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Profiling of the Three Circulating Monocyte Subpopulations in Human Obesity

Estelle F. Deveâvre,*†‡§,1 Mariana Renovato-Martins,*†‡§,1 Karine Clément,‡§,8,**,1 Catherine Sautès-Fridman,*†‡§ Isabelle Cremer,*†‡§,1 and Christine Poitou†‡§,8,**,**,1

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 non-diabetic volunteers (C) and 40 obese non-diabetic (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 FcyRIII (CD16) receptors (8, 9).

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

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The online version of this article contains supplemental material.
Materials and Methods

Subjects

Two groups of subjects were included in this study (Table I). The first group included 40 obese non-diabetic (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 Pitié-Salpêtrière Hospital, Paris, France). Patients met the criteria for bariatric surgery as described earlier (12). The second group comprised 26 healthy normal-weight and non-diabetic volunteers (C), living in the same region as the Ob subjects. The Ethics Committee (CPIE Ile de France I) 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 × 10^6 to 4 × 10^6 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 (PAAR Laboratories). Monocytes were then enriched by a centrifugation on a Hyper-osmotic PercollGradient (GE Healthcare) (18). Monocyte-enriched cell fraction was further subdivided by FACS. For the gene expression experiment, cells were stained with allophycocyanin, CD14 and FITC-CD16. Doublets and dead cells were excluded, monocytes were selected based on their light scatters and CD14 and CD16 expressions, 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 with PE-CD3, PE-CD19, and PE-CD56 to exclude potential contaminant from the monocyte gate. A total of 10,000 monocytes per subset were sorted using a FACSIAria III (BD Biosciences) into RPMI 1640 medium. Sorted monocytes were cultivated immediately. 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 Biosystems).

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 Density 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 × 10^6 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/Ly6/ine; 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-6 (CXC/L) by the human 9-plex cytometric bead array. Each flex set are given in Supplemental Table I. Samples were processed according to the manufacturer’s instructions; 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-CR5, PerCP/Cy5.5-CR2, or PE/Cy7-CX3CR1 and negative cells (Supplemental Fig. 2). Expression of TLR4 and TLR8 was performed 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 allophycocyanin-CD14, allophycocyanin/PE/CD14, V450-CD3, V450-CD19, V450-CD56, 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-TLR8 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). Acquisitions were standardized over time by the use of both BD Cytometer Setup and Tracking software and the application setting functions available in BD FACSDiv software. We used the mean fluorescence intensity (MFI) to evaluate the expression of CSF1R, CR5, CCR2, CX3CR1, TLR4, and TLR8. Abs description and dead cell markers are given in Supplemental Table I.

Statistical analyses

Data are expressed as means ± SEM. Comparisons of mean for quantitative variables between groups were performed using the non-parametric Mann–Whitney U test. Relationship among the different quantitative variables 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 Software).

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 expression 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 expression 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 genes 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. Indeed, TLR4 and TLR8 overexpression could be considered as a molecular signature of monocytes in obese patients.

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 IL-1β 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.

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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, IL-1\( \beta \), 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 \( \mu \)g/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 in CM and CCR2 was negatively correlated with high-density lipoprotein–cholesterol (HDL-c), a protective marker of cardiovascular risk. The expression of CCR5 and CSF-1R in NCM correlated with obesity could be linked to cardiovascular risk. The expression of CCR5 was positively correlated with BMI and metabolic traits of insulin resistance. Overexpression of CCR5 and CSF-1R in NCM subset. This suggests that increased NCM subpopulation 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, Polýák 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

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**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>Insulinaemia (mean ± SEM), μU/ml</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>&lt;5</td>
<td>0.001</td>
</tr>
<tr>
<td>Total WBCs (mean ± SEM), 10⁹/l</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), 10⁹/l</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 high levels of CX3CR1, CCR2, or CCR5 in response to chemotactic stimuli. This could be a result of increased migration of monocytes expressing high levels of macrophages found among adipose tissue during obesity (19). These findings together with our data suggest that higher chemokine and cytokine responses to CCL2 and CCL5 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.

We observed increased expression of CCR2 and CCR5, which have been involved in the recruitment of monocytes in atherosclerotic plaques in murine models (31, 32), in CM and IM 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 CM subset. TLR4 could be stimulated by circulating bacterial LPS or fatty acids, whose levels are commonly increased in obesity (40, 41). An interesting finding from this study is a higher activation of TLR4 signaling in response to LPS and TLR4 in metabolic functions, as well as in innate immune pathways (39). Studies highlighted the involvement of TLR2 and TLR4 in innate immune responses, in obesity (40, 41). An interesting finding from this study is a higher activation of TLR4 and TLR6 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.
In conclusion, our findings demonstrate that the three monocyte subsets from obese patients display a more inflammatory profile and macrophage infiltration in human white adipose tissue. Nature Immunol. 11: 1141–1147.


