Morbidly Obese Human Subjects Have Increased Peripheral Blood CD4$^+$ T Cells With Skewing Toward a Treg- and Th2-Dominated Phenotype

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Obesity is associated with local T-cell abnormalities in adipose tissue. Systemic obesity-related abnormalities in the peripheral blood T-cell compartment are not well defined. In this study, we investigated the peripheral blood T-cell compartment of morbidly obese and lean subjects. We determined all major T-cell subpopulations via six-color flow cytometry, including CD8$^+$ and CD4$^+$ T cells, CD4$^+$ T-helper (Th) subpopulations, and natural CD4$^+$CD25$^+$FoxP3$^+$ T-regulatory (Treg) cells. Moreover, molecular analyses to assess thymic output, T-cell proliferation (T-cell receptor excision circle analysis), and T-cell receptor-β (TCRB) repertoire (GeneScan analysis) were performed. In addition, we determined plasma levels of proinflammatory cytokines and cytokines associated with Th subpopulations and T-cell proliferation. Morbidly obese subjects had a selective increase in peripheral blood CD4$^+$ naive, memory, natural CD4$^+$CD25$^+$FoxP3$^+$ Treg, and Th2 T cells, whereas CD8$^+$ T cells were normal. CD4$^+$ and CD8$^+$ T-cell proliferation was increased, whereas the TCRB repertoire was not significantly altered. Plasma levels of cytokines CCL5 and IL-7 were elevated. CD4$^+$ T-cell numbers correlated positively with fasting insulin levels. The peripheral blood T-cell compartment of morbidly obese subjects is characterized by increased homeostatic T-cell proliferation to which cytokines IL-7 and CCL5, among others, might contribute. This is associated with increased CD4$^+$ T cells, with skewing toward a Treg- and Th2-dominated phenotype, suggesting a more anti-inflammatory set point. ***Diabetes*** **61:401–408, 2012**

Obesity is a major cause of preventable death in the Western world (1), and its prevalence is rapidly increasing (2). Type 2 diabetes mellitus and cardiovascular disease are responsible for the majority of obesity-related morbidity and mortality (2). Obesity is primarily considered to be a metabolic disease. However, in recent years, it has become clear that obesity is also associated with immunological abnormalities (3). These abnormalities probably result from intricate adipose-immune interactions (4) and contribute a great deal to obesity-related morbidity (5).

Immunological abnormalities associated with obesity are often seen as a state of chronic low-grade inflammation. This state of chronic low-grade inflammation is nowadays considered to be crucial in the development of long-term complications of obesity, such as diabetes (6,7) and atherosclerosis (8). The state of chronic low-grade inflammation has long been thought to be primarily due to an accumulation of proinflammatory macrophages within the adipose tissue and the production of proinflammatory cytokines by adipocytes and macrophages, such as tumor necrosis factor (TNF)-α and interleukin (IL)-6 (9). However, T-cell accumulation was demonstrated recently in both mouse and human obese adipose tissue (10–12), which even preceded macrophage accumulation (13,14). Therefore, T cells are thought to be important participants in the initiation of adipose tissue inflammation (9). This idea is further supported by the finding that T-cell depletion reduced adipose tissue macrophage accumulation and improved insulin sensitivity in mice fed a high-fat diet (13,15). Altogether, several lines of evidence suggest a direct link between obesity and a deregulated T-cell accumulation within adipose tissue (9).

Given the systemic nature of obesity, it can be anticipated that the peripheral blood T-cell compartment is affected as well. So far, however, only a limited number of studies have investigated the composition of the peripheral blood immune system in obesity. Positive correlations have been reported between BMI and total white blood cell count (16–19) and T-cell numbers in peripheral blood (16–18,20), but conflicting data have been published as well (21). In the peripheral blood T-cell compartment, increased CD4$^+$ and normal CD8$^+$ T-cell numbers have been found (16,17), whereas both subpopulations were found to be decreased in another study (21). To date, however, studies on CD4$^+$ T-cell subpopulations, T-cell proliferation history, and T-cell diversity are lacking.

In this study, we performed a detailed analysis of the peripheral blood T-cell compartment in morbidly obese and lean subjects. For this purpose, we determined the absolute counts and relative frequencies of all major T-cell subpopulations via six-color flow cytometry, including CD8$^+$ T cells; CD4$^+$ T cells; the CD4$^+$ T-cell subpopulations T-helper (Th)1, Th2, and Th17 cells; and natural CD4$^+$CD25$^+$FoxP3$^+$ T-regulatory (Treg) cells. These numerical analyses were combined with molecular analyses to assess thymic output, T-cell proliferation (T-cell receptor excision circle [TREC] analysis), and T-cell receptor-β (TCRB) repertoire usage

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(GeneScan analysis). In addition, we determined plasma levels of proinflammatory cytokines (IL-6 and TNF-α); cytokines associated with Th1, Th2, or Th17 subpopulations (γ-interferon [IFN-γ], IL-4, and IL-17A); and cytokines involved in T-cell proliferation, survival, and recruitment (CCL5, IL-2, and IL-7).

RESEARCH DESIGN AND METHODS

A total of 13 morbidly obese (BMI >40 kg/m^2) and 25 lean (BMI <25 kg/m^2) healthy control subjects were included in this study. Subjects with overt type 2 diabetes mellitus or liver enzyme test abnormalities were excluded. The presence of concomitant medical illness was excluded by medical history assessment in morbidly obese and lean subjects. All subjects gave their written informed consent. The study was approved by the medical ethical committee of Erasmus University Medical Center.

Blood was obtained using vacuette sodium heparin–containing tubes (Greiner Bio-one, Alphen a/d Rijn, the Netherlands) and further processed within 1 h after collection. Plasma was isolated by centrifugation and frozen for further analyses. Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll density separation and viably frozen for further analyses.

Flow cytometry and cell sorting. Total leukocyte count was measured in freshly collected blood using a Coulter Counter (Beckman Coulter B.V., Woerden, the Netherlands), and leukocyte subpopulations were determined by flow cytometry based on CD45 expression and sideward scatter. For immunophenotyping of T-cell subpopulations, viably frozen PBMCs were used. Antibodies used for flow cytometric analyses and sorting experiments are summarized in Supplementary Table 1. T-cell subpopulations were defined as naive (CD45RA$^+$ and CD27$^+$), central memory (CD45RO$^+$ and CD27$^+$), effector memory (CD45RO$^+$ and CD27$^-$; although it is known that a small population of effector memory cells does still express CD27) (22), and terminally differentiated (CD45RA$^+$ and CD27$^-$). Natural Treg cells were defined as CD4$^+$CD25$^+$FoxP3$^+$. For intracellular cytokine detection, PBMCs were stimulated with phorbol-12-myristate-13-acetate (PMA; 50 ng/mL; Sigma-Aldrich, St. Louis, MO) and ionomycin (500 ng/mL; Invitrogen Ltd., Paisley, UK) for 4 h in the presence of GolgiStop (BD Biosciences, San Jose, CA). Thereafter, cells were stained for extracellular markers, fixed with 2% paraformaldehyde, and permeabilized with 0.5% saponin, followed by intracellular staining for IFN-γ, IL-4, and IL-17A. Stained cells were measured using a FACS LSR-II (BD Biosciences), and data were analyzed with FlowJo software (TreeStar, Ashland, OR). Th1 cells were defined as CD4$^+$IFN-γ$^+$, Th2 cells as CD4$^+$IL-4$^+$, and Th17 cells as CD4$^+$IL-17A$^+$.

The following T-cell subpopulations were sorted with a purity of >90% in all samples using a FACSAria (BD Biosciences): CD4$^+$ naive (CD3$^+$TCR-γδ$^-$CD4$^+$CD45RO$^-$CD27$^+$), CD4$^+$ memory (CD3$^+$TCR-γδ$^-$CD4$^+$CD45RO$^+$), CD8$^+$ naive (CD3$^+$TCR-γδ$^-$CD8$^+$CD45RO$^-$CD27$^+$), and CD8$^+$ memory (CD3$^+$TCR-γδ$^-$CD8$^+$CD45RO$^+$) T cells. Total αβ-T cells were isolated with a purity of >90% in all samples using AutoMACS with allophycocyanin (APC)–labeled anti–TCR-αβ antibodies and anti-APC cell beads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany).

Signal joint TREC analysis. Signal joint TREC (sjTREC) analysis was used to evaluate thymic output and peripheral T-cell proliferation. The sjTREC is a stable circular DNA structure that is formed during TCR-α rearrangements in developing thymocytes (Supplementary Fig. 1*A*) (23). Because the sjTREC, in contrast to genomic DNA, is not duplicated during cell proliferation, it will dilute out during consecutive cell divisions, making it a useful marker to determine proliferation history in αβ-T cells (Supplementary Fig. 1*B*) (24).

To determine sjTREC dilution, DNA was extracted from different T-cell subpopulations using the GeneElute Mammalian Genomic DNA miniprep kit (Sigma-Aldrich). The sjTREC was detected by real-time quantitative PCR using an ABI Prism 7900 machine (Applied Biosystems, Foster City, CA) and the following primer and probe combination: forward primer 5′-CCATGCTGACACCTGGTT-3′, reverse primer 5′-TCGTGAGAACGGTGAATGAAG-3′, and probe 5′-CACGGT GATGCATAGGCACCTGC-3′ as described previously (24). To correct for total DNA input, the amount of albumin gene was determined (forward primer 5′-TGAACAGGCGACCATGCTT-3′, reverse primer 5′-CTCTCCTTCTCAGAAAGTGT-GCATAT-3′, and probe 5′-TGCTGAAACATTCACCTTCCATGCAGA-3′) (25). Cycle threshold values for sjTREC were normalized to albumin cycle threshold values (25). For copy number quantification, serial dilutions of a pGEM-T Easy vector (Promega Benelux BV, Leiden, the Netherlands), in which the sjTREC sequence was cloned, were used (24).

GeneScan analysis for in-frame TCRB gene rearrangements. To determine TCRB diversity, we performed GeneScan analysis for Vβ–Jβ gene rearrangements on DNA of sorted T-cell subpopulations with multiplex PCR as previously described (26).

Cytokine analysis. Plasma levels of IL-2 (sensitivity >16.4 pg/mL), IL-4 (sensitivity >20.8 pg/mL), IL-6 (sensitivity >1.2 pg/mL), IL-10 (sensitivity >1.9 pg/mL), IL-12p70 (sensitivity >1.5 pg/mL), IL-17A (sensitivity >2.5 pg/mL), IFN-γ (sensitivity >1.6 pg/mL), CCL5 (sensitivity >25 pg/mL), and TNF-α (sensitivity >3.2 pg/mL) were measured simultaneously using bead-based FlowCytomix simplex kits (Bender Medsystems GmbH, Vienna, Austria). Plasma levels of IL-7 were measured by ELISA (Invitrogen Ltd).

Glucose and insulin measurements. Fasting blood glucose levels were measured with a Hitachi 917 Chemistry Analyzer (Roche Diagnostics, Almere, the Netherlands). Fasting insulin levels were measured using a chemiluminescent immunometric assay (Immulite 2000; Siemens Medical Solutions Diagnostics, Los Angeles, CA).

Statistical analysis. Subject characteristics are described as mean ± SD. The exact Mann-Whitney *U* test was used for statistical comparisons between morbidly obese and lean subjects. Statistics are displayed as median (range). All statistical analyses were performed with SPSS software version 15.0 (SPSS Inc., Chicago, IL). $P < 0.05$ (two-tailed) was considered statistically significant. Box-and-whisker plots display the 2.5 to 97.5 percentiles. Error bars are expressed as the SEM.

RESULTS

Peripheral blood CD4$^+$ T-cell numbers are increased in morbid obesity. An initial general examination of the blood samples did not reveal clear differences in the total leukocyte number or the numbers of distinct leukocyte subpopulations between morbidly obese and lean subjects (characteristics of the subjects are summarized in Table 1). However, a trend toward increased lymphocyte numbers was present in morbidly obese subjects (Fig. 1*A*). Detailed flow cytometric analyses on isolated PBMCs revealed that NK- and B-cell numbers did not differ between the morbidly obese and lean subjects (Fig. 1*B*). T-cell numbers, however, were significantly ($P < 0.01$) increased in morbidly obese subjects. This was mainly due to a twofold increase in CD4$^+$ T cells ($P < 0.01$), whereas CD8$^+$ T-cell numbers remained normal ($P = 0.35$) (Fig. 1*C*). This resulted in an increased CD4-to-CD8 ratio (morbidly obese 2.82 [1.62–6.17] vs. lean 1.54 [1.29–5.23], $P = 0.03$, data not shown).

Peripheral blood CD4$^+$ T-cell subpopulations that display an anti-inflammatory phenotype are increased in morbid obesity. Next, we performed extensive flow cytometric analyses to determine whether distinct T-cell subpopulations are affected in morbidly obese subjects. Within the CD8$^+$ T-cell compartment, cell numbers within the different subpopulations were similar in morbidly obese and lean subjects (Fig. 2*A*). Within the CD4$^+$ T-cell compartment, increased numbers of naive ($P = 0.04$), central memory ($P < 0.01$), and terminally differentiated ($P = 0.03$) T cells were found in morbidly obese subjects, whereas no differences were found in the effector memory subpopulation between morbidly obese and lean subjects (Fig. 2*A*). Also, absolute counts of natural CD4$^+$CD25$^+$FoxP3$^+$ Treg cells were

TABLE 1
Characteristics of morbidly obese and lean healthy subjects

	Subjects	
	Lean	Morbidly obese
Flow cytometric and cytokine analysis		
n	11	8
BMI (kg/m^2)	23.2 ± 1.4	42.4 ± 6.7
Age (years)	34 ± 9	45 ± 10
Female/male	9/2	8/0
TREC and GeneScan analysis		
n	14	13
BMI (kg/m^2)	23.9 ± 1.9	42.1 ± 5.9
Age (years)	31 ± 7	48 ± 11
Female/male	12/2	13/0

Data are mean ± SD.

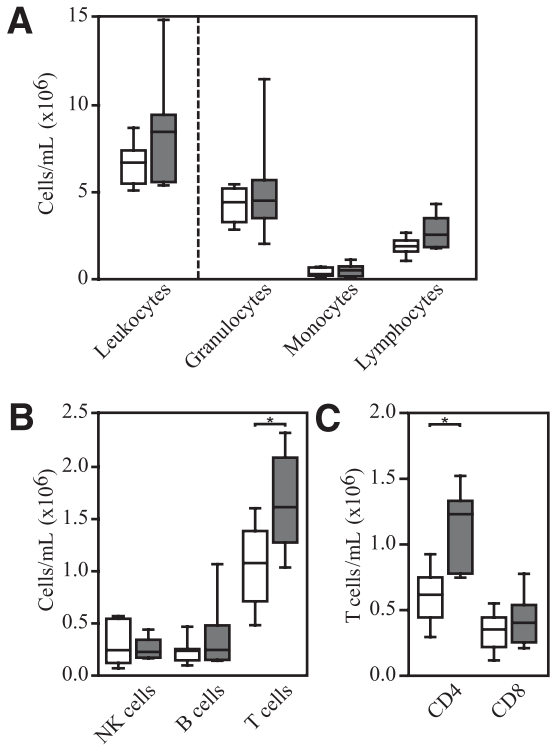

FIG. 1. Absolute counts of leukocyte subpopulations (*A*), PBMC subpopulations (*B*), and CD4$^+$ and CD8$^+$ T cells (*C*) in peripheral blood of morbidly obese and lean subjects. CD4, CD4$^+$ T cells; CD8, CD8$^+$ T cells. White bars represent lean subjects (n = 11, BMI 23.2 ± 1.4 kg/m^2); gray bars represent morbidly obese subjects (n = 8, BMI 42.4 ± 6.7 kg/m^2). *P < 0.05.

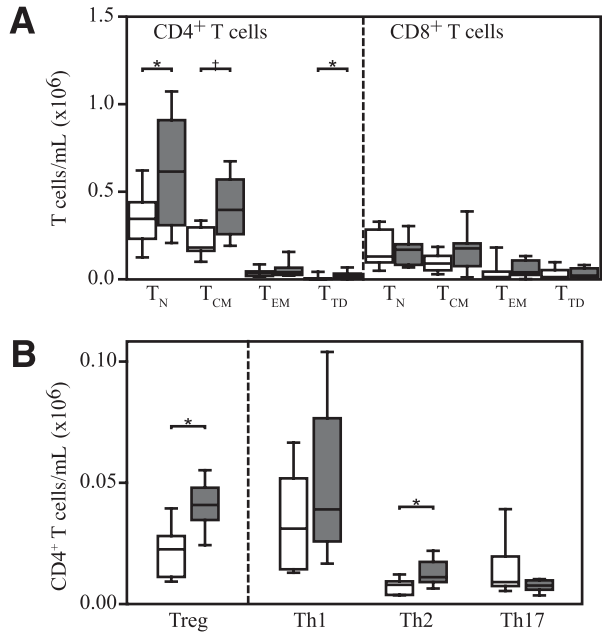

FIG. 2. *A*: Absolute counts of CD4$^+$ and CD8$^+$ T-cell subpopulations in peripheral blood of morbidly obese and lean subjects. *B*: Absolute counts of natural CD4$^+$CD25$^+$FoxP3$^+$ Treg cells, IFN-γ–producing (Th1) cells, IL-4–producing (Th2) cells, and IL-17A–producing (Th17) cells in peripheral blood of morbidly obese and lean subjects within the CD4$^+$ T-cell gate. T_N, naive T cells; T_{CM}, central memory T cells; T_{EM}, effector memory T cells; T_{TD}, terminally differentiated T cells. White bars represent lean subjects (n = 11, BMI 23.2 ± 1.4 kg/m^2); gray bars represent morbidly obese subjects (n = 8, BMI 42.4 ± 6.7 kg/m^2). *P < 0.05, †P < 0.01.

increased ($P < 0.01$) (Fig. 2*B*). Effector CD4$^+$ T-cell subpopulations were determined by measuring the intracellular cytokine profile after stimulation with PMA and ionomycin. The numbers of IFN-γ–producing T cells (Th1) and IL-17A–producing T cells (Th17) were similar in morbidly obese and lean subjects, whereas the number of IL-4–producing T cells (Th2) was increased in morbidly obese subjects (P = 0.03) (Fig. 2*B*).

Additional correlation analyses demonstrated a significant correlation between BMI and the number of total T cells as well as the numbers of CD4$^+$ T cells, naive CD4$^+$ T cells, terminally differentiated CD4$^+$ T cells, central memory CD4$^+$ T cells, natural CD4$^+$CD25$^+$FoxP3$^+$ Treg cells, and Th2 cells. For age, only a significant correlation was observed with the number of Th2 cells (Supplementary Table 2).

Taken together, these data demonstrate that morbid obesity is associated with increased naive and memory CD4$^+$ T cells and with increased numbers of anti-inflammatory natural CD4$^+$CD25$^+$FoxP3$^+$ Treg cells and Th2 cells.

Proliferation of CD4$^+$ and CD8$^+$ T cells is increased in morbid obesity. Several mechanisms, including increased thymic output, increased peripheral proliferation, decreased apoptosis, or altered redistribution, can account for the observed increased CD4$^+$ T-cell numbers found in morbidly obese subjects. To distinguish between increased thymic output and increased peripheral proliferation or survival, the sjTREC content in peripheral blood T-cell subpopulations was determined (Supplementary Fig. 1*C*). A significantly lower sjTREC content, which together with increased cell numbers resembles increased proliferation (Supplementary Fig. 1*C*), was found in total αβ-T cells ($P < 0.01$) and CD4$^+$ naive (P = 0.03), CD4$^+$ memory (P = 0.02), and CD8$^+$ naive (P = 0.02) T-cell subpopulations of morbidly obese subjects (Fig. 3*A*). Moreover, a significant negative correlation ($P < 0.01$) was found between αβ-T cell sjTREC content and BMI (Fig. 3*B*, *left*).

A negative correlation between sjTREC content and age has been reported previously (25), but although the morbidly obese group was significantly older than the lean control group (morbidly obese aged 45 years [28–62] vs. lean aged 31 years [25–51]; P = 0.02), the sjTREC content in αβ-T cells did not correlate significantly with age (Fig. 3*B*, *right*), although we cannot exclude that this might be due to the relatively limited number of subjects studied. Moreover, in multiple regression analysis, the BMI was the only variable significantly associated with the TREC content (R = 0.8, R^2 = 0.58, P_{total} = 0.001, P_{age} = 0.28, P_{BMI} = 0.002), demonstrating that the decreased sjTREC content in morbid obesity is mainly determined by obesity and not by age.

Overall, the decreased sjTREC content together with the increased T-cell numbers in morbidly obese subjects is indicative of increased proliferation within the T-cell compartment of these subjects.

Increased T-cell proliferation in morbid obesity is not driven by dominant antigens. Several studies describe a reduced diversity within the TCRB repertoire of T cells isolated from adipose tissue of obese mice, suggesting a local antigen-driven immune response toward the main antigens present within adipose tissue (13,27). We determined TCRB diversity in peripheral blood T-cell subpopulations. We observed a diverse TCRB repertoire in CD4$^+$ and CD8$^+$ naive and memory T cells from both morbidly obese and lean subjects (Fig. 4*A* and *B*). Minor alterations in the

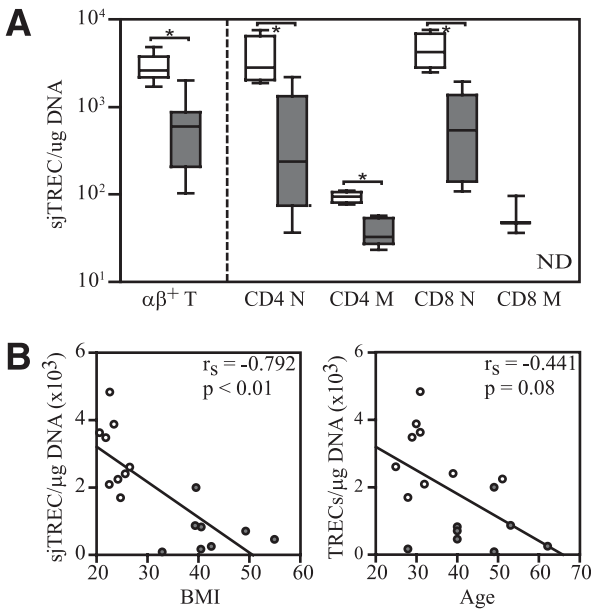

FIG. 3. *A*: sjTREC content in total αβ$^+$-T cells and T-cell subpopulations of morbidly obese and lean subjects. αβ$^+$ T, αβ$^+$-T cells; CD4 N, CD4$^+$ naive T cells; CD4 M, CD4$^+$ memory T cells; CD8 N, CD8$^+$ naive T cells; CD8 M, CD8$^+$ memory T cells; ND, not detectable. *B*: Correlation between sjTREC content and BMI (*left*) and age (*right*) in morbidly obese and lean subjects. White bars or dots represent lean subjects, αβ$^+$-T cells (n = 10, BMI 24.0 ± 2.2 kg/m^2), T-cell subpopulations (n = 4, BMI 23.6 ± 1.0 kg/m^2); gray bars or dots represent morbidly obese subjects, αβ$^+$-T cells (n = 8, BMI 42.4 ± 6.7 kg/m^2), T-cell subpopulations (n = 5, BMI 41.6 ± 5.1 kg/m^2). *P < 0.05.

(normally Gaussian distributed) TCRB repertoire were found to a limited extent in the CD4$^+$ memory T cells and CD8$^+$ naive and memory T cells of morbidly obese subjects, suggesting the existence of a slightly skewed TCRB repertoire in morbidly obese subjects (Fig. 4*A* and *B*). In addition, we obtained fat tissue that was removed during surgery from five other morbidly obese subjects. No peripheral blood T cells were available from these patients because only adipose tissue and plasma samples were stored. Nevertheless, it gave us an opportunity to investigate the TCR repertoire of adipose tissue T cells. Hence, we performed GeneScan analysis on the T cells present in the adipose tissue. We observed a polyclonal TCRB repertoire (Supplementary Fig. 2), indicating that there was no strong skewing toward particular T-cell clones. Instead a rather broad TCR repertoire was present in the adipose tissue T cells.

T-cell growth factors in plasma are elevated in morbid obesity. Because obesity is characterized by abnormal production of proinflammatory cytokines (28), we hypothesized that cytokines involved in T-cell proliferation, survival, and recruitment might also be produced in excess in morbidly obese subjects. Therefore, we determined a broad panel of cytokines in plasma from morbidly obese and lean subjects.

Plasma levels of the proinflammatory cytokines IL-6 and TNF-α did not differ between morbidly obese and lean subjects (Fig. 5*A*). Also, plasma levels of IFN-γ, IL-4, and IL-17A, cytokines respectively associated with Th1, Th2, or Th17 subpopulations, were similar in morbidly obese and lean subjects (Fig. 5*B*).

The cytokines CCL5, IL-2, and IL-7 enhance T-cell proliferation, survival, and recruitment (29–31). Plasma levels of CCL5 ($P < 0.01$) and IL-7 ($P < 0.01$) were significantly elevated in morbidly obese subjects (Fig. 5*C*) and correlated positively with BMI (Supplementary Table 3). IL-2 plasma levels were similar in morbidly obese and lean subjects (Fig. 5*C*).

As expected, IL-7 and CCL5 plasma levels positively correlated with total CD4$^+$ T-cell numbers but not with total CD8$^+$ T-cell numbers (Fig. 5*D* and *E*). In the CD4$^+$ T-cell compartment, a positive correlation was found between IL-7 plasma levels and the number of naive CD4$^+$ T cells, terminally differentiated CD4$^+$ T cells, central memory CD4$^+$ T cells, and natural CD4$^+$CD25$^+$FoxP3$^+$ Treg cells; CCL5 plasma levels correlated positively with the number of terminally differentiated CD4$^+$ T cells, central memory CD4$^+$ T cells, and natural CD4$^+$CD25$^+$FoxP3$^+$ Treg cells (Supplementary Table 4).

Increased CD4$^+$ T-cell numbers correlate with fasting insulin levels in morbid obesity. Although the morbidly obese subjects did not have type 2 diabetes mellitus, we investigated the correlations between the increased CD4$^+$ T-cell numbers and metabolic measures. Fasting glucose and insulin levels were determined in the morbidly obese group only (32). A significant correlation was found between fasting insulin levels and CD4$^+$ T-cell numbers (Fig. 6*A*). Moreover, the glucose-to-insulin ratio was calculated as a measure of insulin sensitivity. This ratio also correlated with CD4$^+$ T-cell numbers (Fig. 6*B*). Fasting insulin levels and insulin sensitivity did not correlate with CD8$^+$ T-cell numbers (Fig. 6*C* and *D*). No significant correlations were found between fasting blood glucose levels and T-cell subpopulations (data not shown).

DISCUSSION

This study is the first to comprehensively investigate the peripheral blood T-cell compartment of morbidly obese subjects. Our main finding was a selective increase in CD4$^+$ T-cell numbers within the peripheral blood T-cell compartment of morbidly obese subjects. Peripheral blood CD8$^+$ T-cell numbers were normal in morbidly obese subjects. This latter observation is in contrast with the increased numbers of local effector and memory CD8$^+$ T cells described in adipose tissue of obese subjects (12,13).

In mice, diet-induced obesity results in reduced sjTREC content in splenic CD4$^+$ T cells (33). This is accompanied by a reduction in naive T cells and a more restricted TCRB repertoire, suggesting that in this mouse model, the decrease in sjTREC content is mainly the result of reduced thymic output (33). Because ageing also is associated with a reduction in thymic output, resulting in a reduced TREC content and a reduction in naive T-cell numbers, it was suggested that obesity is related to accelerated ageing of the T-cell compartment (33,34).

In our study, we found a decreased sjTREC content in peripheral blood T-cell subpopulations of morbidly obese subjects. However, in contrast to observations in ageing studies (35,36), the decreased sjTREC content was accompanied by increased numbers of naive as well as memory CD4$^+$ T cells and only an insignificant skewing of the TCRB repertoire. Therefore, despite the limitation of the significant age difference between morbidly obese and lean subjects in our study, we conclude from the increased naive T-cell numbers that the decrease in sjTREC content in morbidly obese subjects predominantly results from increased proliferation rather than accelerated ageing and decreased thymic output. This notion is further supported by the decreased telomere length observed in leukocytes of obese subjects (37).

The increased proliferation within the peripheral blood T-cell compartment is more likely of homeostatic nature

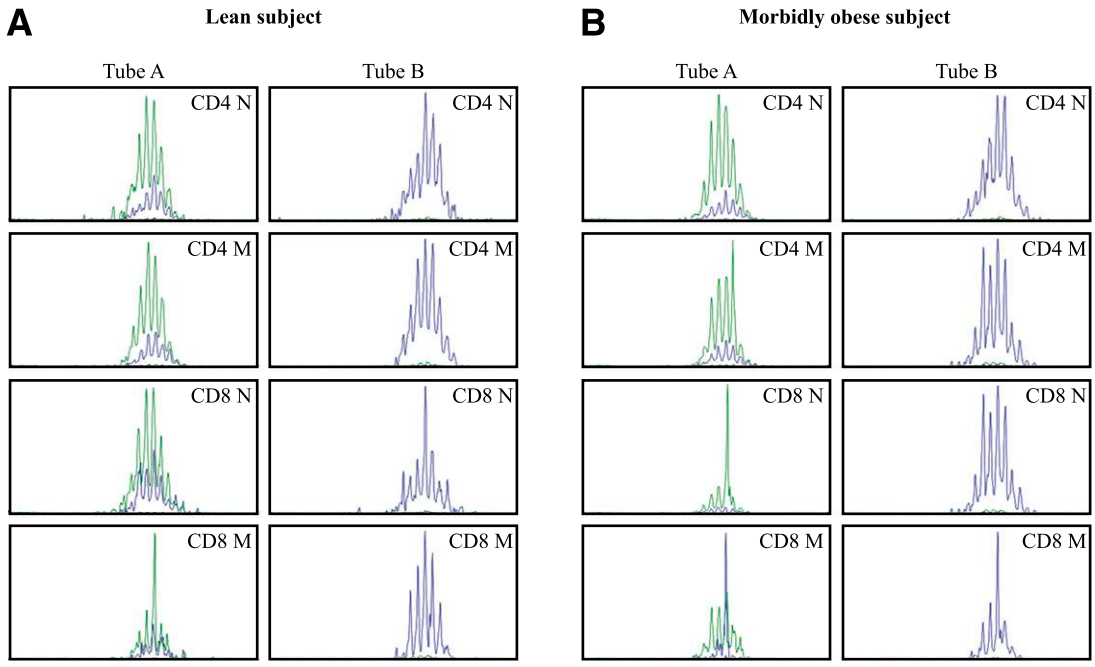

FIG. 4. GeneScan analysis of Vβ–Jβ rearrangements in CD4$^+$ and CD8$^+$ naive and memory T-cell subpopulations in a representative lean (*A*) and morbidly obese (*B*) subject. Tube A: Vβ+Jβ1.1 to Jβ1.6+Jβ2.2+Jβ2.6+Jβ2.7; Tube B: Vβ+Jβ2.3+Jβ2.4+Jβ2.5. Primers for the Jβ1 cluster were HEX-labeled (green line); primers for the Jβ2 cluster were FAM-labeled (blue line). CD4 N, CD4$^+$ naive T cells; CD4 M, CD4$^+$ memory T cells; CD8 N, CD8$^+$ naive T cells; CD8 M, CD8$^+$ memory T cells. Lean subjects (n = 4, BMI 23.6 ± 1.0 kg/m^2); morbidly obese subjects (n = 5, BMI 41.6 ± 5.1 kg/m^2).

rather than driven by dominant antigens because the latter would result in increased memory and effector T-cell subpopulations with prominent skewing of the TCRB repertoire, whereas the naive T-cell compartment would remain unaffected. In our cohort, we do not see such changes in the peripheral blood T-cell compartment. Moreover, in an additional analysis, a rather polyclonal TCR repertoire was observed in adipose tissue T cells of morbidly obese subjects. It therefore seems likely that there is no vast change in TCR repertoire in the adipose tissue T cells in our cohort. However, we formally cannot exclude the possibility of some skewing of the TCRB repertoire within adipose tissue T cells.

Several T-cell mitogenic factors, such as adipokines, fatty acids, or bacterial products, can be elevated in plasma of morbidly obese subjects (38–40). Also, increased levels of IL-7 and CCL5, cytokines capable of stimulating homeostatic T-cell proliferation, survival, and recruitment (29–31), have been found in adipose tissue of obese mice and men (41–43). We also found highly elevated plasma levels of IL-7 and CCL5 in morbidly obese subjects in this study, which positively correlated with peripheral blood CD4$^+$ T-cell numbers. On the basis of these data, we hypothesize that IL-7 and CCL5, as well as the other T-cell mitogenic factors, might contribute to the increased homeostatic CD4$^+$ T-cell proliferation in morbidly obese subjects.

Despite the selective increase in CD4$^+$ T-cell numbers in peripheral blood, CD8$^+$ T cells also displayed decreased sjTREC content, indicating that CD8$^+$ T cells also undergo increased homeostatic proliferation due to the increased IL-7 and CCL5 cytokine levels in morbidly obese subjects. However, peripheral blood CD8$^+$ T-cell numbers were not increased, suggesting a selective redistribution of CD8$^+$ T cells into adipose tissue, which is in line with the described preferential accumulation of CD8$^+$ T cells in obese adipose tissue (12,13). Also, CCL5 is a more potent chemoattractant for CD4$^+$ T cells than for CD8$^+$ T cells (44), and in obesity, systemic levels of CCL5 are ~100-fold higher than those locally produced within adipose tissue (41). Therefore, the elevated CCL5 plasma levels that we observed may contribute to the selective retention of CD4$^+$ T cells in peripheral blood of morbidly obese subjects.

With regard to the increased numbers of peripheral blood CD4$^+$ T cells, we observed that this was accompanied by a selective increase in natural CD4$^+$CD25$^+$FoxP3$^+$ Treg cell numbers. In addition, stimulation of PBMCs with PMA/ionomycin specifically induced a Th2 phenotype within the CD4$^+$ T-cell compartment of morbidly obese subjects. This indicates that the numerically elevated peripheral blood CD4$^+$ T-cell compartment of morbidly obese subjects is skewed toward a Treg- and Th2-dominated phenotype, suggesting a more anti-inflammatory set point. Despite this clear skewing, plasma levels of cytokines associated with the Th2 phenotype were mostly undetectable in plasma from both morbidly obese and lean subjects, as was the case for cytokines associated with the Th1 and Th17 phenotypes.

Natural CD4$^+$CD25$^+$FoxP3$^+$ Treg cells and Th2 cells are capable of polarizing monocytes/macrophages toward an anti-inflammatory M2 phenotype, which is characterized by the production of anti-inflammatory mediators such as IL-1 receptor antagonist, IL-10, and transforming growth factor-β (45,46). We hypothesize that the preferential skewing of the CD4$^+$ T-cell compartment toward a Treg- and Th2-dominated phenotype can be considered as a mechanism to counterregulate the proinflammatory activity that exists systemically and locally within the monocyte/macrophage compartment (47–50) in obesity. The absence of increased IL-6 and TNF-α plasma levels in our cohort of morbidly obese subjects supports this notion.

We demonstrated this anti-inflammatory T-cell set point in a morbidly obese cohort that was selected on the basis of being nondiabetic and, thus, relatively healthy and free

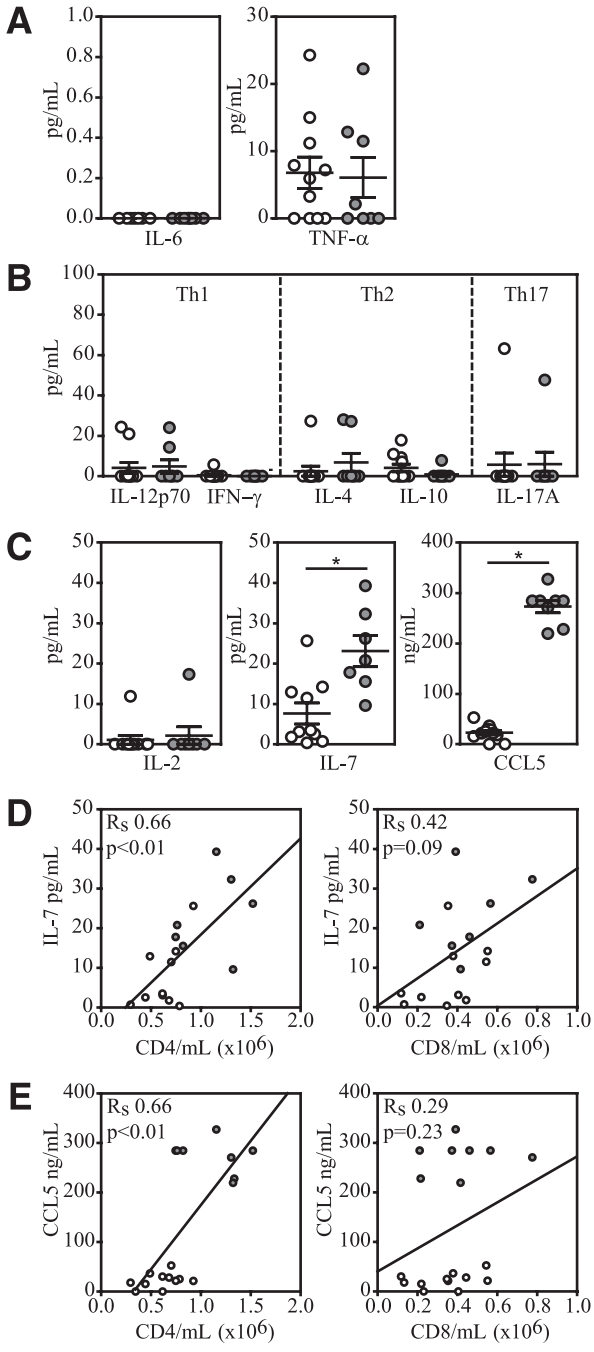

FIG. 5. Plasma levels of IL-6 and TNF-α (*A*); Th1 cytokines IL-12p70 and IFN-γ, Th2 cytokines IL-4 and IL-10, and the Th17 cytokine IL-17A (*B*); and IL-2, IL-7, and CCL5 in morbidly obese and lean subjects (*C*). *D*: Correlation between IL-7 plasma levels and CD4$^+$ or CD8$^+$ T-cell counts in peripheral blood. *E*: Correlation between CCL5 plasma levels and CD4$^+$ or CD8$^+$ T-cell numbers in peripheral blood. White dots represent lean subjects (n = 11, BMI 23.2 ± 1.4 kg/m^2); gray dots represent morbidly obese subjects (n = 8, BMI 42.4 ± 6.7 kg/m^2). *P < 0.05.

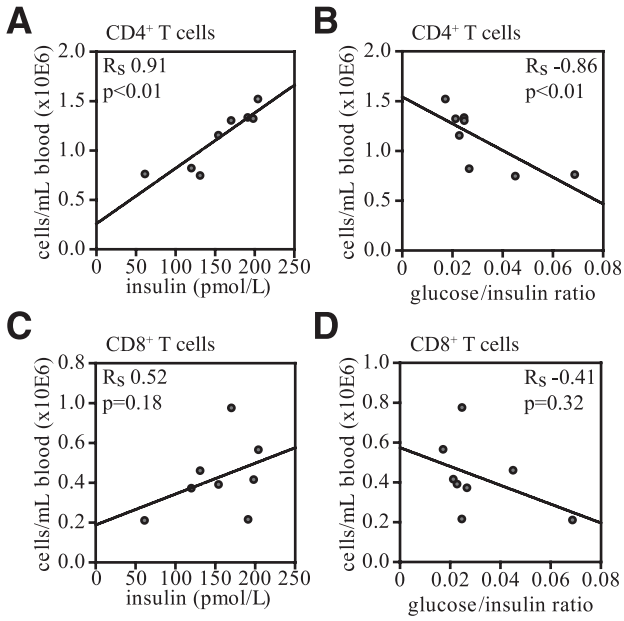

FIG. 6. Correlation between CD4$^+$ T-cell count in peripheral blood and fasting insulin levels (*A*) and insulin sensitivity (fasting glucose–to–fasting insulin ratio) (*B*). Correlation between CD8$^+$ T-cell count in peripheral blood and fasting insulin levels (*C*) and insulin sensitivity (fasting glucose–to–fasting insulin ratio) (*D*). Gray dots represent morbidly obese subjects (n = 8, BMI 42.4 ± 6.7 kg/m^2).

of comorbidities (although we are not informed on the atherosclerotic state of our patients). To date, it is unknown whether changes in this set point away from the anti-inflammatory phenotype are associated with the development of obesity-related comorbidities.

Atherosclerosis, which frequently occurs during obesity, is characterized by accumulation of Th1 CD4$^+$ T cells within the plaques (51), whereas CD4$^+$ T-cell depletion reduces the development of atherosclerosis in mice (52). Also, the development of type 2 diabetes mellitus is delayed in mice with diet-induced obesity when T cells are depleted (13,15). In addition, we also demonstrated that CD4$^+$ T-cell numbers positively correlated with fasting insulin levels.

On the basis of these literature data and our own data presented herein, it is thus tempting to speculate that changes away from the Treg- and Th2-dominated phenotype toward a more proinflammatory Th1- or Th17-dominated set point may prove an important indicator, or even mediator, for the development of atherosclerosis or diabetes in morbidly obese subjects. Longitudinal studies in morbidly obese subjects will be important to further address these issues.

In conclusion, the peripheral blood T-cell compartment of morbidly obese subjects is characterized by an increased homeostatic proliferation of both CD4$^+$ and CD8$^+$ T cells to which cytokines such as IL-7 and CCL5 probably contribute. This increased homeostatic proliferation is associated with an increase in peripheral blood CD4$^+$ T-cell numbers, with a skewing toward a Treg- and Th2-dominated phenotype, suggesting an anti-inflammatory set point of the peripheral blood CD4$^+$ T-cell compartment.

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K.v.d.W. researched data and wrote the manuscript. W.A.D. contributed to discussion and wrote, reviewed, and

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REFERENCES

1. Danaei G, Ding EL, Mozaffarian D, et al. The preventable causes of death in the United States: comparative risk assessment of dietary, lifestyle, and metabolic risk factors. PLoS Med 2009;6:e1000058
2. Flegal KM, Carroll MD, Ogden CL, Curtin LR. Prevalence and trends in obesity among US adults, 1999-2008. JAMA 2010;303:235–241
3. Moulin CM, Marguti I, Peron JP, Rizzo LV, Halpern A. Impact of adiposity on immunological parameters. Arq Bras Endocrinol Metabol 2009;53:183–189
4. Dixit VD. Adipose-immune interactions during obesity and caloric restriction: reciprocal mechanisms regulating immunity and health span. J Leukoc Biol 2008;84:882–892
5. Wellen KE, Hotamisligil GS. Inflammation, stress, and diabetes. J Clin Invest 2005;115:1111–1119
6. Gregor MF, Hotamisligil GS. Inflammatory mechanisms in obesity. Annu Rev Immunol 2011;29:415–445
7. Kahn SE, Hull RL, Utzschneider KM. Mechanisms linking obesity to insulin resistance and type 2 diabetes. Nature 2006;444:840–846
8. Rocha VZ, Libby P. Obesity, inflammation, and atherosclerosis. Nat Rev Cardiol 2009;6:399–409
9. Sell H, Eckel J. Adipose tissue inflammation: novel insight into the role of macrophages and lymphocytes. Curr Opin Clin Nutr Metab Care 2010;13:366–370
10. Rausch ME, Weisberg S, Vardhana P, Tortoriello DV. Obesity in C57BL/6J mice is characterized by adipose tissue hypoxia and cytotoxic T-cell infiltration. Int J Obes (Lond) 2008;32:451–463
11. Kintscher U, Hartge M, Hess K, et al. T-lymphocyte infiltration in visceral adipose tissue: a primary event in adipose tissue inflammation and the development of obesity-mediated insulin resistance. Arterioscler Thromb Vasc Biol 2008;28:1304–1310
12. Duffaut C, Zakaroff-Girard A, Bourlier V, et al. Interplay between human adipocytes and T lymphocytes in obesity: CCL20 as an adipochemokine and T lymphocytes as lipogenic modulators. Arterioscler Thromb Vasc Biol 2009;29:1608–1614
13. Nishimura S, Manabe I, Nagasaki M, et al. CD8+ effector T cells contribute to macrophage recruitment and adipose tissue inflammation in obesity. Nat Med 2009;15:914–920
14. Duffaut C, Galitzky J, Lafontan M, Bouloumié A. Unexpected trafficking of immune cells within the adipose tissue during the onset of obesity. Biochem Biophys Res Commun 2009;384:482–485
15. Winer S, Chan Y, Paltser G, et al. Normalization of obesity-associated insulin resistance through immunotherapy. Nat Med 2009;15:921–929
16. Womack J, Tien PC, Feldman J, et al. Obesity and immune cell counts in women. Metabolism 2007;56:998–1004
17. Nieman DC, Henson DA, Nehlsen-Cannarella SL, et al. Influence of obesity on immune function. J Am Diet Assoc 1999;99:294–299
18. Kim JA, Park HS. White blood cell count and abdominal fat distribution in female obese adolescents. Metabolism 2008;57:1375–1379
19. Panagiotakos DB, Pitsavos C, Yannakoulia M, Chrysohoou C, Stefanadis C. The implication of obesity and central fat on markers of chronic inflammation: The ATTICA study. Atherosclerosis 2005;183:308–315
20. O'Rourke RW, Kay T, Scholz MH, et al. Alterations in T-cell subset frequency in peripheral blood in obesity. Obes Surg 2005;15:1463–1468
21. Tanaka S, Isoda F, Ishihara Y, Kimura M, Yamakawa T. T lymphopaenia in relation to body mass index and TNF-alpha in human obesity: adequate weight reduction can be corrective. Clin Endocrinol (Oxf) 2001;54:347–354
22. Sallusto F, Geginat J, Lanzavecchia A. Central memory and effector memory T cell subsets: function, generation, and maintenance. Annu Rev Immunol 2004;22:745–763
23. Hazenberg MD, Verschuren MC, Hamann D, Miedema F, van Dongen JJ. T cell receptor excision circles as markers for recent thymic emigrants: basic aspects, technical approach, and guidelines for interpretation. J Mol Med (Berl) 2001;79:631–640
24. Hazenberg MD, Otto SA, Cohen Stuart JW, et al. Increased cell division but not thymic dysfunction rapidly affects the T-cell receptor excision circle content of the naive T cell population in HIV-1 infection. Nat Med 2000;6:1036–1042
25. Zubakov D, Liu F, van Zelm MC, et al. Estimating human age from T-cell DNA rearrangements. Curr Biol 2010;20:R970–R971
26. van Dongen JJ, Langerak AW, Brüggemann M, et al. Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: report of the BIOMED-2 Concerted Action BMH4-CT98-3936. Leukemia 2003;17:2257–2317
27. Yang H, Youm YH, Vandanmagsar B, et al. Obesity increases the production of proinflammatory mediators from adipose tissue T cells and compromises TCR repertoire diversity: implications for systemic inflammation and insulin resistance. J Immunol 2010;185:1836–1845
28. Monteiro R, Azevedo I. Chronic inflammation in obesity and the metabolic syndrome. Mediators Inflamm 2010;2010:289645
29. Overwijk WW, Schluns KS. Functions of gammaC cytokines in immune homeostasis: current and potential clinical applications. Clin Immunol 2009;132:153–165
30. Wong MM, Fish EN. Chemokines: attractive mediators of the immune response. Semin Immunol 2003;15:5–14
31. Bacon KB, Premack BA, Gardner P, Schall TJ. Activation of dual T cell signaling pathways by the chemokine RANTES. Science 1995;269:1727–1730
32. Kiewiet RM, van Aken MO, van der Weerd K, et al. Effects of acute administration of acylated and unacylated ghrelin on glucose and insulin concentrations in morbidly obese subjects without overt diabetes. Eur J Endocrinol 2009;161:567–573
33. Yang H, Youm YH, Vandanmagsar B, et al. Obesity accelerates thymic aging. Blood 2009;114:3803–3812
34. Lynch HE, Goldberg GL, Chidgey A, Van den Brink MR, Boyd R, Sempowski GD. Thymic involution and immune reconstitution. Trends Immunol 2009;30:366–373
35. Goronzy JJ, Weyand CM. T cell development and receptor diversity during aging. Curr Opin Immunol 2005;17:468–475
36. Gruver AL, Hudson LL, Sempowski GD. Immunosenescence of ageing. J Pathol 2007;211:144–156
37. Valdes AM, Andrew T, Gardner JP, et al. Obesity, cigarette smoking, and telomere length in women. Lancet 2005;366:662–664
38. Kim SY, Lim JH, Choi SW, et al. Preferential effects of leptin on CD4 T cells in central and peripheral immune system are critically linked to the expression of leptin receptor. Biochem Biophys Res Commun 2010;394:562–568
39. Stentz FB, Kitabchi AE. Palmitic acid-induced activation of human T-lymphocytes and aortic endothelial cells with production of insulin receptors, reactive oxygen species, cytokines, and lipid peroxidation. Biochem Biophys Res Commun 2006;346:721–726
40. Cani PD, Bibiloni R, Knauf C, et al. Changes in gut microbiota control metabolic endotoxemia-induced inflammation in high-fat diet-induced obesity and diabetes in mice. Diabetes 2008;57:1470–1481
41. Madani R, Karastergiou K, Ogston NC, et al. RANTES release by human adipose tissue in vivo and evidence for depot-specific differences. Am J Physiol Endocrinol Metab 2009;296:E1262–E1268
42. Wu H, Ghosh S, Perrard XD, et al. T-cell accumulation and regulated on activation, normal T cell expressed and secreted upregulation in adipose tissue in obesity. Circulation 2007;115:1029–1038
43. Maury E, Ehala-Aleksejev K, Guiot Y, Detry R, Vandenhooft A, Brichard SM. Adipokines oversecreted by omental adipose tissue in human obesity. Am J Physiol Endocrinol Metab 2007;293:E656–E665
44. Schall TJ, Bacon K, Toy KJ, Goeddel DV. Selective attraction of monocytes and T lymphocytes of the memory phenotype by cytokine RANTES. Nature 1990;347:669–671

45. Biswas SK, Mantovani A. Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. Nat Immunol 2010;11:889–896
46. Gordon S. Alternative activation of macrophages. Nat Rev Immunol 2003;3:23–35
47. Ghanim H, Aljada A, Hofmeyer D, Syed T, Mohanty P, Dandona P. Circulating mononuclear cells in the obese are in a proinflammatory state. Circulation 2004;110:1564–1571
48. Degasperi GR, Denis RG, Morari J, et al. Reactive oxygen species production is increased in the peripheral blood monocytes of obese patients. Metabolism 2009;58:1087–1095
49. Lumeng CN, Bodzin JL, Saltiel AR. Obesity induces a phenotypic switch in adipose tissue macrophage polarization. J Clin Invest 2007;117:175–184
50. Aron-Wisnewsky J, Tordjman J, Poitou C, et al. Human adipose tissue macrophages: m1 and m2 cell surface markers in subcutaneous and omental depots and after weight loss. J Clin Endocrinol Metab 2009;94:4619–4623
51. Andersson J, Libby P, Hansson GK. Adaptive immunity and atherosclerosis. Clin Immunol 2010;134:33–46
52. Steffens S, Burger F, Pelli G, et al. Short-term treatment with anti-CD3 antibody reduces the development and progression of atherosclerosis in mice. Circulation 2006;114:1977–1984