, high levels of CX3CR1, CCR2, or CCR5 in response to chemotactic signals could be a result of increased migration of monocytes expressing these receptors from visceral adipose tissue of obese subjects or the addition of obese subjects. We previously observed that conditioned medium from visceral adipose tissue of obese subjects or the addition of CCL5 induced adhesion and transmigration of human blood monocytes through adipose tissue endothelium (33). A recent study on obese women demonstrated higher chemotactic activity of monocytes from obese subjects in response to CCL2 and CCL5 (27–29). The highest expression of CSF-1R in NCM could reflect a longer life span compared with the other subsets. Interestingly, we found a positive association between overexpression of CSF-1R and CX3CR1 and CCR2 expression. These data suggest that decreased HDL, a common abnormality observed in obesity, could be directly linked to changes in monocyte phenotype and function.

We observed that CSF-1R was overexpressed in the NCM of obese subjects, however not at a significant level. CSF-1R (also known as CD115, c-fms, and M-CSF receptor) is a tyrosine kinase protein high-affinity receptor for CSF-1 and IL-34. Its ligands, IL-34 and CSF-1, have a pivotal role in regulating survival, proliferation, and differentiation of mononuclear phagocytes (9, 35, 36). CSF-1R is notably expressed by adipose tissue in obese subjects (37, 38). The highest expression of CSF-1R in the NCM could reflect a longer life span compared with the other subsets. Interestingly, we found a positive association between overexpression of CSF-1R in NCM and homeostasis model assessment–estimated insulin resistance, a surrogate of insulin resistance, suggesting that CSF-1R could be considered as characteristic of metabolic alteration, especially insulin resistance, in obese subjects. TLRs are members of a family of pattern-recognition receptors that detect components of microorganisms, playing a critical role in the innate immune system by activating inflammatory signaling pathways (39). Studies highlighted the involvement of TLR2 and TLR4 in metabolic functions, as well as in innate immune responses, in obesity (40, 41). An interesting finding from this study is a higher activation of TLR4 signaling in response to LPS of CM subset. TLR4 could be stimulated by circulating bacterial LPS or fatty acids, whose levels are commonly increased in obesity (40). We demonstrate for the first time, to our knowledge, a higher activation of TLR8 signaling in response to ssRNA in NCM and CM from obese patients compared with lean subjects. TLR8 is an endosomal TLR that binds viral ssRNAs (17). Although there are no evidences showing increased levels of circulating nucleic acids in obesity, we can suggest that TLR8 present in monocytes from obese subjects could detect circulating nucleic acids or some not yet known ligands other than viral ssRNAs. Moreover, in accordance with increased TLR8 overexpression and stimulation, we found that monocytes display high levels of proinflammatory cytokines in obese patients, particularly IL-1β for NCM.

**FIGURE 5.** Correlation among CCR2, CCR5, CX3CR1, and CSF-1R protein expressions of each monocyte subset with obesity-related clinical parameters. Data are from 10 normal-weight and 12 obese subjects. Correlations were estimated by the nonparametric Spearman test. Distance between protein expression of each subset was estimated by complete linkage clustering. Heat-map colors are representations of each correlation coefficient, and numbers indicate p values (*p < 0.05, **p < 0.01, ***p < 0.001). CRP, C-reactive protein; HOMA-IR, homeostasis model assessment–estimated insulin resistance; LDL-c, low-density lipoprotein-cholesterol.

**FIGURE 6.** Presumed functions of monocyte subsets in obesity-related low-grade inflammation. We propose a model where proinflammatory monocytes of obese subjects would sense circulating factors through TLR4 and TLR8. In response, the CM and NCM subsets secrete more inflammatory cytokines.Expressing more CCR5 and CCR2, the IM and CM could be able to migrate more toward tissues where they could differentiate into macrophages. Both phenomena contribute to sustain low-grade inflammation associated with obesity, whereas NCM could stay longer in the circulation and assume “patrolling” function.
In conclusion, our findings demonstrate that the three monocyte subsets from obese patients display a more inflammatory phenotype with a higher response to TLR4 and TLR8 stimulation, and a chemokine receptor expression profile that suggests a higher capacity to migrate for CM and IM and increased survival for NC (Fig. 6). Indeed, therapeutic strategies targeting inflammatory processes or TLR signaling could be a promising approach to reduce inflammatory, metabolic, and cardiovascular complications linked to obesity.

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Disclosures
The authors have no financial conflicts of interest.

References