Increased monocytic CD14+HLADR^{low/-} myeloid-derived suppressor cells in obesity

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Abstract. Obesity is associated with numerous immunological disorders. The present study investigated the proportion and phenotype of myeloid-derived suppressor cells (MDSCs) in the plasma of obese subjects and the association of these cells with the level of liver enzymes. Certain features of the immune response in obese subjects were examined by analyzing the expression of T cell receptor-ζ (TCRζ) molecules on the surface of T cells. The expression and secretion of S100A9, a possible marker for MDSCs, were detected in the peripheral blood of obese subjects and compared with levels in lean controls. Results showed that the percentage of monocytic MDSCs, with the phenotype CD33+CD11b+CD14+HLADR^{low/-}, was significantly increased in obese subjects compared with lean controls. The circulating level of monocytic MDSCs was positively correlated with the levels of liver enzymes in serum. The expression of the TCRζ molecule in the resting T cells was significantly lower in obese individuals than that of lean controls. The expression of S100A9 was detected in the majority of monocytes in peripheral blood mononulear cells, but no difference was identified in the frequency of CD14+S100A9+ cells between the obese and lean groups. However, the plasma level of S100A8/9 was significantly increased in obese compared with lean subjects. These observations suggested that the increased frequency of CD33+CD11b+CD14+HLADR^{low/-} cells may be responsible for the impaired T-cell function and liver injury observed in obesity.

Introduction

Obesity may be regarded as a low-level chronic inflammatory state, and is associated with type 2 diabetes mellitus, cardiovascular disease and a number of cancer types, such as breast cancer, colorectal cancer and prostate cancer (1,2).

Adipose tissue is viewed as an endocrine organ, which produces a wide range of pro-inflammatory cytokines, such as monocyte chemoattractant protein-1 (MCP-1), interlukin-6 (IL-6) and tumor necrosis factor-α (TNF-α). These molecules are collectively termed adipokines (3). Immunological dysregulation in obese subjects may result from interactions between white adipose tissue and the immune system (4). Obesity affects macrophage accumulation in fat stores, as well as T cell-mediated responses (5). Evidence has arisen suggesting that a reduction in levels of circulating T regulatory cells (Tregs) has an impact on the increased risk of metabolic and cardiovascular dysfunctions observed in obese patients (6). Myeloid-derived suppressor cells (MDSCs) are a population of myeloid cells, which act to suppress the proliferation and function of effector T cells (7) via alteration of the signal-transducing molecule, TCRζ, on the surface of T cells (8). Decreased TCRζ chain expression in circulating T cells has been reported in a number of types of cancer, including melanoma, and ovarian, breast, renal and oral squamous cell carcinoma, and is associated with a compromised antitumor response as a result of reduced T cell proliferation and cytokine production (9,10). MDSCs have been investigated in numerous types of cancer. However, there have been few studies into the phenotype and functions of these cells in obese patients.

The S100A8 and S100A9 proteins have been identified as members of the S100 Ca^{2+}-binding protein family, and are known to form a heterodimeric complex, S100A8/A9, which is involved in mediating the inflammatory response (11). Recently, it has been suggested that S100A8/A9 proteins may serve as a novel marker for monocytic MDSCs (12). However, whether S100A8/A9 proteins are also elevated in obese subjects, and thus affect the frequency of monocytic MDSCs in these individuals, requires further investigation.

The present study characterized the proportion and phenotypes of MDSCs in the circulation of obese subjects compared with those in lean subjects and examined whether MDSCs correlate with levels of liver enzymes. In addition, the levels of S100A9/9 were measured and the proportion of CD14+S100A9+ cells in obese and lean subjects was examined.

Materials and methods

Subject recruitment and blood sample preparation. A total of 16 adult Chinese men, including eight

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Table I. Description of clinical information of the two groups.

	Lean subjects (n=8)	Overweight/obese subjects (n=8)
Age (years)	30.10±2.714	32.55±2.413
BMI (kg/m^2)	21.01±0.80	28.81±0.99[a]
Monocyte number	0.418±0.063	0.407±0.0256

[a]P<0.05 compared with lean subjects; BMI, body mass index.

overweight/obese subjects (BMI >25 kg/m^2) and eight lean healthy controls (BMI <25 kg/m^2) were recruited between February and June 2013. Subjects with type 2 diabetes mellitus, hepatitis or other medical comorbidities likely to impact on the immune system were excluded. The clinical information from the two groups is summarized in Table I. The study was approved by the Medical Ethical Committee of the Second Hospital of Jiaxing, (Jiaxing, China). Blood samples were collected from all study subjects using vacuette sodium heparin-containing tubes (BD Biosciences, Franklin Lakes, NJ, USA) and further processed within 24 h of collection. Plasma was isolated by centrifugation at 1000 x g for 10 min and stored at -20˚C for further analyses. Liver serum parameters, alanine transaminase (ALT) and aspartate transaminase (AST), were determined by an automated chemistry analyzer (D2400/P800; Roche Diagnostics, Basel, Switzerland).

Ultrasonography. Ultrasonography was performing using a B-mode of Ultrasound (iU22 xMATRIX, Philips Healthcare, Andover, MA, USA). Screening for fatty liver was based on a B ultrasonic diagnosis according to the standard guidelines of the Chinese Medical Association (13).

Flow cytometric analysis. To determine the proportion and phenotypes of MDSCs in total plasma leukocytes, multicolor fluorescence-activated cell sorting (FACS) analysis was conducted using freshly-collected whole blood and fluoro-chrome-conjugated mouse anti-human monoclonal antibodies against CD11b, CD14, CD16, CD15, CD33 and HLADR, as well as isotype control antibodies (BD Pharmingen, San Diego, CA, USA). S100A9 was obtained from BioLegend Inc. (San Diego, CA, USA) and staining was performed according to the manufacturer's instructions. Intracellular staining of TCRζ (BD Pharmingen) was conducted using the methods described previously (14). In this experiment, peripheral blood mononulcear cells (PBMCs) were isolated from 4 ml whole blood. Blood in a vacuette tube was transferred to a 15 ml conical tube (BD Falcon, Franklin Lakes, NJ), diluted to a volume of 8 ml with 1 x PBS (Solarbio, Beijing, P.R.China), and under-layed with 4 ml of Ficoll Paque (Sigma-Aldrich, St. Louis, MO, USA). The PBMCs were collected at the interface layer following centrifugation of the conical tube at 400 x g for 30 min. Data were collected using a BD FACSCanto II flow cytometer, and analyzed with DIVA software (version V6.1.3, BD Pharmingen).

ELISA assay. Plasma was obtained from obese and lean subjects, and stored at -20˚C. The plasma concentration of
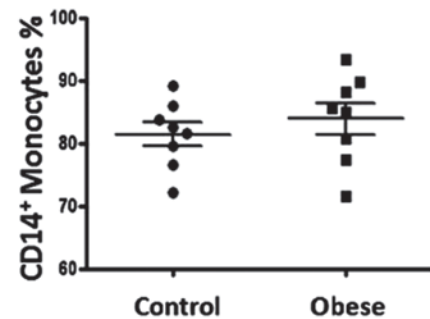

Figure 1. Percentage of CD14$^+$ cells in the monocyte fraction from the peripheral blood. Using flow cytometry, the monocyte fraction from whole blood cells was gated and the percentage of CD14$^+$ cells was analyzed. The was no significant difference in the percentage of CD14$^+$ monocytes in the whole blood from obese and lean subjects (83.91±2.490 compared with 81.36±1.893%, respectively; P>0.05). Data are expressed as the mean ± standard deviation.

S100A8/9 was measured using a commercially available ELISA kit, according to the manufacturer's instructions (Cuisabio Biotech Co., Ltd., Wuhan, China).

Statistical analysis. Data are presented as the mean ± standard error of the mean. Differences in means between groups of data were analyzed using Student's unpaired t-test. Correlation analysis was performed using Pearson's correlation with the coefficient of correlation (r^2). Statistical analysis was performed, and figures created, using Prism Version 5.0 software (GraphPad Software Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

CD14$^+$ monocyte numbers in the peripheral blood of obese subjects. The total number of monocytes in the peripheral blood in each group was analyzed. The absolute number of monocytes in peripheral blood was not significantly different in obese individuals compared with lean controls (P>0.05; Table I). The percentage of CD14$^+$ monocytes was further analyzed in whole blood by flow cytometry. No significant difference was observed between the obese and lean groups (83.91±2.490 compared with 81.36±1.893%, respectively; P>0.05; Fig. 1).

Phenotypical characterization of myeloid-derived suppressor cells in obese subjects. To determine whether the proportion of granulocytic and monocytic MDSCs, two previously identified subtypes of MDSCs (15), was increased in obese
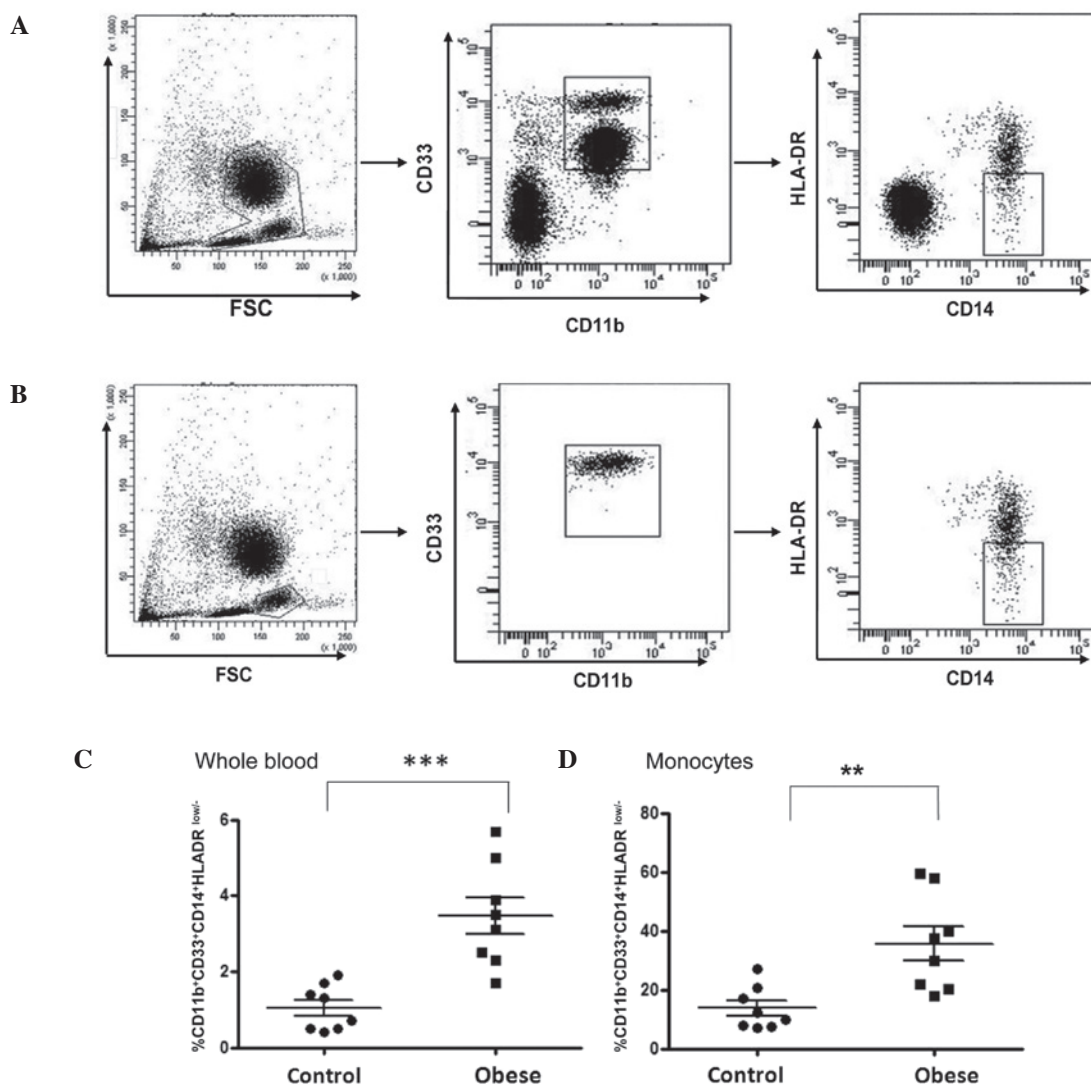
Figure 2. Identification and characterization of a population of CD14$^+$HLADR$^{low/-}$ in whole white blood cells, including lymphocytes, monocytes and granulocytes, and in monocytes only from obese and lean control individuals. (A) Results of flow cytometry. Cells were gated on the whole white cells fraction and then, based on the higher expression of CD11b and CD33, the CD11b$^+$CD33$^+$ fraction of the ,CD14$^+$ and HLADR$^{low/-}$ population were further gated and analyzed. (B) Results of flow cytometry with cells gated on the monocytic fraction only and subsequently gated on CD11b$^+$CD33$^+$ fraction. The CD14$^+$ and HLADR$^{low/-}$ population were further gated and analyzed. (C) Percentage of CD11b$^+$CD33$^+$CD14$^+$HLADR$^{low/-}$ MDSCs in the whole white cell fraction in obese and lean individuals (3.46±0.48 versus 1.05±0.21; ***P<.001). (D) Percentage of CD11b$^+$CD33$^+$CD14$^+$HLADR$^{low/-}$ MDSCs in the monocytic fraction in obese and lean individuals (35.56±5.77 and 13.69±2.57, **P <0.01). Data are expressed as the mean ± standard deviation. MDSCs, myeloid-derived stem cells; FSC, forward scatter.

subjects, these two populations were analyzed using flow cytometric analysis. MDSCs have been defined as a heterogeneous cell population with a granulocytic phenotype CD33$^+$CD11b$^+$CD14$^-$HLADR$^-$ in renal cell carcinoma, glioblastoma and gastric cancer (16-18). In addition, monocytic MDSCs, with a phenotype of CD14$^+$CD11b$^+$HLADR$^{low/-}$, have also been described in circulating leukocytes of patients with melanoma (19). In the present study, granulocytic MDSCs were defined as those expressing CD33 and CD11b, but that were CD14$^-$, with low/- HLADR. Monocytic MDSCs have alternatively been described as a population of cells with a typical CD33$^+$CD11b$^+$CD14$^+$HLADR$^{low/-}$ phenotype. The gating strategy used is shown in Fig. 2. Cells were gated on the whole blood cell fraction (Fig. 2A) as well as the monocytic fraction only (Fig. 2B). The percentage of monocytic MDSCs was significantly increased in the obese group compared with that in the
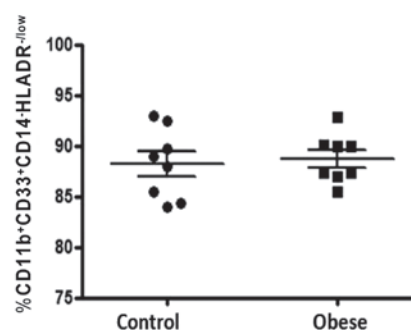

Figure 3. Percentage of CD11b$^+$ CD33$^+$CD14$^-$HLADR$^{low/-}$ in whole white cell fraction. Cells were gated on whole blood cells as shown in Figure 2A. Subsequently, CD14$^-$ and HLADR$^{low/-}$ population was further analyzed in the CD11b$^+$ CD33$^+$ fraction. The frequency of CD11b$^+$ CD33$^+$CD14$^-$HLADR$^{low/-}$-MDSCs was not significantly different in the whole white cell fraction between obese and lean individuals (88.71±0.84 and 88.19±1.25, P>0.05). Data are expressed as the mean ± standard deviation.
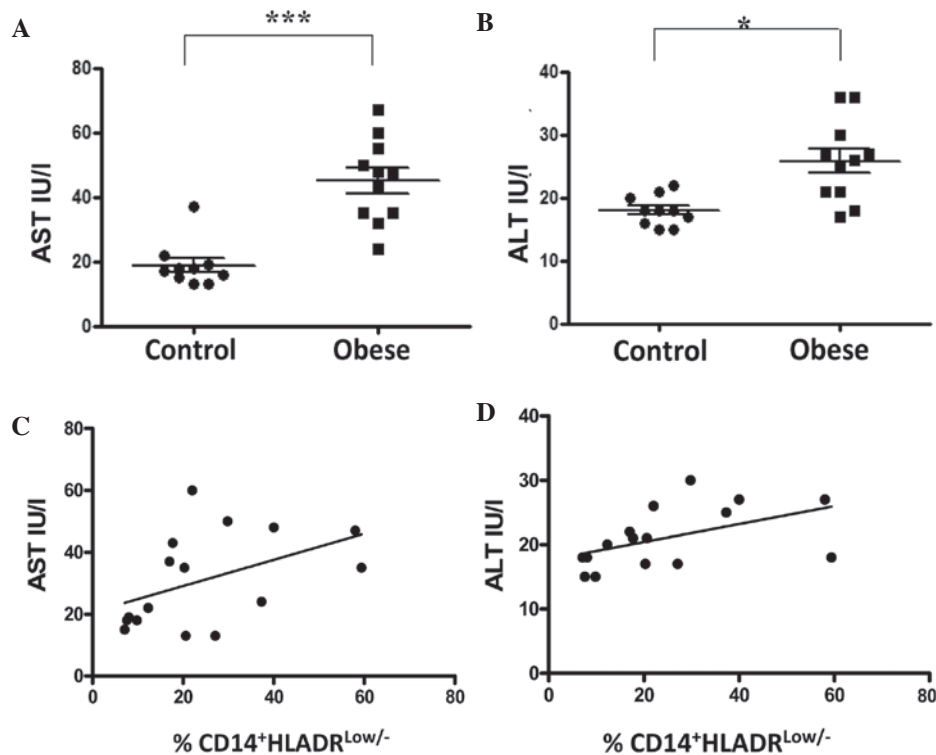
Figure 4. Plasma concentration of liver enzymes in obesity and the association with proportion of CD14⁺HLADR^{low/-} monocytes. (A) Serum AST levels in the obese and lean groups (42.75±3.94 and 19.38±2.75 IU/l, respectively). ***P<0.001. (B) Serum ALT levels in the obese and lean groups (23.88±1.49 and 18.25±0.92 IU/l, respectively). *P<0.05. The frequency CD14⁺HLADR[Low/-] monocytes was positively correlated with (C) AST levels and (D) ALT levels. All correlations had P<0.05. ALT, alanine transaminase; AST, aspartate transaminase.

age-matched lean control group when gated on the whole cell fraction (3.46±0.48 compared with 1.05±0.21%, respectively; P<0.001; Fig. 2C) as well as when gated on the monocytic fraction only (35.56±5.77 compared with 13.69±2.57, respectively; P<0.01*; Fig. 2D). However, there was no significant difference in the percentage of CD33⁺CD11b⁺CD14⁻HLADR^{low/-} between obese and lean control groups when gated on whole blood cell fraction (88.71±0.84 compared with 88.19±1.25%, respectively; P>0.05; Fig. 3).

Serum levels of liver enzymes are correlated with the proportion of CD14⁺HLADR^{low/-} cells in obese subjects. Elevated serum levels of ALT and AST are markers of liver injury. In the present study, it was observed that the overweight obese individuals had elevated circulating levels of ALT compared with lean control subjects (23.88±1.49 vs. 18.25±0.92 IU/l, respectively; P<0.05; Fig. 4A). Levels of AST were also significantly higher in the obese group than in the lean group (42.75±3.94 compared with 19.38±2.75 IU/l, respectively; P<0.0001; Fig. 4B). The association between serum levels of ALT and AST and the proportion of CD14⁺HLADR^{low/-} monocytes in peripheral blood was examined. A positive correlation was observed between AST and CD14⁺HLADR^{low/-} monocytes (r=0.50, 95% CI -0.009 to 0.803; P<0.05; Fig. 4C); as well as between ALT and CD14⁺HLADR^{low/-} monocytes (r=0.58, 95% CI 0.102 to 0.840; P<0.05; Fig. 4D).

Expression of TCRζ is decreased in the PBMCs of obese subjects. To evaluate the expression of TCRζ in the peripheral T cell population, flow cytometric analysis was performed
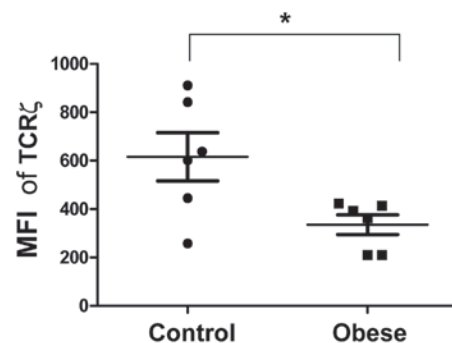

Figure 5. Expression of TCRζ molecules in resting peripheral T cells. Intracellular staining was performed to detect the TCRζ molecule in peripheral blood mononuclear cells of obese and lean subjects (335.35±40.57 and 616±99.36 MFI, respectively; P=0.0259). TCR, T-cell receptor; MFI, mean fluorescence intensity.

to measure TCRζ mean fluorescence intensity (MFI) in the CD3⁺ resting T cell population. The expression of TCRζ was reduced in obese subjects compared with lean controls (335.35±40.57 compared with 616±99.36 MFI, respectively; P=0.0259; Fig. 5)

Expression of S100A9 in CD14⁺ monocytes. The pro-inflammatory heterodimeric S100A8/9 complex is elevated in a number of chronic inflammatory diseases, including rheumatoid arthritis, cystic fibrosis and inflammatory bowel disease (20,21). S100A9 has been proposed as a novel marker for monocytic human MDSCs in certain types of malignancy (12). To
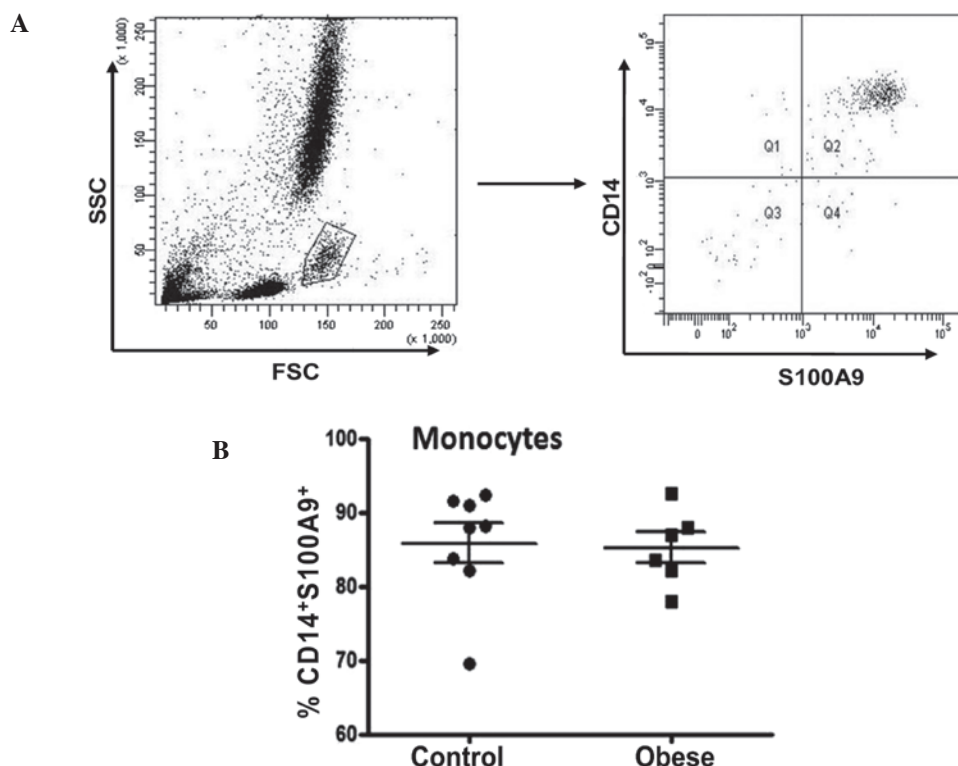
Figure 6. Analysis of S100A9 expression in CD14$^+$ monocytes of whole blood cells from obese and lean subjects. (A) Cells were gated on monocytes and a quadrum was then used on the basis of CD14 and S100A9 expression. Q2 population represents CD14$^+$ and S100A9$^+$ monocytes. (B) Frequency of CD14$^+$S100A9$^+$ in obese and lean subjects (85.20±2.07 and 85.80±2.67%, respectively; P>0.05). Data are expressed as the mean ± standard deviation. SSC, side scatter.
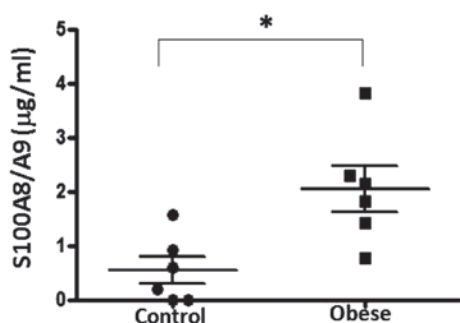

Figure 7. Plasma levels of S100A8/9 in obese and lean subjects (2.04±0.423 and 0.548±0.252 µg/ml, respectively; *P<0.05). Data are expressed as the mean ± standard deviation.

investigate whether S100A9 serves as a marker for monocytic MDSCs in obesity, S100A9-specific antibodies were used to detect this intracellular molecule. As shown in Fig. 6A, cells were gated on the monocytic fraction following flow cytometry using whole peripheral blood. The majority of CD14$^+$ monocytes were S100A9$^+$ (Fig. 6A), and there was no difference in the proportion of CD14$^+$S100A9$^+$ between obese subjects and lean controls (85.20±2.07 compared with 85.80±2.67%, respectively; P>0.05; Fig. 6B). No statistically significant correlation was identified between CD14$^+$HLADR$^{low/-}$ MDSC and CD14$^+$S100A9$^+$ cells in the monocyte population (data not shown). In monocytes, a small population with a CD16$^+$CD14$^-$ phenotype has previously been identified (22). The current study found that S100A9 was present in CD14$^+$ cells but not in CD16$^+$ cells (data not shown).

Plasma levels of S100A8/A9 in obese subjects. The plasma levels of S100A8/A9 were significantly increased in obese subjects compared with age-matched lean controls (2.04±0.423 compared with 0.548±0.252 µg/ml, respectively; P<0.05; Fig. 7).

Discussion

Monocytes respond to immune-stimulating cytokines (23). These are derived from pluripotent hematopoietic stem cells in the bone marrow, and can rapidly and efficiently differentiate into dendritic cells (23). The impaired immune response observed in obesity may be associated with the accumulation of macrophages in adipose tissue as a result of the attraction of monocytes from the circulation (23). The present study reports that an increased frequency of monocytic MDSCs appears to be amongst the immunosuppressive responses promoted by obesity.

The current study reports an increased population of CD14$^+$HLADR$^{low/-}$-monocytes in obese subjects compared with lean subjects. CD14$^+$HLADR$^{low/-}$ monocytic MDSCs have been documented in patients with melanoma (19), and Non-Hodgkin lymphoma (NHL) (24). The increased proportion of CD14$^+$HLADR$^{low/-}$ monocytes leads to immunosuppression by impairing T cell functions, including inhibiting T cell proliferation, reducing their response to stimuli and reducing production of interferon γ (24).

Decreased monocyte expression of HLADR has been observed in a range of malignancies, such as hepatocellular carcinoma, prostate cancer and glioblastoma (25). In addition

to tumors, an increased frequency of CD14⁺HLADR$^{low/-}$ cells has also been reported in non-malignant conditions, such as sepsis (26), pancreatitis (27) and acute liver failure (28). Furthermore, a low percentage of monocytic HLADR expression serves as a predictor of poor clinical outcome in acute liver failure (29).

White adipose tissue is now regarded as a key endocrine organ, secreting a wide range of adipokines including leptin, adiponectin, TNF-α and IL-6. These molecules act locally or distantly to regulate energy balance and other physiological processes, such as insulin sensitivity and inflammatory responses (30,31). Pro-inflammatory cytokines may contribute to the loss of HLADR expression in monocytes in obese subjects. Obesity is associated with a spectrum of liver abnormalities and is known to cause nonalcoholic fatty liver disease (NAFLD) (32). In addition to regulating energy balance, the liver is also involved in mediating the immune response. Although no liver biopsies were performed in the current study, ultrasound examination indicated fatty livers in all obese subjects. In the present study, a significant positive correlation was observed between the proportion of CD14⁺HLADR$^{low/-}$ monocytes and levels of the liver enzymes, AST and ALT. This data led to the hypothesis that the induction of these immunosuppressive monocytes may contribute to liver dysfunction in obesity. Recently, data has shown that human activated hepatic stellate cells are able to convert mature peripheral blood monocytes into MDSCs, and that this action may prevent ensuing liver injury (33). Therefore, further studies are required to investigate whether the elevated number CD14⁺HLADR$^{low/-}$ monocytes originates from the injured liver or from the circulation. Furthermore, the question of how those immunosuppressive cells interact with hepatocytes in NAFLD should also be addressed.

MDSCs have been reported to suppress autologous T cell proliferation and reduce the expression of TCRζ on the surfaces of CD8⁺ cells. The ζ chain of TCR is a key component responsible for the initiation of immune responses mediated by T cells and natural killer cells (34). Reduced TCRζ expression in circulating lymphocytes has been reported in patients with ovarian cancer, breast cancer, oral cancer and melanoma (35). The reduced expression of TCRζ molecule in obese subjects indicates that CD14⁺HLADR$^{low/-}$ monocytes may lead to immunosuppression via regulation of TCRζ expression. The upregulation of CD14⁺HLADR$^{low/-}$ and downregulation of T cell function may be associated with the increased incidence of cancer in obese patients.

S100A8/9 is produced by myeloid and tumor cells (34), and elevated S100A8/9 can in turn induce the production of MDSCs in patients with cancer (36). Circulating levels of S100A8/9 have been shown to be upregulated in a number of tumor types, including gastric cancer (18). Blocking S1008/9 and its receptor, RAGE, on the surface of MDSCs may restore T cell proliferation (18). Recently, S100A9 has been proposed to be a novel marker for monocytic MDSCs in colon cancer (12). In the present study, S100A9 was found to be highly expressed in the majority of CD14⁺ monocytes and no association was found between CD14⁺S100A9⁺ and CD14⁺HLADR$^{low/-}$ cells. Thus, whether S100A9 should be used as a marker for monocytic MDSCs requires further clarification. The elevation of S100A8/9 in the plasma of

obese subjects may further induce the production of monocytic MDSCs in obesity.

In conclusion, to the best of our knowledge, the current study reports for the first time that the proportion of an immunosuppressive monocytic population with a CD14⁺HLADR$^{low/-}$ phenotype is significantly elevated in the circulation of obese subjects compared with that of lean control subjects. The increased frequency of this cell population is positively correlated with the concentration of liver enzymes in the plasma. The expression of the TCRζ molecule was found to be downregulated in obese patients. In addition CD14⁺HLADR$^{low/-}$ monocytes are not correlated with CD14⁺S100A9⁺ expression. The plasma concentration of S100A8/9 was found to be increased in obesity. These findings suggest that the upregulation of CD14⁺HLADR$^{low/-}$ may be responsible for the impaired T-cell function and liver injury observed in obesity.

Acknowledgements

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