Peripheral monocytes of obese women display increased chemokine receptor expression and migration capacity

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Context: The activation of peripheral immune cells and the infiltration of immune cells into adipose tissue in obesity are implicated in the development of type 2 diabetes mellitus.

Objective: Aim of the study was to compare peripheral immune cells from obese and normal-weight women with regard to composition of immune cell subpopulations, surface expression of the chemokine receptors CCR2, CCR3, CCR5 and CXCR3 and cell-intrinsic migration capacity.

Design: Case-Control Study.

Setting: University Clinical Study Centre.

Patients: Obese females and normal-weight females were included for fluorescence activated cell sorting (FACS) analysis and migration assays.

Main Outcome Measures: Peripheral blood mononuclear cells (PBMCs) were prepared from fasting blood samples and used for FACS analysis and migration assays.

Results: An increase in the percentages of $CD14^+CD16^+$ monocytes was observed in obese subjects compared to controls. The chemokine receptor (CCR) profile of monocytes differed significantly in the obese state, particularly CCR2 levels were increased. In addition, a higher chemotactic activity of monocytes from obese subjects was observed in a migration assay, which was associated with both insulin resistance and CCR2 expression.

Conclusion: Our results suggest that the enhanced intrinsic migratory capacity of peripheral monocytes in obese women may be due to the increased chemokine receptor expression, further supporting a link between peripheral immune cell dysfunction and obesity.

Obesity is known to represent a state of chronic low-grade inflammation with increased accumulation of leukocytes within adipose tissue (AT). Numerous lines of evidence associate both, obesity and insulin resistance, with the activation of AT-resident immune cells (1), which have been shown to be derived from bone marrow pre-

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Abbreviations: PBMCs, Peripheral blood mononuclear cells; FACS, Fluorescence activated cell sorting; AT, Adipose tissue; BMI, Body mass index; HOMA-IR, Homeostatic model assessment of insulin resistance; MFI, Median fluorescence intensity.

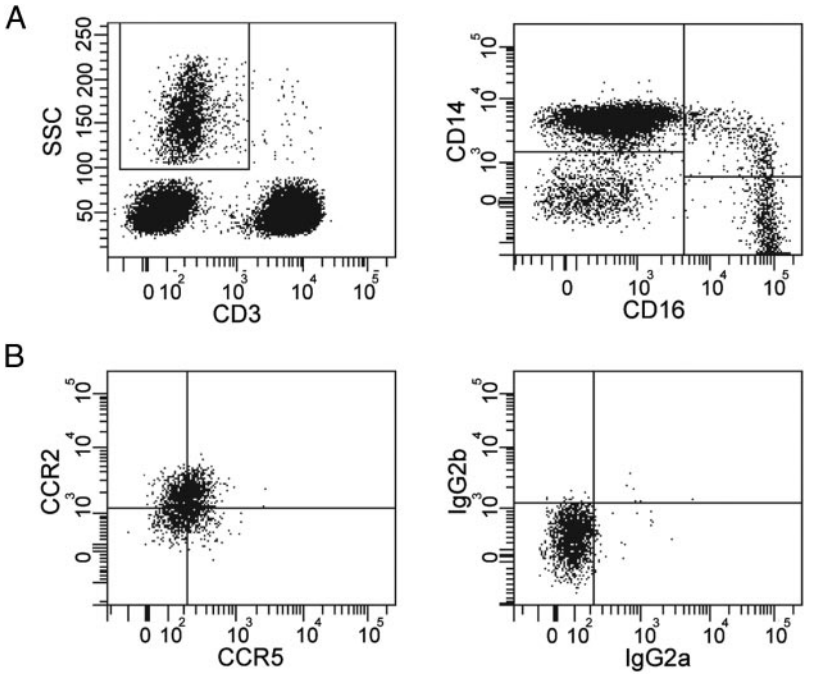

Figure 1. Representative FACS dot plots of a representative normal-weight subject. Monocytes were identified by high granularity (SSC, side scatter) and absence of CD3 expression (A, left panel), and monocyte subsets further characterized by the expression patterns of the LPS coreceptors CD14 and FcγIII receptor CD16 gated as $CD14^+CD16^-$ and / or $CD14^+CD16^+$ cells (A, right panel). Representative plot showing the CCR2 and CCR5 expression levels on $CD14^+CD16^+$ monocytes (B, left panel) and corresponding isotype controls (B, right panel).

cursors that migrate via the peripheral circulation into AT (2). Furthermore, infiltration of peripheral immune cells has consistently been associated with the pathogenesis of atherosclerotic plaques (3).

Excessive energy intake induces both oxidative stress and an increase of NF-κB activity in peripheral blood mononuclear cells (PBMCs) (4). NF-κB activation and secretion of proinflammatory cytokines was also observed in PBMCs from obese individuals (5), whereas weight loss in individuals with Metabolic Syndrome was shown to result in a decreased expression of inflammatory genes in PBMCs together with an increase of systemic insulin sensitivity (6). Therefore, in obesity PBMCs might respond to intra- and extracellular signals in various ways including a change of leukocyte subsets as well as an increased activation and infiltration of leukocytes into target tissues such as AT or arterial intima media.

For monocyte migration into AT, it has been supposed to be induced by numerous chemokines, which are upregulated in obese AT (2, 7 – 11), and an upregulation of the chemokine receptors CCR1, CCR2, CCR3, and CCR5 expression was described for AT of obese individuals (12). However, it remains unclear whether chemokine receptor up-regulation is restricted to AT or can be also observed in peripheral blood leukocytes. Here, we were interested to investigate whether leukocytes from obese subjects might differ in their chemokine receptor expression, chemokine-responsiveness and ability to migrate compared to monocytes from normal-weight subjects. Our results further support the concept of an increased systemic inflammatory response as a characteristic feature of obesity.

Subjects and Methods

Participants

Female subjects were recruited between October 2008 and July 2009 as part of the AdipoRiSc case-control study. Written informed consent was obtained prior to the start of the study. Recruitment, clinical phenotyping and biosample collection were performed at the study unit of the Else Kroener-Fresenius Centre for Nutritional Medicine (Technische Universität München). Two groups of women were classified by body mass index (BMI) as lean ($\geq$ 18.5 to $\leq$ 24.9 kg/m^2) and obese ($\geq$ 30.0 kg/m^2). Blood samples of 29 obese and 19 lean women were investigated by migration assay analysis. For a subcohort of 14 obese and 16 lean women, we additionally performed FACS analysis to determine chemokine receptor levels on PBMCs. Statistical analysis was performed for both subcohorts separately. Due to the ethical limitations relating to the entire AdipoRiSc study, blood withdrawal for PBMC isolation available for FACS analysis and migration assay was restricted to 8 ml per patient. As donor dependent isolation efficiency was highly variable (average number of PBMCs isolated per patient = 4.5×10^6 ($3.9 \times 10^6 - 6.4 \times 10^6$), median and 25th – 75th quartile, n = 48), we could not perform both FACS and migration assays (requiring 3.0×10^6 and 2.0×10^6 cells/assay, respectively) for each donor. Please note that for two obese subjects in the FACS cohort no migration data were available and that the RANTES-induced migration assay could only be performed for 8 lean and 12 obese subjects. From these, 4 lean and 8 obese women had enough PBMCs to also allow for FACS analyses.

The weight of the included subjects had been stable for at least 3 months (variation of less than $\pm$ 3 kg). Subjects with any evidence of inflammatory or metabolic disease including type 2 diabetes mellitus (assessed by oral glucose tolerance test (OGTT), data not shown) or other chronic diseases and cancer were excluded. Acute infectious and inflammatory diseases were ruled out by analyzing the leukocyte counts (< 4 and $> 10 \times 10^3/\mu$l), and by history and thorough clinical examination. Further exclusion criteria were smoking, medication with metabolic adverse effects, pregnancy, lactation and chronic alcohol consumption. Subjects in the obese group fulfilling the criteria for the Metabolic Syndrome were defined (13, 14). Additional subgroup analyses were performed after excluding obese women who had hsCRP levels $>$ 10 mg/l. This study was approved by the Ethics Committee of the Technische Universität München.

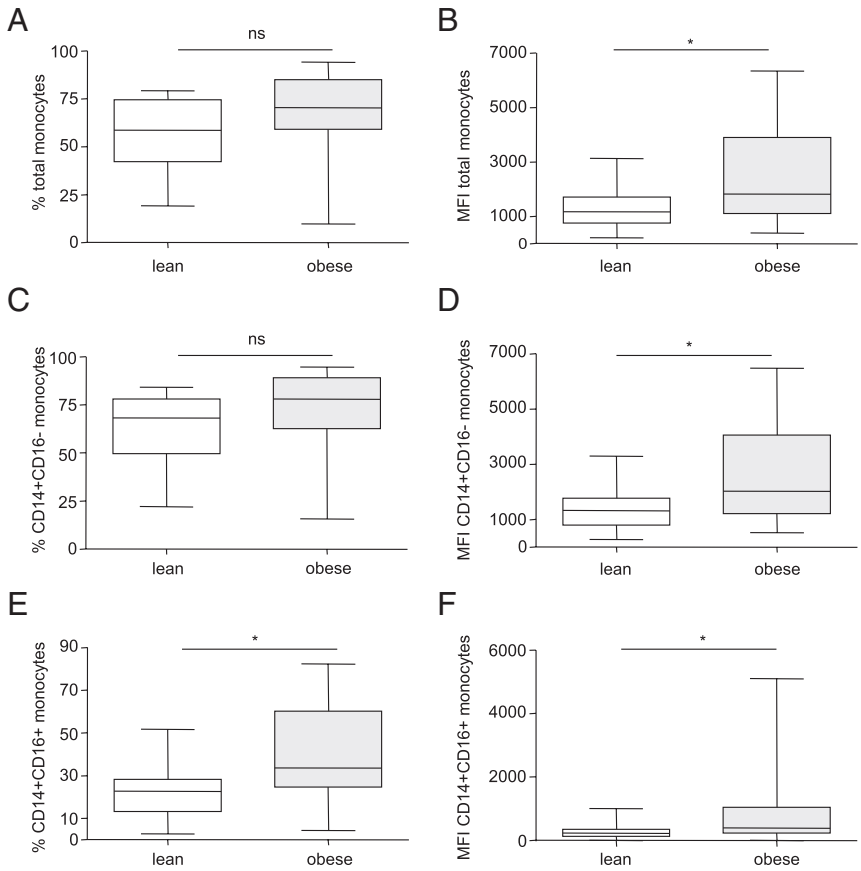

Figure 2. Surface expression and density of chemokine receptor CCR5 on monocytes. Peripheral blood total monocytes (A, B), $CD14^+CD16^-$ monocytes (C, D) and $CD14^+CD16^+$ monocytes (E, F) of obese subjects (n = 14) were compared to lean control subjects (n = 16). Percentages of positive cells (A, C, E) and the receptor surface density (MFI) (B, D, F) are given. Data are presented as median (horizontal bar) with 25th and 75th percentiles (boxes) and extreme values (whiskers). Analysis was performed using independent-sample *t* test with $*P < .05$, $**P < .01$; ns, not significant.

Anthropometric, clinical and plasma chemokine measurements

Weight was determined using an electronic scale (BC 418 segmental body composition analyzer, Tanita, München, Germany). Body circumference assessments were carried out according to WHO criteria. Total body fat mass was determined by dual-energy X-ray absorptiometry (DXA) (Hologic Explorer S/N 90417, QDR-Series, Hologic Inc.; software version 12.4). Venous blood samples were collected in the fasting state (overnight fast of at least 12 hours) for determination of routine biochemical parameters (Laboratory Becker, Olgemöller & Colleagues, Munich, Germany). Insulin resistance was estimated using the homeostatic model assessment of insulin resistance (HOMA-IR) calculated as insulin (mg/dl) x glucose (mg/dl) / 405 (15). Plasma MCP-1, RANTES and IP-10 chemokine levels (interand intra-assay CVs 5.7%/5.8%, 9.1%/3.2%, and 6.7%/3.6%, respectively) were measured using commercially available ELISAs (R&D Systems, Wiesbaden, Germany).

Flow cytometric analysis

PBMCs were isolated from whole venous blood using BD Vacutainer CPTs (Becton Dickinson, Heidelberg, Germany) containing sodium heparin according to the manufacturer's instructions and suspended in RPMI 1640 (Gibco, Darmstadt, Germany), 0.5% heat-inactivated FCS and 1000 mg/l glucose. FACS analysis was performed similar to a previously described protocol (16). The percentages of chemokine-receptor positive cells were determined within total lymphocytes or monocytes after defining a cut-off value according to the isotype control by using anti-CD3-fluorescein isothiocyanate (FITC) mouse IgG1, κ isotype, anti-CD14-APC-H7 mouse IgG2b, κ isotype, anti-CD14-Peridinin-chlorophyll-protein (PerCP) mouse IgG2b, κ isotype, anti-CD16-phycoerythrine (PE) mouse IgG2b, κ isotype, anti-CD16-PE-Cy7 mouse IgG1, κ isotype, anti-CCR2- Alexa Fluor 647 (A647) mouse IgG2b, κ isotype, anti-CCR3-A647 mouse IgG2b, κ isotype, anti-CCR5-PE mouse IgG2a, κ isotype and anti-CXCR3-PE mouse IgG1, κ isotype (all antibodies were from BD Bioscience, Heidelberg, Germany). Cells were incubated with antibodies for 25 minutes at 4°C, washed two times, and analyzed by flow cytometry (FACS Canto with FACS DIVA software, Version 5.0.3, BD Bioscience, Heidelberg, Germany). Results are expressed as percentages of stained cells and median fluorescence intensity (MFI) representing the receptor surface density.

Chemotactic assay

To assess migration capacity of human monocytes, we adopted a previously reported protocol (17) as follows. Monocyte chemotaxis was evaluated using 24-well transwell plates (Costar, Bodenheim, Germany) with polycarbonate membranes of 6.5 mm diameter and a pore size of 5 μm. Chemoattractants (100 ng/ml MCP-1, 100 ng/ml IP-10, 1 ng/ml RANTES and a 'chemokine-mix' consisting of 50 ng/ml MCP-1, 50 ng/ml IP-10, 0.5 ng/ml RANTES, Pepro Tech, Hamburg, Germany; the chemokine concentrations sufficient to induce a maximal migration response were determined by assessing the dose-response for each chemokine) were diluted in 600 μl assay medium (RPMI 1640, 0.5% heat-inactivated FCS, 1000 mg/l glucose), and were placed in the lower wells of the chemotaxis chamber. 100 μl aliquots of PBMC suspension containing 4×10^6 monocytes/ml were placed in the upper wells. Migration was quantified by counting monocytes in the lower chamber after 150 minutes at 37°C and 5% CO_2. Results are expressed as chemotactic index (fold increase of monocyte response to stimulants over the response to medium alone which was set as one).

Statistical analysis

Statistical analyses were performed using SPSS v2.0 (IBM, Ehningen). Comparisons between groups were performed using *t* test or Wilcoxon signed rank test as indicated. Correlations between variables were expressed as Pearson's correlation coef-

ficients. A p-value < 0.05 was considered as statistically significant.

Results

Anthropometric and metabolic characteristics of the obese and normal-weight women

Blood samples from 14 obese and 16 lean women were subjected to chemokine receptor analysis of PBMCs by FACS, and samples from 29 obese and 19 lean women were analyzed using a migration assay. Clinical parameters of both subgroups are summarized in Table 1 and Supplemental Table 1. Both obese subgroups had significantly elevated levels of low-density lipoprotein (LDL) cholesterol, C-reactive protein (CRP) (hsCRP), uric acid, γ-glutamyltransferase (GGT), insulin and HOMA-IR (Table 1), and significantly decreased concentrations of high-density lipoprotein (HDL) cholesterol. Fasting plasma glucose was increased in obese of the migration assay subgroup, when compared to lean controls. For blood cell counts, creatinine, total cholesterol, triglycerides (TG), liver transaminases and thyroid-stimulating hormone (TSH), no significant differences were observed (Table 1, Supplemental Table 1). Overall, the analyzed study populations represented two homogenous and healthy groups without indication of acute or chronic diseases, but with the expected obesity-related alterations, allowing for analysis of the effects of obesity on functional variables in peripheral immune cells. Four of the total of 29 (14%) obese women fulfilled the criteria for Metabolic Syndrome (13, 14). Nine of 29 and 4 of 14 obese subjects in the migration assay and FACS analysis population, respectively, had moderately elevated hsCRP levels ranging from 11 to 22 mg/l.

Composition of monocyte subpopulations in obese and lean women

To obtain insight into the proportions of monocyte subpopulations in peripheral blood from lean and obese women, FACS analyses were performed (Figure 1). Comparing both groups, no differences were detected in total monocyte percentages between both groups. Monocytes were further subdivided into classical monocytes characterized by high expression level of the LPS coreceptor $CD14^+CD16^-$ and nonclassical monocytes with expression of CD14 and coexpression of the FcγIII receptor CD16 receptor, the $CD14^+CD16^+$ monocytes. The latter secrete proinflammatory cytokines upon activation (18). The percentage of $CD14^+CD16^+$ cells in obese subjects was significantly increased as compared to lean subjects, the percentage of classical $CD14^+CD16^-$ monocytes was significantly decreased (Table 2).

Chemokine receptor expression on peripheral monocytes

To compare cell surface protein expression levels of the chemokine receptors CCR2, CCR3, CCR5 and CXCR3 on distinct monocyte subsets in lean vs obese, we assessed both, the percentage of receptor positive cells and the receptor protein surface density as median fluorescence intensity (MFI) (Supplemental Table 2 and 3, respectively). Both the percentage of $CCR5^+$ total monocytes as well as receptor surface density for CCR5 was significantly increased in the obese group (Figures 2A and 2B). In addition, an increase of the receptor surface density of $CCR2^+$ total monocytes was observed in the obese (Figure 3B), but no significant change in the percentages of $CCR2^+$ monocytes (Figure 3A). Taking monocyte subpopulations into account, we found a significantly increased percentage of $CCR5^+$ cells solely for the $CD14^+CD16^-$ monocyte population (Figure 2C), whereas the percentage of $CCR2^+$ positive cells was specifically increased in the $CD14^+CD16^+$ monocytes of obese women (Figure 3E). Note that we found a concomitant upregulation of CCR5 and CCR2 receptor surface densities on both, $CD14^+CD16^-$ and $CD14^+CD16^+$ monocytes (Figures 2D, 2F, 3D, and 3F; a representative plot of CCR2 and CCR5 staining on $CD14^+CD16^+$ monocytes is shown in Figure 1B). Differences in CCR3 and CXCR3 expression on monocytes as well as on monocyte subsets $CD14^+CD16^-$ and $CD14^+CD16^+$ did not reach statistical significance (Supplemental Tables 2 and 3).

Migration of monocytes

The observed increase of chemokine receptor levels on monocytes from obese as compared to lean subjects suggested an enhanced responsiveness to chemokine signaling. Using a migration assay, we analyzed the migratory response of monocytes towards the chemokines MCP-1, RANTES and IP-10, all of which have been previously reported to be regulated in human AT (17, 19 - 21). To mimic a more physiological situation, we also included a combination of all three chemokines termed chemokine-mix. Monocytes efficiently migrated in response to all chemokines (Figure 4A). Comparing the migration indices of lean vs obese, we found a significantly enhanced migration of monocytes from obese towards MCP-1 by 1.5-fold, towards RANTES by 2-fold, towards the chemokine-mix by 1.5-fold in average (Table 3, Figures 4B, D, E, respectively) and with a trend for increased migration upon IP-10 exposure (Table 3, Figure 4C). We found no significant increase of migration indices in obese subjects with Metabolic Syndrome ($n = 4$) compared to obese subjects without Metabolic Syndrome ($n = 25$) (data not shown). We additionally assessed plasma chemokine lev-

Table 1. Age, body composition and metabolic parameters of study participants included into migration assay and FACS analysis.

	Migration assay population			FACS analysis population		
	Lean (n = 19) mean ± SD	Obese (n = 29) [a] mean ± SD	P-value*	Lean (n = 16) mean ± SD	Obese (n = 14) [b] mean ± SD	P-value*
Age (years)	35.2 ± 6.9	35.5 ± 6.5	0.862	34.9 ± 6.4	35.7 ± 5.0	0.716
Weight (kg)	60.1 ± 7.8	99.9 ± 12.3	<0.001	58.2 ± 7.3	101.1 ± 12.8	<0.001
BMI (kg/m^2)	21.8 ± 1.9	35.6 ± 4.1	<0.001	21.5 ± 2.0	36.0 ± 4.7	<0.001
WHR	0.78 ± 0.04	0.85 ± 0.05	<0.001	0.77 ± 0.13	0.84 ± 0.09	<0.001
Fat mass DEXA (%)	30.2 ± 5.3	45.3 ± 3.9	<0.001	30.3 ± 4.6	45.9 ± 4.1	<0.001
Fasting blood glucose (mg/dl)	78.6 ± 5.5	83.8 ± 8.2	0.020	79.1 ± 6.7	83.9 ± 6.8	0.065
Cholesterol (mg/dl)	193.8 ± 41.1	209.9 ± 32.7	0.138	193.8 ± 39.6	215 ± 26.6	0.108
HDL cholesterol (mg/dl)	70.6 ± 10.4	60.0 ± 13.0	0.004	70.4 ± 9.9	57.5 ± 12.2	0.003
LDL cholesterol (mg/dl)	116.6 ± 31.4	135.3 ± 25.9	0.030	115.1 ± 29.3	140.6 ± 23.3	0.014
Triglycerides (mg/dl)	90.5 ± 43.5	119.2 ± 54.2	0.059	90.8 ± 39.0	117.8 ± 54.7	0.127
hsCRP (mg/liter)**	1.0 (1.0–1.5)	5.0 (2.0–12.0)[c]	<0.001***	1.0 (1.0-1.3)	7.0 (2.5-12.8)[d]	0.001***
Insulin (mU/liter)	4.9 ± 1.4	9.7 ± 3.2	<0.001	5.2 ± 2.4	9.8 ± 3.5	<0.001
HOMA-IR	0.95 ± 0.29	2.01 ± 0.71	<0.001	0.93 ± 0.19	1.98 ± 0.82	<0.001

* Independent-sample t test. ** Data are given as median (25–75th percentile) for skewed hsCRP levels, *** Wilcoxon signed rank test. [a] 4 of 29 (14%) obese women fulfill the criteria for Metabolic Syndrome (13, 14), i.e. they had at least three of five risk factors which constitute a diagnosis of Metabolic Syndrome, including central obesity, arterial hypertension, dyslipidemia (low HDL cholesterol, high triglycerides), fasting hyperglycemia and/or respective drug treatment. [b] The FACS analysis population comprised 3 of the 4 obese women with Metabolic Syndrome. [c] 9 of 29 obese women had hsCRP levels between 11 and 22 mg/liter. [d] 4 of 14 obese women had hsCRP levels between 12 and 22 mg/liter. BMI, body mass index; DEXA, dual-energy x-ray absorptiometry; HDL cholesterol, high-density lipoprotein cholesterol; HOMA-IR, homeostatic model assessment of insulin resistance; hsCRP, high-sensitive CRP; LDL cholesterol, low-density lipoprotein cholesterol; WHR, waist-to-hip ratio.

els of MCP-1, RANTES and IP-10 and found no significant differences comparing obese and lean (Supplemental Table 4). Overall, monocytes from obese subjects exhibited an increased migratory capacity to diverse chemokines compared to monocytes from normal weight subjects.

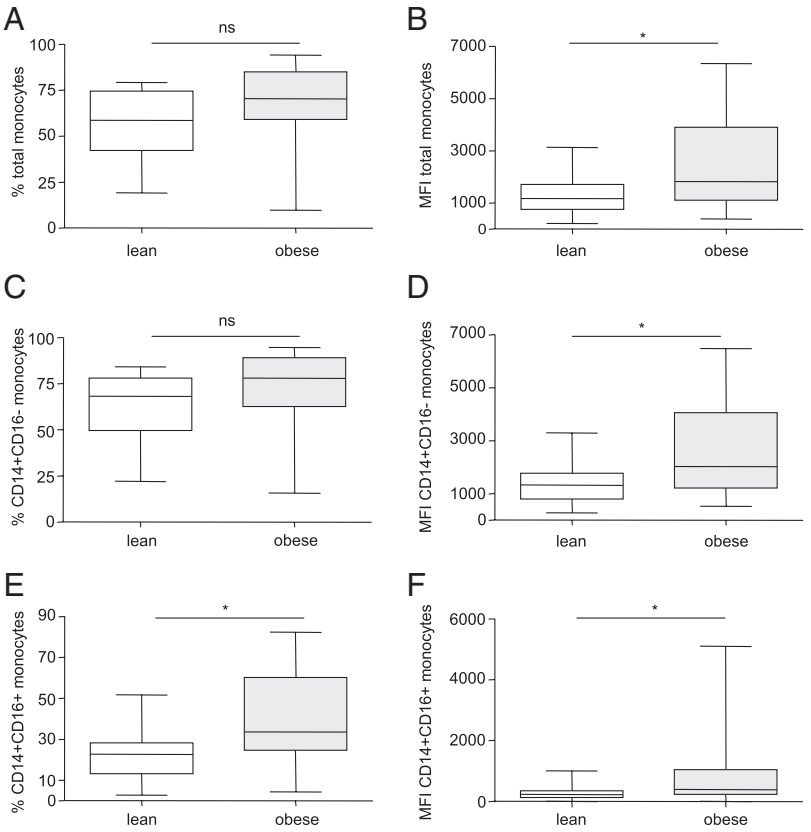

Figure 3. Surface expression and density of chemokine receptor CCR2 on monocytes. Peripheral blood total monocytes (A, B), $CD14^+CD16^-$ monocytes (C, D) and $CD14^+CD16^+$ monocytes (E, F) of obese subjects (n = 14) were compared to lean control subjects (n = 16). Percentages of positive cells (A, C, E) and the receptor surface density (MFI) (B, D, F) are given. Data are presented as median (horizontal bar) with 25th and 75th percentiles (boxes) and extreme values (whiskers). Analysis was performed using independent-sample *t* test with $*P < .05$; ns, not significant.

Correlation of monocytes migration capacity with obesity-related phenotypes and chemokine receptor levels

We performed a correlation analysis to identify obesity-related phenotypes that are associated with monocyte migration capacity in obese vs lean (Supplemental Table 5). The migration responsiveness towards MCP-1, RANTES and the chemokine-mix was significantly positively correlated with BMI and fat mass. No associations were found for the inflammatory marker hsCRP with any of the chemotactic indices. Moreover, no correlation was observed for MCP-1, IP-10 and RANTES plasma levels with anthropometric measures, migration index and chemokine receptor levels (data not shown). Notably, the chemotactic indices of MCP-1, RANTES and the chemokine-mix revealed a significant correlation with fasting insulin and HOMA-IR.

Next, we investigated whether there is a relationship between the increased migratory capacity of monocytes upon the induction with MCP-1, IP-10 and chemokine-mix and enhanced chemokine receptor expression on monocytes from obese women. No correlation could be observed for monocyte migration with CCR5, CCR3 and CXCR3 expression on monocytes (Supplemental Tables 6 and 7). Note that for RANTES-induced migration such correlation analysis was not possible due to the small overlap of the study participants, in whom sufficient material was available to perform both FACS analyses and migration assays (see also materials and methods). We observed a significant correlation for CCR2 expression on monocytes and monocyte subtypes with migration towards MCP-1 for both, the percentages of $CCR2^+$ cells and receptor surface density (Figure 5A-C, Supplemental Tables 6 and 7, respectively). Correspondingly, migration towards the chemokine-mix correlated with the percentages of $CCR2^+$ cells and receptor surface density (Figure 5D-F, Supplemental Tables 6 and 7).

Finally, we assessed monocyte migration and chemokine receptor expression levels in a subcohort where subjects with hsCRP1 levels > 10 mg/ml were excluded. Clinical characteristics of this subcohort were comparable to the entire obese population (data not shown). Notably, we found an increased migration capacity in response to all chemokines in the obese group (Supplemental Table 8), a correlation of both, chemokine mix and MCP-1 induced migration with BMI, fat mass and HOMA-IR (data not shown), and a correlation of chemokine-mix induced migration with the percentage of $CCR2^+$ total monocytes ($r = 0.451$, $P = .035$, Pearson's correlation, n = 10 obese and n = 16 lean subjects). An increase of CCR5 surface density was observed on $CD14^+CD16^+$ monocytes, whereas for CCR2 we observed an increased receptor surface density on total monocytes, on $CD14^+CD16^-$ monocytes, and notably solely for CCR2 expression we found a significant increase in the percentage of receptor positive $CD14^+CD16^+$ monocytes (Supplemental Table 9).

Together, results indicate that the cell-intrinsic migratory capacity of monocytes towards the different tested chemokines and the chemokine-mix seems to be associated with expression of CCR2 on monocytes as well as with anthropometric markers and insulin resistance.

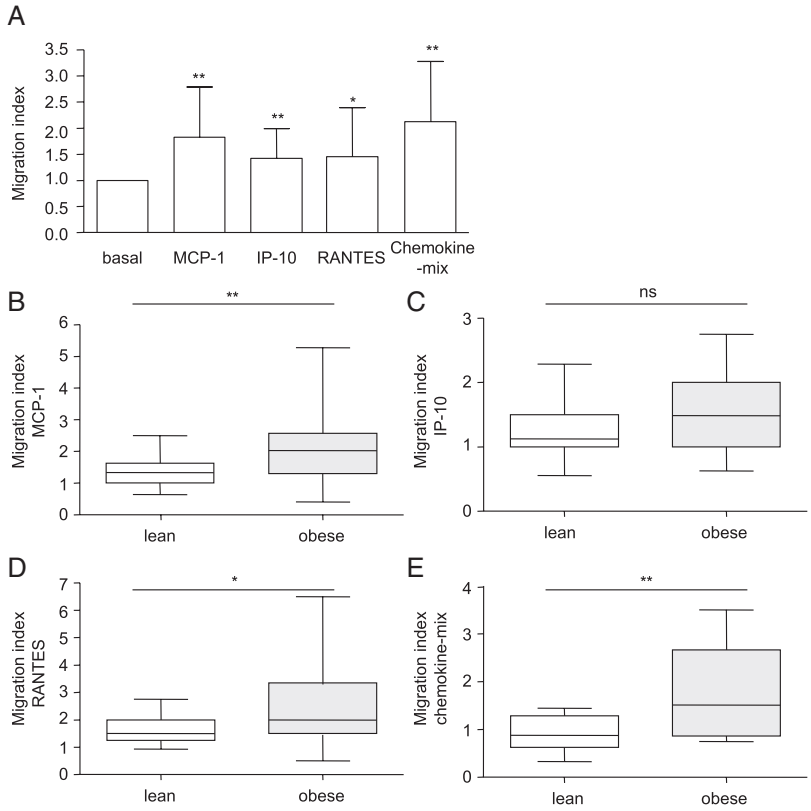

Figure 4. Chemokine induced monocyte migration. (A) The chemoattractants MCP-1, IP-10, RANTES and a chemokine-mix (MCP-1, IP-10, RANTES) efficiently induced monocyte migration (n = 48, n = 20 for RANTES). Data are presented as mean ± SD. Analysis was performed using sample *t* test with $*P < .05$, $**P < .001$. (B-E) Migration indices of monocytes from lean and obese women according to the chemokines MCP-1 (B), IP-10 (C), RANTES (D) and the chemokine-mix (E). Lean/obese groups in (B-E) with n = 19/29, 19/29, 8/12 and 19/29 subjects, respectively; results are expressed as chemotactic activity (fold increase of monocyte response to stimulants over the basal response to medium alone). Data are presented as median (horizontal bar) with 25th and 75th percentiles (boxes) and extreme values (whiskers). Analysis was performed using independent-sample *t* test with $*P < .05$, $**P < .001$; ns, not significant.

Discussion

In the present study we report an enhanced cell-intrinsic migration capacity of monocytes from obese as compared to lean women and a correspondingly increased surface expression of chemokine receptors, in particular for CCR2, on peripheral blood monocytes in obese vs normal-weight women. Overall, our results provide further evidence for the close link between peripheral immune cell function and obesity.

In the first part of the study, we were interested to elucidate whether peripheral monocytes differ in their inflammatory status according to BMI. We found a significant increase of $CD14^+CD16^+$ monocytes in obese subjects compared to lean subjects suggesting that peripheral monocytes from obese are activated. These data are consistent with recent findings demonstrating an increased frequency of activated $CD14^+CD16^+$ monocytes in obesity (22, 23).

The investigation of chemokine receptor expression patterns may help to extend our knowledge on cell type-specific function of immune cells in obesity-associated diseases like T2D or atherosclerosis. Regarding AT infiltration, to date, most studies were dealing with chemokine and chemokine receptor expression in tissue resident cells (12). However, it remains unclear whether this up-regulation is restricted to AT or can also be observed in peripheral blood. It was therefore one specific aim of our study to investigate the expression of chemokine receptors on monocyte subsets. The chemokine receptor profile on monocytes differed significantly between lean and obese subjects, in particular CCR2 and CCR5 showed a significantly increased expression on monocytes of obese women. Taking monocyte heterogeneity into account, in obese we found an upregulation in both, the percentage and surface density of $CCR2^+$ cells for the $CD14^+CD16^+$ subpopulation of activated monocytes, and of $CCR5^+$ cells for the $CD14^+CD16^-$ subpopulation. A low expression of these receptors was previously described in the respective monocyte subpopulations in healthy humans (24). Diverse studies established the essential role of CCR2 for monocyte recruitment into AT and an association with insulin resistance (2, 7 – 10, 25, 26), more recent studies the function of CCR5 in AT (11, 27), and moreover the importance of both receptors in atherosclerosis (28, 29). In addition to the well-established role of chemokine receptor expression in adipose tissue from obese subjects, our finding of increased chemokine receptor levels on distinct monocyte subpopulations corroborates an obesity-related functional role of these receptors also on peripheral leukocytes. The mechanisms leading to enhanced chemokine receptor expression in monocytes remain unknown.

The most prominent finding was the increased migratory ability of peripheral monocytes in human obesity. It has been previously reported that mice harboring a myeloid-specific IKKβ or JNK1 deletion and fed a high-fat diet develop obesity similar to wild-type animals, but are relatively protected from obesity-induced glucose intolerance and hyperinsulinemia (30, 31). In addition, IKKβ and

Table 2. Percentages of peripheral blood monocyte sub-populations from obese compared with lean controls.

	Lean (n = 16)	Obese (n = 14)	
% positive cells	mean ± SD	mean ± SD	P-value*
Total monocytes	81.28 ± 5.81	82.83 ± 4.61	0.430
$CD14^+CD16^-$ monocytes	87.30 ± 4.56	80.72 ± 7.94	0.009
$CD14^+CD16^+$ monocytes	12.70 ± 4.56	19.3 ± 7.94	0.009

* Independent-sample *t* test.

Table 3. Migrated monocytes from lean *vs.* obese women.

	Lean		Obese		
Migration index	mean ± SD	n	mean ± SD	n	P-value*
MCP-1	1.4 ± 0.5	19	2.1 ± 1.1	29	0.005
IP-10	1.2 ± 0.5	19	1.6 ± 0.6	26	0.059
RANTES	0.9 ± 0.4	8	1.8 ± 1.0	12	0.017
Chemokine-mix	1.6 ± 0.5	19	2.4 ± 1.3	29	0.006

* Independent-sample *t* test. Migration of monocytes from lean and obese was quantified by counting monocytes in the lower chamber. The results are expressed as migration index (fold increase of monocyte response to stimulants over the response to medium alone).

JNK deficient mice have lower levels of AT macrophages and a reduced expression of genes related to inflammatory pathways with decreased tissue cytokine levels in AT, liver, and even muscle tissue. Thus, these knock-out mice are protected from systemic insulin resistance, which might be partly due to a reduced migration of monocytes into AT. Moreover, chemokine hyperresponsiveness of lymphocytes derived from an obesity mouse model was recently implicated in lymphocyte homing to the liver (32). These results together with the increased chemokine receptor surface expression on monocytes of obese subjects shown here imply that monocytes from obese human individuals may have increased migratory properties. In order to address this hypothesis, a chemotactic assay was established to assess the effect of chemokines with different cell-type- and chemokine-receptor-specificity on monocyte migration. IP-10-induced migration revealed no differences between both groups reflecting both the reported predominant action on T cell migration (33, 34) and our finding that expression of the IP-10 receptor CXCR3 was not altered in monocytes from obese and lean women. In contrast, a significantly higher chemotactic activity of monocytes was observed in the migration assay towards the chemokines MCP-1, RANTES and the chemokine-mix in the obese group. For both chemokines, increased expression levels were reported in AT of obese (2, 7 – 10, 20, 21) and in atherosclerotic lesions (34). The increased migratory capacity of monocytes from obese is in accordance with the enhanced expression of the chemokine receptors. Therefore, the elevated expression of these receptors in obese subjects might cause the increased monocyte migration-capacity compared to normal weight subjects. This activation of peripheral monocytes may contribute to the increased AT monocyte infiltration in obesity and monocyte infiltration involved in the pathophysiology of atherosclerosis. Moreover, adoptive transfer experiments in mice recently suggested a predominant role of AT signals vs monocyte preactivation for AT infiltration (35), thus future experiments are needed to further elucidate the contribution of monocyte preactivation for AT infiltration in humans.

Plasma hsCRP levels above 10 mg/l may indicate clinically significant inflammation (36), the number of subjects with increased hsCRP levels are higher in obese as compared to nonobese humans (37), and CRP induces both, CCR2 expression and MCP1-induced migration in monocyte cell lines in vitro (38, 39). Here, even when we excluded subjects with plasma hsCRP levels above 10 mg/l, we found an enhanced chemokine induced monocyte migration and an increase particularly for CCR2 levels on diverse monocyte subpopulations in obese. However, considering the small sample size in the subanalysis, detailed studies on the interaction of inflammatory status and migration capacity and the specific role of CCR2 and CCR5 receptors in obesity require larger populations. Similarly, to assess whether the increase of migration capacity may be accentuated in obese subjects with Metabolic Syndrome, for which an increased inflammatory gene expression in PBMCs was reported (6), the number of subjects in our cohort was too small and should be analyzed in future studies.

Correlation analyses were performed to dissect whether the increased migratory ability of monocytes from obese subjects is associated with anthropometric and metabolic variables and chemokine receptor expression. Both BMI and fat mass were found to correlate positively with the migratory activity towards MCP-1, RANTES and the chemokine-mix. WHR did not show any association with these parameters indicating that fat mass might play a more prominent role than body fat distribution in this context. Furthermore, a relationship between insulin resistance and proinflammatory PBMCs as well as recruitment of macrophages into AT has been proposed (2, 10, 25, 40). In our study, the migration capacity of monocytes towards the chemoattractants MCP-1, RANTES and the chemokine-mix were also positively correlated with HOMA-IR, a marker of insulin resistance. In addition, the migratory ability towards all tested chemokines showed an association with CCR2 expression levels on mono-

cytes. Even after excluding obese women with moderately elevated plasma hsCRP levels, a correlation of chemokine-mix induced migration with monocyte CCR2 levels was evident. Therefore, it might be speculated that macrophage accumulation in obese AT could be, at least in part, due to an enhanced chemotactic response caused by up-regulation of CCR2 expression on monocytes. The mechanisms of the association of enhanced monocyte migration capacity with insulin resistance are still poorly understood, and it is an open question whether these processes are causally interconnected or independent events. Of note, as we have only investigated adult females, the findings of our study cannot be generalized for males.

In contrast to a previous report (41), we did not observe an increase of plasma chemokine levels. However, Huber et al showed increased MCP1 and CCR2 expression levels in adipose tissue, without increased systemic levels of plasma MCP1 (12). Thus, a local plasma-tissue chemokine gradient may contribute to increased infiltration of peripheral monocytes into adipose tissue, independent of increased plasma chemokine levels. Moreover, the effect of other chemokines, which are implicated in obesity or atherosclerosis-related monocyte homing, on peripheral monocytes with increased migration-capacity and possible AT infiltration remains elusive.

In conclusion, monocytes from obese compared to normal-weight women are in an inflammatory state with enhanced expression of chemokine receptors, in particular CCR2 on peripheral monocytes. Thus, the increased infiltration of macrophages into AT from obese women may not only be caused by an increased secretion of chemokines from AT, but may also be due to the enhanced migratory capacity of peripheral monocytes, probably caused by the increased chemokine receptor expression. Thus, our results provide further evidence of an altered function of peripheral immune cells in obese subjects further provoking a systemic inflammatory response in obesity.

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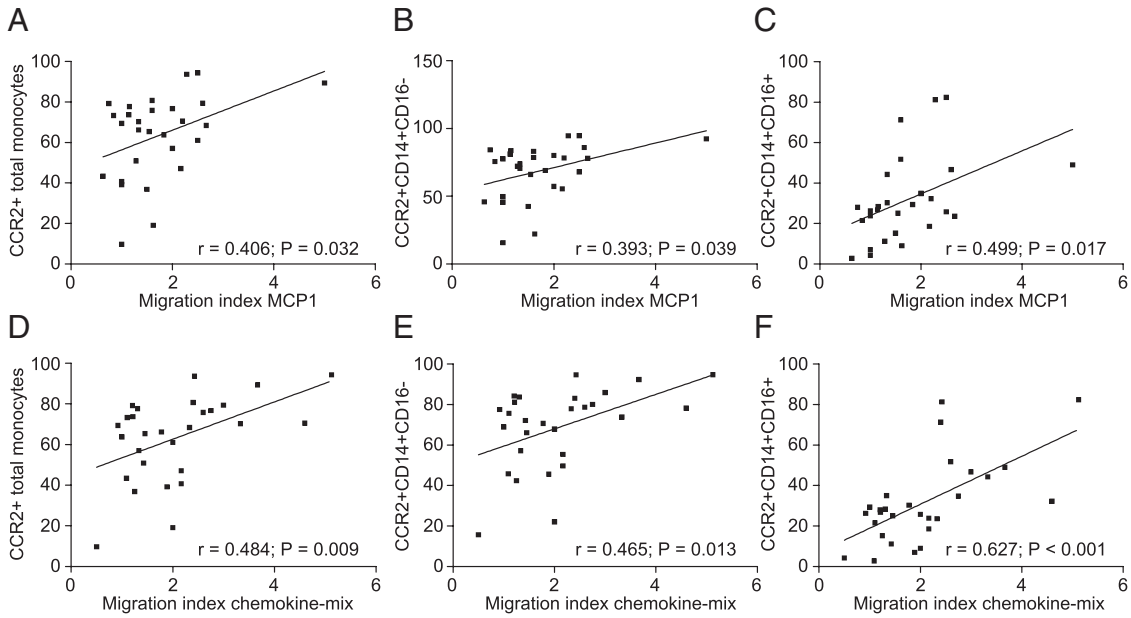

Figure 5. Correlation of CCR2 expression levels with monocyte migration capacity. The correlation of CCR2 chemokine receptor expression (number of $CCR2^+$ cells) with migration capacity (shown as migration index) in response to both, MCP-1 (A-C) and to chemokine-mix (D-F) is shown for total monocytes (A, D), $CD14^+CD16^-$ monocytes (B, E), and $CD14^+CD16^+$ monocytes (C, F). Data from 16 lean and 14 obese women; the regression coefficient was obtained from linear regression analysis and correlations between variables are expressed as Pearson's correlation coefficients.

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