Systemic and white adipose tissue inflammation in obesity and insulin resistance

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2 Increased systemic and adipose tissue inflammation differentiates obese women with T2DM from obese women with normal glucose tolerance

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Abstract

Aim: Obesity is strongly related to type-2 diabetes (T2DM), but there is a subset of obese individuals that remains relatively insulin sensitive and metabolically healthy. This study determined to what extent differences in metabolic health in obese women are associated with differences in adipose tissue and/or systemic inflammation.

Methods: The subject group consisted of age-matched lean (n=12) and obese women either with T2DM (n=28) or normal glucose tolerance (NGT; n=26). Number of crown like structures (CLS) and adipocyte size were measured in subcutaneous and visceral adipose tissue of the obese women. Circulating cytokine and free fatty acid (FFA) levels, as well as number and activation status of peripheral leukocytes were determined.

Results: Obese T2DM subjects showed higher circulating levels of IL-6, FFA and glycerol as compared to obese NGT subjects. Obese T2DM subjects had higher absolute numbers of peripheral leukocytes which was mainly due to an increase of T helper cells. Activation status of circulating cytotoxic T (CD8+CD25+) and B (CD19+CD38+) cells was significantly increased in obese NGT subjects as compared to lean but was not different between the two obese groups. Subcutaneous adipose tissue of obese T2DM subjects contained more CLS than adipose tissue of obese NGT subjects.

Conclusion: Obese T2DM subjects show higher FFA levels and adipose tissue macrophage infiltration in addition to higher levels of circulating IL-6 and numbers of CD4+T cells. Hence, obese T2DM subjects show a higher extent of inflammation both at the systemic and adipose tissue level than obese NGT subjects.

Introduction

The metabolic syndrome comprises a combination of risk factors that increase the risk of developing type-2 diabetes and cardiovascular disease (1). Obesity, in particular abdominal obesity, is one of the main risk factors of the metabolic syndrome. The majority of obese individuals (~80%) will eventually develop metabolic abnormalities associated with a reduced life expectancy. However, there is a subset of obese individuals who remain relatively insulin sensitive and metabolically healthy throughout life (2). The reason why these individuals are unaffected is still not completely understood.

The pathological metabolic consequences of obesity are closely linked to the expanding adipose tissue that at a certain level responds with stress signals to the energy overload (3). Adipose tissue functions as a metabolic and endocrine organ releasing fatty acids and adipokines, both of which have immune modulatory activities as reviewed in (4, 5). Obesity induces adipose tissue dysfunction with increased secretion of pro-inflammatory cytokines and chemokines. Adipose tissue acquires a chronic inflammatory state which is characterized by macrophage accumulation in crown like structures that surround stressed and dying adipocytes (6, 7). Adipose tissue inflammation may affect systemic immune responses that contribute to the initiation and progression of obesity induced metabolic and cardiovascular dysfunctions. Several studies in obese subjects have shown elevated levels of adipose tissue released pro-inflammatory cytokines -such as leptin, TNF-α and IL-6- in contrast to a decreased level of the anti-inflammatory cytokine adiponectin (8). Also, the levels of the acute phase protein, C-reactive protein (CRP), are higher in subjects with obesity (9), indicating that obesity is associated with (low grade) systemic inflammation.

Numerous studies have investigated the effects of obesity or type 2 diabetes on systemic inflammation (8-17), but they have not considered differences between obese individuals that develop T2DM and those that remain relatively healthy. We hypothesize that in obese individuals that have developed type 2 diabetes, the intensity of adipose tissue inflammation and/or the systemic inflammatory state may be higher as compared to obese individuals that still have normal glucose tolerance (NGT). To this end, we compared the extent of abdominal subcutaneous and visceral adipose tissue inflammation between age matched severely obese women with T2DM and NGT. Moreover, we compared systemic inflammation between lean and obese women either with T2DM or NGT by determining number and activation- or memory status of peripheral leukocytes in addition to circulating levels of pro-inflammatory cytokines, CRP and free fatty acids (FFA).

Materials and methods

Subjects

The study group consisted of 12 lean and 54 obese women of whom 28 had type-2 diabetes. The three groups were comparable in age and the obese groups in BMI. All the obese women had been morbidly obese (mean BMI=42.8 ± 4.7 kg/m2) for at least five years. Subjects who reported the use of weight loss medications within 90 days prior to enrollment in the study were excluded. Body weight of all subjects had been stable for at least 3 months prior to inclusion. All subjects were non-smokers, had

no signs of any infections nor had any history of auto immune diseases. The subjects were investigated in the morning after an overnight fast. Venous blood samples were taken for determination of number of leukocytes as well as determination of glucose, insulin, lipids, cholesterol and cytokines in serum. Moreover, ~50-ml of venous blood was taken for subsequent flow cytometry analysis (see below). Around 4 weeks after the first examination a subgroup (n=35 of whom 14 had T2DM) of the obese individuals underwent bariatric surgery (gastric bypass or banding). Within 1h after opening the abdominal wall adipose tissue specimens were taken from the epigastric region of the abdominal wall (subcutaneous) and from the major omentum (visceral). These samples were used for determination of cell size and extent of adipose tissue inflammation. The study was approved by the Ethics Committee of Leiden University. All subjects gave informed consent to participate in the study.

Medication

For obvious reasons we could not restrict to obese subjects not using any type of medication. All diabetic subjects were treated with oral medication only (metformin or sulfonylurea derivatives). Participants were allowed to use cholesterol lowering statins and antihypertensive medication. The use of drugs such as statins and antihypertensive drugs was slightly higher in the diabetic subjects. At baseline, statins were used by 60% of T2DM patients and 25% of NGT patients. Of T2DM patients 50% used anti-hypertensives (diuretics n=7, ACE-inhibitors n=5, β-blockers n=6) against 33% in NGT patients (diuretics n=4, ACE-inhibitors n=3, β-blockers n=4). The patients were neither using any medication that affects lipid or glucose metabolism nor any anti-inflammatory agents (i.e. thiazolidinediones steroids (prednisone) or NSAIDS).

Blood measurements

Serum Glucose, Total cholesterol, High Density Lipoprotein cholesterol (HDL-C), Triglycerides (TG) and C-reactive protein (CRP) as well as total number of leukocytes in blood were measured at the laboratory for Clinical Chemistry at the Leiden University medical Center, using a fully automated Hitachi 704/911 system. Low Density Lipoprotein (LDL) cholesterol was calculated according to the Friedewald equation. Serum insulin was measured by an IRMA (Medgenix, Fleurus, Belgium). FFA were determined by a colorimetric method (Wako Chemicals, Neuss, Germany). Glycerol was measured using the Free Glycerol determination kit of Sigma Aldrich (St Louis, MO, USA). Leptin was measured by radioimmunoassay (RIA) (Leptin HL-81K, Millipore, Billerica, USA) with an inter-assay precision of 3.6–6.2% and an intra-assay precision of 3.4-8.3%. IL-6 and adiponectin were measured using a commercially available kit (Mesoscale Discovery MSD, Maryland, USA). IL-1β, IL-2, IL-4, IL-5, IL-8, IL-10, IL-13, tumor-necrosis factor α (TNF-α) and interferon-γ (IFN-γ) were measured with a multisport commercially available kit (Mesoscale Discovery MSD, Maryland, USA).

Flow cytometry analysis on peripheral blood mononuclear cells

Fresh heparinized whole blood was stained with solutions containing mixes of the following antibodies (50µl blood/mix): PE-conjugated CD3, CD19, CD16, FITC-conjugated CD45RA, CD27, CD56, CD3, CD8, APC-conjugated CD8, PE-Cy-7-conjugated CD25, CD14, Percp-Cy5.5-conjugated CD38 (all Abs were from BD biosciences, CA, USA). Red blood cells were lysed using BD Lysis solution and remaining cells were fixed with 1% paraformaldehyde and analyzed with a LSR II flow cytometer using Diva 6 software (BD Biosciences, CA, USA). T cells were determined by selecting the CD3+ population, B cells by selecting the CD19+ population, NK cells by selecting the CD56+CD16+ population plus CD56brightCD16- population and monocytes by selecting the CD14+population. Granulocytes were determined by selecting their distinct population in the forward site scatter. Absolute numbers of leukocyte subsets were calculated using the absolute leukocyte numbers (determined as described above under Blood Measurements) and percentages determined by flow cytometry.

Peripheral blood mononuclear cells (PBMCs) were isolated by a Ficoll gradient and kept in liquid nitrogen. Subjects with highest and lowest CD8+CD25+ levels were selected for intracellular cytokine stainings. These groups included six individuals each, both from the obese with T2DM and the obese with NGT. The range of CD8+CD25+ in the low group was 0 to 1%, whereas for the high group the range was 2.8 to 8.5%. PBMC were stimulated for 5hrs with 20 ng/ml phorbol myristate acetate (PMA)/ 1000 ng/ml Ionomycin. Ten µg/ml Brefeldin A was added for the last 4hrs. Intracellular cytokines were detected using a Cytofix/Cytoperm Fixation/Permeabilization Solution Kit (BD biosciences) and cytokine-specific antibodies: PE-conjugated Abs to IFN-γ, IL-10, TNF-α, IL-6 (all BD biosciences except the Ab to IL-6 which was from eBioscience, CA, USA), Alexa fluor 647-conjugated antibody to IL-17 (eBioscience, CA, USA) and the appropriate isotype controls.

Proliferation assay on peripheral blood mononuclear cells

In the above mentioned subjects with highest and lowest CD8+CD25+, T cell proliferation was measured using the 3H-Thymidine incorporation assay. Proliferation assays were performed in triplicate using 50,000 PBMCs in 150 µl Iscove's Modified Dulbecco's Medium (IMDM, Gibco Life Technologies, Bleiswijk, The Netherlands) with 10% pooled normal human serum per well of 96 well round-bottom plates. Proliferation was stimulated with Phytohaemagglutinin (PHA; 0.5 µg/ml), IL-2 (20 units/ml), IL-7 (5 ng/ml), or IL-15 (5 ng/ml). After three days at 37°C, 3 H-thymidine (0.5 µCi/well) was added to the cultures. Twenty-four hours thereafter the proliferation was stopped by putting the cells at 4°C, and within one week the cells were harvested using vacuum aspiration onto glass matrix filters. 3 H-thymidine incorporation was determined with a liquid scintillation counter.

Immunohistochemistry of crown like structures

One explant of the adipose tissue specimens was fixed in 3.7% paraformaldehyde overnight and subsequently dehydrated in increasing concentrations of ethanol. The pieces were embedded in paraffin. For the IHC of CD68, antigens were retrieved using citrate buffer. The primary antibody

was mouse-anti-human CD68 (1:800 dilution, clone KP1, from Dako, Glostrup, Denmark). Staining and counterstaining was done with Nova Red (Vector labs, Brunschwig Chemie, Amsterdam, The Netherlands) and haematoxylin, respectively. Both solitary CD68 positive cells and CLS were counted using a light microscope. The criterion for a CLS was three or more CD68 positive cells surrounding an adipocyte (Fig. 1A). The number of CLS was expressed as number of CLS per area of adipose tissue section on the slide.

Determination of adipocyte cell size

The remaining adipose tissue specimens were minced and digested in 0.5g/l collagenase in DMEM/F12 (pH 7.4) with 20 g/l of dialyzed bovine serum albumin (BSA, fraction V, Sigma, St Louis, MO, USA) for 1 h at 37oC. The disaggregated adipose tissue was filtered through a nylon mesh with a pore size of 236 m. For the isolation of mature adipocytes, cells were obtained from the surface of the filtrate and washed several times. Using direct microscopy, the diameter of 100 adipocytes was determined from which mean cell diameter was calculated.

Statistical analysis

Values in Tables and Figures are given as mean ± SD. Groups were compared using one-way ANOVA, with Bonferroni's post-hoc multiple comparison test. The cytokine data (Table 2) was not normally distributed and therefore the data was log transformed to obtain a normal distribution in the data. The data shown in Table 2 is the untransformed data, the ANOVA however is performed on the log transformed data. The linear regression method was used to analyze correlations.

Results

Obese with T2DM have elevated circulating levels of IL-6, FFA and glycerol

Table 1 shows subject characteristics of the three groups included in this study. Neither waist circumference nor total body fat percentage did differ between the two obese groups. As expected, fasting glucose and HOMA-index was significantly higher in the obese group with T2DM (Table 1).

We tested the presence of pro-inflammatory cytokines in the circulation of obese subjects with NGT and whether these levels were different in the obese with T2DM. As expected the levels of the pro-inflammatory cytokines, leptin, TNF-α and IL-6 levels, as well as CRP, and glycerol (i.e. a measure of adipose tissue lipolysis) were all significantly elevated in obese NGT subjects, whereas the levels of the anti-inflammatory cytokine adiponectin was decreased (Table 2). IFN-γ, IL-2, IL-4, IL-5, IL-8, IL-10 and IL-13 levels were equal between the lean and the obese with NGT (Table 2). When comparing cytokines between the obese NGT and obese T2DM subjects, the only difference was IL-6 which was significantly increased and leptin which was significantly decreased in the obese T2DM subjects (Table 2). Both glycerol and FFA tended to be higher in the obese T2DM subjects as compared to the obese NGT subjects (Table 2, $p<0.10$). Comparing glycerol and FFA between obese with NGT and obese with T2DM using an unpaired t-test showed significant higher levels of both in the obese with T2DM ($p<0.05$).

Table 1. Anthropometric and metabolic measurements in lean and obese women with NGT or T2DM

				Statistics		
				P-values		
	Lean	Obese with NGT	Obese with T2DM	Anova	Post-hoc test Lean vs. obese with NGT	Post-hoc test Obese with NGT vs. obese with T2DM
N	12	26	28			
Age (y)	50±5	48±6	51 ±7	NS		
BMI (kg/m²)	21.7±1.6	44.0±3.4	41.7±5.5	<0.0001	<0.0001	NS
Waist (cm)	78.0±5.9	122.5±9.3	122.6±11.2	<0.0001	<0.0001	NS
Fat percentage (% of bw)	35.5±2.4	56.4±2.1	56.1±3.4	<0.0001	<0.0001	NS
Fasting glucose (mmol/l)	4.7±0.3	5.0±0.6	8.7±2.5	0.0001	NS	<0.0001
Fasting insulin (mU/l)	1.6±0.2	10.5±7.9	12.0±7.8	<0.0001	<0.001	NS
HOMA-IR	0.3±0.06	2.4±1.9	4.4±3.0	<0.0001	<0.05	<0.001
Total cholesterol (mmol/L)	4.9±0.92	4.6±1.0	4.36±0.8	NS		
HDL cholesterol (mmol/L)	1.7±0.3	1.1±0.3	1.1±0.3	<0.0001	<0.0001	NS
LDL cholesterol (mmol/L)	2.9±0.9	2.9±0.9	2.5±0.6	NS		
Triglycerides (mmol/L)	1.0±0.3	1.4±0.6	1.8±0.7	<0.001	NS	<0.05

Groups were compared using one-way ANOVA. A statistically significant ANOVA was followed with a post-hoc Bonferroni's Multiple Comparison Test to compare lean vs. obese with NGT or obese with NGT vs. obese with T2DM. BW=body weight.

Table 2. Serum levels of cytokines, CRP as well as FFA and glycerol in lean and obese women with NGT or T2DM

	Lean	Obese with NGT	Obese with T2DM	Statistics		
				P values		
				Anova	Post-hoc test Lean vs. obese with NGT	Post-hoc test Obese with NGT vs. obese with T2DM
N	12	26	28			
Leptin (µg/L)	9.9±5.2	83.6±30.1	55.6±33.1	<0.0001	<0.0001	<0.001
TNF-α (pg/ml)	5.9±1.3	7.6±2.1	8.1±2.7	<0.05	<0.05	NS
IFN-γ (pg/ml)	2.7±2.7	2.5±2.4	3.3±4.3	NS		
IL-1β (pg/ml)	0.49±0.84	0.74±0.81	1.14±1.93	NS		
IL-2 (pg/ml)	0.73±0.33	0.81±0.51	0.98±0.38	NS		
IL-4 (pg/ml)	0.61±0.55	1.19±0.98	0.92±0.86	NS		
IL-5 (pg/ml)	6.3±15.2	5.2±16.8	1.5±2.0	NS		
IL-6 (pg/ml)	0.43±0.21	1.62±0.77	2.82±1.73	<0.0001	<0.05	<0.05
IL-8 (pg/ml)	8.4±3.3	8.2±3.0	9.6±4.2	NS		
IL-10 (pg/ml)	1.8±1.0	3.0±2.8	3.9±3.9	NS		
IL-13 (pg/ml)	23.7±22.2	16.1±43.2	6.5±8.8	NS		
Adiponectin (µg/ml)	18.6±6.0	11.8±3.6	9.8±4.2	<0.0001	<0.0001	NS
CRP (mg/L)	1.9±1.3	7.6±6.9	6.9±5.4	<0.05	<0.05	NS
Glycerol (µmol/L)	180.4±63.3	398.5±153.8	470.5±119.9	<0.0001	<0.0001	0.08
FFA (mmol/L)	0.86±0.31	1.00±0.38	1.180±0.32	<0.05	NS	0.06

As the cytokine data was not normally distributed, the data was log transformed to obtain a normal distribution. The ANOVA was performed on log transformed data. A statistically significant ANOVA was followed with a post-hoc Bonferroni's Multiple Comparison Test to compare lean vs. obese with NGT or obese with NGT vs. obese with T2DM.

Obese with T2DM have more crown like structures in subcutaneous adipose tissue

We compared the extent of adipose tissue inflammation between the obese individuals with NGT and with T2DM and used the number of crown like structures per area adipose tissue on immunohistochemistry coupes as an index of adipose tissue inflammation. We found that the subcutaneous but not the omental adipose tissue of the obese with T2DM had significantly more CLS and more solitary macrophages than the obese with NGT (Fig.1B-C). It should be noted that the single macrophages were located within the adipose tissue and not near or in a blood vessel. The number of CLS was not significantly related to any of the plasma cytokines in the circulation (data not shown). No differences were found in mean adipocyte size between the adipose tissue regions or between the two groups of obese women (Fig. 1D).

Obese with T2DM have higher circulating T cell numbers

Percentages of circulating T cells (CD3+), T helper cells (CD3+CD4+), cytotoxic T cells (CD3+CD8+), B cells (CD19+), NK cells (CD56+CD16+ population plus CD56brightCD16- population) and monocytes out of leukocytes did not differ between the three subject groups (Table 3). In contrast, percentages of

granulocytes were significantly higher in the obese NGT subjects as compared to lean and obese T2DM subjects (Table 3). Absolute numbers of leukocytes in the circulation were significantly enhanced in the obese with T2DM as compared to the lean and the obese with NGT (Table 3). Absolute numbers of B cells, NK cells or monocytes did not differ significantly between the three subject groups (Table 3). The absolute number of T cells was significantly higher in the obese T2DM subjects as compared to the obese NGT subjects (Table 3). There was a tendency to higher absolute numbers of T helper cells in the obese T2DM subjects as compared to the obese NGT subjects, whereas the absolute numbers of cytotoxic T cells did not differ (Table 3). There was a tendency to higher absolute granulocyte numbers in the obese groups as compared to the lean, but no differences between the two obese groups (Table 3). For the three groups together, leukocyte number associated positively and significantly with both IL-6 and FFA levels in the circulation (leukocyte number vs. IL-6, $r=0.35$, $p<0.01$, leukocyte number vs. FFA, $r=0.28$, $p<0.05$).

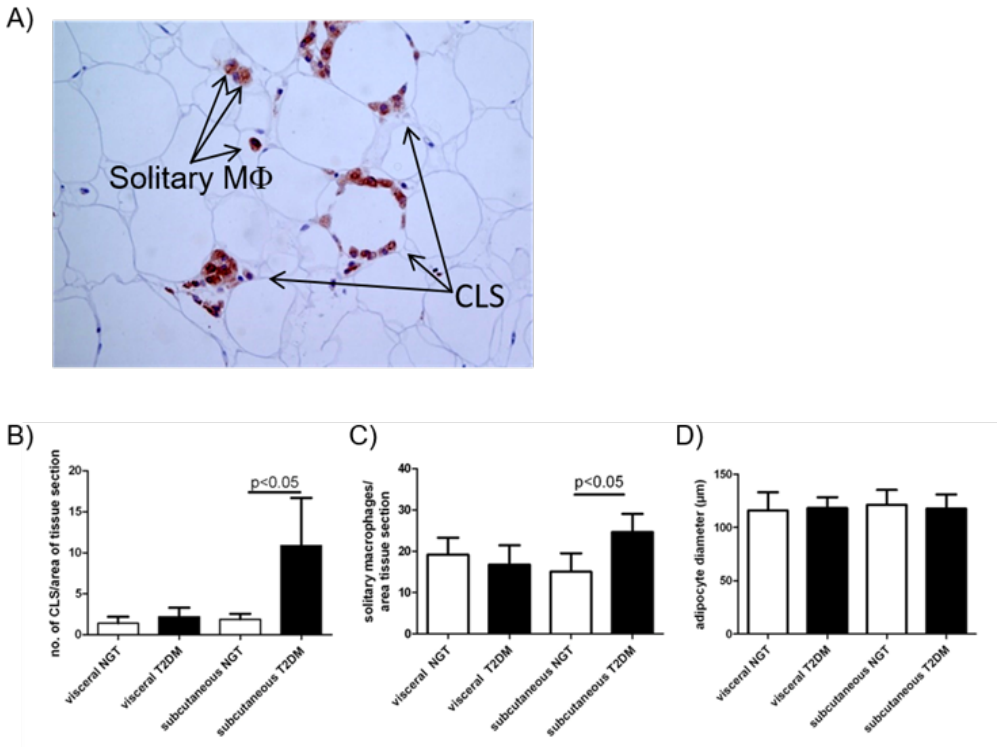

Figure 1. Quantification of macrophages and fat cell size determination in visceral and subcutaneous adipose tissue of obese women with NGT or T2DM. A) CD68 staining in adipose tissue showing solitary macrophages (MΦ**)** and crown like structures (CLS). B) Number of CLS and C) solitary or single macrophages per area of adipose tissue section as determined by immunostaining of CD68, D) Fat cell size, expressed as mean adipocyte diameter.

Table 3. Comparison of leukocyte subtypes in circulation between lean and obese women with NGT or T2DM

	Lean	Obese with NGT	Obese with T2DM	Statistics		
				P values		
				Anova	Post-hoc test Lean vs. obese with NGT	Post-hoc test Obese with NGT vs. obese with T2DM
Number of subjects	12	26	28			
Percentage of cells within leukocytes						
T cells (%)	29.5±10.9	25.0±6.4	26.3±10.8	NS		
CD4+ T cells (%)	21.6±8.4	18.4±4.9	20.5±9.4	NS		
CD8+ T cells (%)	6.6±2.4	5.4±2.4	5.0±1.9	NS		
B cells (%)	5.0±2.0	4.0±2.2	4.0±1.9	NS		
NK cells (%)	2.4±0.3	2.4±0.4	2.2±0.3	NS		
Monocytes (%)	2.7±1.3	2.7±1.4	2.6±0.9	NS		
Granulocytes (%)	35.0±12.0	51.5±13.4	39.4±13.7	<0.001	<0.01	<0.01
Absolute number of cells in the circulation						
All leukocytes (cell no. x10^9/L)	6.3±1.4	6.6±1.4	7.8±1.4	<0.005	NS	<0.05
T cells (x10^9/L)	1.8±0.9	1.5±0.4	2.1±0.9	<0.05	NS	<0.05
CD4+ T cells (x10^9/L)	1.2±0.5	1.2±0.4	1.6±0.4	0.06		
CD8+ T cells (x10^9/L)	0.4±0.1	0.4±0.2	0.4±0.2	NS		
B cells (x10^9/L)	0.29±0.12	0.28±0.22	0.34±0.14	NS		
NK cells (x10^9/L)	0.16±0.10	0.17±0.17	0.18±0.11	NS		
Monocytes (x10^9/L)	0.18±0.08	0.26±0.10	0.21±0.09	NS		
Granulocytes (x10^9/L)	2.2±1.3	3.3±1.1	3.3±1.2	0.06		

For information on the immunological cell markers used in the FACS analyses see Material and Methods. Groups were compared using one-way ANOVA. A statistically significant ANOVA was followed with a post-hoc Bonferroni's Multiple Comparison Test to compare lean vs. obese with NGT or obese with NGT vs. obese with T2DM.

Obesity is associated with elevated activation markers on circulating lymphocytes which is not further increased in the obese with T2DM

The phenotype of the leukocyte subsets in the circulation was further investigated. Regarding NK cells and granulocytes, the expression of the FcgRIII receptor (CD16+) did not differ on both cell types between the three subject groups (Table 4). For the monocytes we determined the presence and abundance of classical (CD14+CD16-), intermediate (CD14+CD16+) and non-classical monocytes (CD14dimCD16+). No significant differences were found in percentages for these monocyte subgroups between the three subject groups (Table 4).

On both the cytotoxic and T helper cells the naivety marker CD45RA did not differ between the lean and the obese (Table 4). The activation marker CD25 (IL-2 receptor) was significantly higher expressed on the circulating cytotoxic T cells in the obese with NGT as compared to lean, but was similar between the obese with NGT and with T2DM (Fig. 2A, Table 4). For the T helper cells there was a tendency for more expression of CD25 in the obese groups as compared to the lean (Fig. 2B,

Table 4). On the circulating B cells the memory marker CD27 did not differ between the groups. The activation marker CD38 was significantly higher expressed on circulating B cells in the obese with NGT as compared to lean but not further affected in the obese with T2DM (Fig 2C, Table 4). There were no significant differences in absolute numbers of activated T or B cells between the three subject groups (data not shown).

Table 4. Phenotypic characterization of T cells, B cells, NK cells, granulocytes and monocytes in lean and obese women with NGT or T2DM

				Statistics		
				P values		
	Lean	Obese with NGT	Obese with T2DM	Anova	Post-hoc test Lean vs. obese with NGT	Post-hoc test Obese with NGT vs. obese with T2DM
N	12	26	28			
Cytotoxic T cells						
CD8+CD25+ (%)	0.3±0.07	1.6±0.41	1.4±0.4	<0.01	<0.01	NS
CD8+CD45RA+ (%)	46.2±12.3	50.4±18.9	49.2±18.4	NS		
T helper cells						
CD4+CD25+ (%)	3.4±2.7	10.3±10.1	9.5±13.6	p=0.10		
CD4+CD45RA+ (%)	35.2±13.2	32.2±18.6	31.6±18.2	NS		
B cells						
CD19+CD38+ (%)	49.3±21.6	70.9±17.7	63.4±15.9	<0.01	<0.01	NS
CD19+CD27+ (%)	27.9±13.4	21.9±7.4	21.7±14.4	NS		
NK cells						
CD56+CD16+ (%)	90.7±4.6	86.7±11.9	85.1±16.1	NS		
Monocytes						
CD14+CD16- (%)	75.9±12.5	77.2±9.9	78.8±9.2	NS		
CD14+CD16+ (%)	10.4±3.2	14.1±7.7	13.3±6.8	NS		
CD14dimCD16+ (%)	13.7±11.4	8.7±6.1	7.9±6.4	NS		
Granulocytes						
CD16+ (%)	82.9±28.0	68.3±39.8	59.0±43.5	NS		

Groups were compared using one-way ANOVA. A statistically significant ANOVA was followed with a post-hoc Bonferroni's Multiple Comparison Test to compare lean vs. obese with NGT or obese with NGT vs. obese with T2DM.

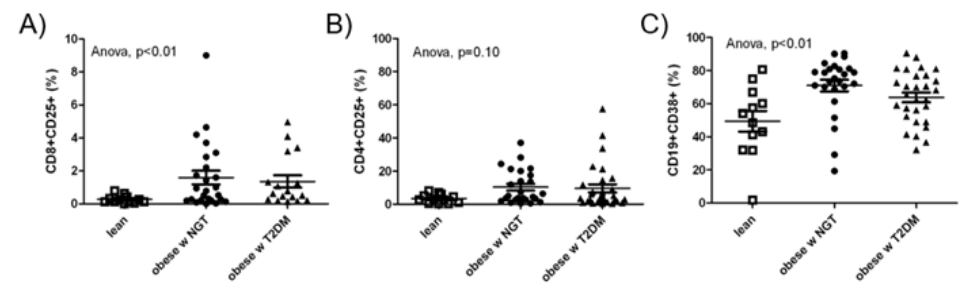

Figure 2. A-B) **Expression of CD25** in CD8 and CD4 T cells (as % of CD8 or CD4 cells) and C) **Expression of CD38** in CD19 B cells (as % of CD19 cells) in lean and obese women with NGT or T2DM.

Large variation in activation status of T cells between obese individuals

Fig. 2 shows that in the both groups of obese individuals there was a large variation in the activation status of the lymphocytes in the circulation, in particular the T cells. There was a subgroup of obese individuals that showed a higher activation status of their T cells. Expression of CD25 on cytotoxic T cells correlated positively with the expression of CD25 on T helper cells in the whole group (data not shown; $r=0.82$, $p<0.0001$) indicating that the same individuals that showed a high activation status of their cytotoxic T cells also showed a high activation status of their T helper cells. Expression of CD25 on both cytotoxic and T helper cells also correlated to the expression of CD38 on the B cells (data not shown; both cytotoxic T cells and T helper cells: $r=0.30$, $p<0.05$). Activation status of T cells did not correlate with any of the measured (adipo)cytokine levels in blood. Activation status of the B cells did not correlate with any of the cytokines either, except with leptin levels (data not shown, $r=0.46$, $p<0.0005$).

We selected six obese individuals with high and six obese individuals with low CD25 expression on their cytotoxic T cells levels and further characterized the phenotype of the T cells by measuring proliferation capacity and cytokine release after non-specific stimulation. We found that the T cells of the individuals with high CD8+CD25+ levels showed a higher proliferation rate after IL-2 or IL-15 stimulation (Fig. 3A). There were no major differences in cytokine release by either the CD4+ or CD8+ cells between the high and the low CD8+CD25+ individuals. There was a tendency to a higher TNF-α ($p=0.11$) and IFN-γ ($p=0.15$) by the CD8+ cells of the individuals with high CD25 (Fig. 3B).

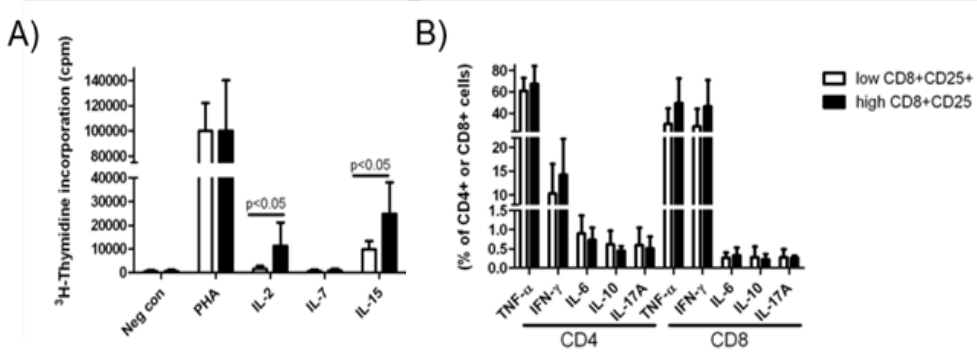

Figure 3. Proliferation capacity and intracellular cytokine staining of PBMCs from obese women with low or high peripheral CD8+CD25+. A) Proliferation of T cells after either PHA, IL-2, IL-7 or IL-15 stimulation. B) Percentage CD4+ or CD8+ cells that are positive for TNF-α, IFN γ, IL-6, IL-10 or IL-17A upon in vitro stimulation with PMA/ionomycin.

Discussion

The current study aimed to explain differences in inflammation between obese individuals that have developed T2DM and obese individuals that were glucose tolerant in a well-defined and entirely matched cohort. As compared to both the lean and the obese with NGT, obese women with T2DM had higher numbers of leukocytes and levels of IL-6 in their circulation as well as more crown like structures and solitary macrophages in the subcutaneous adipose tissue both reflecting a higher state of inflammation in the circulation and in the adipose tissue.

As the two obese groups had the same body fat percentage and waist circumference, the increased inflammatory state in adipose tissue could not be explained by differences in adipose tissue mass or fat distribution. Previously, fat cell size has been shown to be a major determinant of adipose tissue inflammation. Hypertrophic adipocytes become more insulin resistant and release more FFA and pro-inflammatory cytokines attracting pro-inflammatory monocytes to infiltrate the adipose tissue (18-20). However, our results show that adipocyte hypertrophy cannot explain the increased number of subcutaneous CLS in obese subjects with T2DM, as the adipocyte sizes were not significantly different in the obese with T2DM as compared to the obese with NGT. In contrast, our data do implicate that the adipocytes of the obese with T2DM were more insulin resistant as compared to those of obese with NGT despite the equal cell sizes in both groups. FFA and glycerol levels in the circulation were elevated in the obese with T2DM, which suggest that adipocyte lipolysis was higher in the obese with T2DM and that the adipocytes were more insulin resistant. As a consequence adipose tissue inflammation may have been elevated.

Previously Apovian et al (21) have shown that accumulation of macrophages in CLS in subcutaneous adipose tissue of obese subjects was associated with systemic hyperinsulinemia and insulin resistance, which is in line with our study. It is unclear why the differences in adipose tissue inflammation between the obese groups in our study were restricted to the subcutaneous tissue. Xu et al (22) found higher expression of the macrophage marker CD68 in visceral but not subcutaneous adipose tissue of insulin resistant obese as compared to insulin sensitive individuals. Their data suggest a higher content of macrophages in visceral adipose tissue of insulin resistant individuals. Visceral adipose tissue is regarded to have higher inflammatory responses than subcutaneous tissue, at least during the onset of obesity (23, 24). One explanation may be that our subjects were severely obese for a relative long duration which may have altered the regional differences in the inflammatory state between their adipose tissues.

The enlarged number of circulating leukocytes in obese subjects with T2DM was mainly due to a higher number of T helper cells in their leukocyte pool. Previously, elevated numbers of peripheral CD4+T cells were found in morbidly obese as compared to lean individuals (14, 15). Our data may indicate a different T cell activation and/or proliferation status in the obese with T2DM as compared to the obese with NGT. However, although we found an increased activation status of both the B and T cells in obesity, there was no further elevation in individuals who had developed T2DM. As such, the higher number of circulating T cells in the obese with T2DM cannot be explained by a higher activation

status of these cells. Interestingly, peripheral leukocyte number correlated positively with FFA levels. Although this study does not show any direct evidence, one could speculate that the elevated FFA in the obese with T2DM may have contributed to the higher numbers of leukocytes in the circulation. Adipose tissue derived FFA have been shown to stimulate proliferation of T cells before (25).

It has been previously shown that obesity is associated with a higher activation status of peripheral T lymphocytes (16). However, in our study, the increased activation status of the T lymphocytes seemed only restricted to a subgroup of the obese individuals. The T cells with a high CD25 percentage proliferated at a higher rate after IL-2 or IL-15 stimulation, confirming the elevated activation status of these cells. However, these T cells did not show increased cytokine release after non-specific stimulation arguing against an elevated pro-inflammatory phenotype. Our results thus imply that the higher activation status of peripheral T cells in obesity is not accompanied by an elevated pro-inflammatory phenotype. The reason why a subgroup of severely obese individuals have T cells with such a phenotype remains to be investigated.

One of the inclusion criteria for the obese subjects was that they should have a BMI > 40 kg/m2 for more than 5 years. We expected that individuals that are prone to develop T2DM would have developed T2DM within this relative long duration of being severely obese. However, although our NGT obese subjects did not develop T2DM yet and as a consequence were healthier than the obese with T2DM at the time of the experiments, we cannot exclude that some of the these subjects were on the way to develop T2DM. In the studies of Karelis et al (26) and Phillips et al (27), obese individuals were classified as metabolically healthy or at risk based on insulin sensitivity measurements (hyperinulinemic euglycemic clamp) or cardiometabolic parameters, respectively. Both studies found lower CRP levels and in addition Phillips et al found lower circulating TNF-α and IL-6 as well as higher adiponectin levels for the healthy obese. Although our study did not find differences in CRP, TNF-α or adiponectin levels between NGT and T2DM obese subjects, the favourable systemic inflammation profile in the healthy obese is in agreement with our study.

The reason why leptin levels in the obese subjects with T2DM were lower than in the obese with NGT remains unclear. Literature on leptin levels in T2DM patients is conflicting. Several studies did not show any differences in leptin levels in obese with T2DM, but there is also a number of studies that show lower leptin levels in T2DM (28, 29). These lower leptin levels were explained by the more severe chronic inflammatory state in T2DM patients (30). Another explanation for the lower leptin levels could be the hypoglycemic, antihypertensive or hypolipidemic medication taken by these subjects. Although the T2DM patients in our study were not using insulin, some of them were using metformin or sulfonyl urea. Thus we cannot exclude that medication may have influenced leptin levels. Metformin has been shown to have anti-inflammatory effects and to inhibit proliferation of T cells (31-33). We can therefore not exclude that metformin also has influenced the inflammatory state in the obese with T2DM.

In conclusion, obese women with T2DM have elevated absolute numbers of circulating leukocytes and levels of IL-6 as well as more crown-like structures in their adipose tissue. As the activation status

of the leukocyte subtypes did not differ between the obese T2DM and NGT subjects, the higher leukocyte numbers cannot be explained by an increased activation status. The elevated FFA release by insulin resistant adipocytes, however, may play a role in the induction of both adipose tissue and systemic inflammation in obese individuals with T2DM.

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Reference list

1. Duvnjak L, Duvnjak M. The metabolic syndrome - an ongoing story. Journal of physiology and pharmacology : an official journal of the Polish Physiological Society. 2009;60 Suppl 7:19-24.
2. Bluher M. The distinction of metabolically 'healthy' from 'unhealthy' obese individuals. Current opinion in lipidology. 2010;21(1):38-43.
3. Mathieu P, Lemieux I, Despres JP. Obesity, inflammation, and cardiovascular risk. Clinical pharmacology and therapeutics. 2010;87(4):407-16.
4. Harwood HJ, Jr. The adipocyte as an endocrine organ in the regulation of metabolic homeostasis. Neuropharmacology. 2012;63(1):57-75.
5. Boden G. Obesity, insulin resistance and free fatty acids. Current opinion in endocrinology, diabetes, and obesity. 2011;18(2):139-43.
6. Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW, Jr. Obesity is associated with macrophage accumulation in adipose tissue. The Journal of clinical investigation. 2003;112(12):1796-808.
7. West M. Dead adipocytes and metabolic dysfunction: recent progress. Current opinion in endocrinology, diabetes, and obesity. 2009;16(2):178-82.
8. Karalis KP, Giannogonas P, Kodela E, Koutmani Y, Zoumakis M, Teli T. Mechanisms of obesity and related pathology: linking immune responses to metabolic stress. The FEBS journal. 2009;276(20):5747-54.
9. Visser M, Bouter LM, McQuillan GM, Wener MH, Harris TB. Elevated C-reactive protein levels in overweight and obese adults. Jama. 1999;282(22):2131-5.
10. Womack J, Tien PC, Feldman J, Shin JH, Fennie K, Anastos K, et al. Obesity and immune cell counts in women. Metabolism: clinical and experimental. 2007;56(7):998-1004.
11. Nieman DC, Henson DA, Nehlsen-Cannarella SL, Ekkens M, Utter AC, Butterworth DE, et al. Influence of obesity on immune function. Journal of the American Dietetic Association. 1999;99(3):294-9.
12. Kim JA, Park HS. White blood cell count and abdominal fat distribution in female obese adolescents. Metabolism: clinical and experimental. 2008;57(10):1375-9.
13. 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(2):308-15.
14. van der Weerd K, Dik WA, Schrijver B, Schweitzer DH, Langerak AW, Drexhage HA, et al. Morbidly obese human subjects have increased peripheral blood CD4+ T cells with skewing toward a Treg- and Th2-dominated phenotype. Diabetes. 2012;61(2):401-8.
15. Winer S, Paltser G, Chan Y, Tsui H, Engleman E, Winer D, et al. Obesity predisposes to Th17 bias. European journal of immunology. 2009;39(9):2629-35.
16. Viardot A, Heilbronn LK, Samocha-Bonet D, Mackay F, Campbell LV, Samaras K. Obesity is associated with activated and insulin resistant immune cells. Diabetes/metabolism research and reviews. 2012;28(5):447-54.
17. Jagannathan-Bogdan M, McDonnell ME, Shin H, Rehman Q, Hasturk H, Apovian CM, et al. Elevated proinflammatory cytokine production by a skewed T cell compartment requires monocytes and promotes inflammation in type 2 diabetes. Journal of immunology (Baltimore, Md : 1950). 2011;186(2):1162-72.
18. Cinti S, Mitchell G, Barbatelli G, Murano I, Ceresi E, Faloia E, et al. Adipocyte death defines macrophage localization and function in adipose tissue of obese mice and humans. Journal of lipid research. 2005;46(11):2347-55.
19. Murano I, Barbatelli G, Parisani V, Latini C, Muzzonigro G, Castellucci M, et al. Dead adipocytes, detected as crown-like structures, are prevalent in visceral fat depots of genetically obese mice. Journal of lipid research. 2008;49(7):1562-8.
20. Gornicka A, Fettig J, Eguchi A, Berk MP, Thapaliya S, Dixon LJ, et al. Adipocyte hypertrophy is associated with lysosomal permeability both in vivo and in vitro: role in adipose tissue inflammation. American journal of physiology Endocrinology and metabolism. 2012;303(5):E597-606.
21. Apovian CM, Bigornia S, Mott M, Meyers MR, Ulloor J, Gagua M, et al. Adipose macrophage infiltration is associated with insulin resistance and vascular endothelial dysfunction in obese subjects. Arteriosclerosis, thrombosis, and vascular biology. 2008;28(9):1654-9.
22. Xu XJ, Gauthier MS, Hess DT, Apovian CM, Cacicedo JM, Gokce N, et al. Insulin sensitive and resistant obesity

in humans: AMPK activity, oxidative stress, and depot-specific changes in gene expression in adipose tissue. Journal of lipid research. 2012;53(4):792-801.

23. Kintscher U, Hartge M, Hess K, Foryst-Ludwig A, Clemenz M, Wabitsch M, et al. T-lymphocyte infiltration in visceral adipose tissue: a primary event in adipose tissue inflammation and the development of obesity-mediated insulin resistance. Arteriosclerosis, thrombosis, and vascular biology. 2008;28(7):1304-10.
24. Nishimura S, Manabe I, Nagasaki M, Eto K, Yamashita H, Ohsugi M, et al. CD8+ effector T cells contribute to macrophage recruitment and adipose tissue inflammation in obesity. Nature medicine. 2009;15(8):914-20.
25. Ioan-Facsinay A, Kwekkeboom JC, Westhoff S, Giera M, Rombouts Y, van Harmelen V, et al. Adipocyte-derived lipids modulate CD4+ T-cell function. European journal of immunology. 2013;43(6):1578-87.
26. Karelis AD, Faraj M, Bastard JP, St-Pierre DH, Brochu M, Prud'homme D, et al. The metabolically healthy but obese individual presents a favorable inflammation profile. The Journal of clinical endocrinology and metabolism. 2005;90(7):4145-50.
27. Phillips CM, Perry IJ. Does inflammation determine metabolic health status in obese and nonobese adults? The Journal of clinical endocrinology and metabolism. 2013;98(10):E1610-9.
28. Katsiki N, Mikhailidis DP, Gotzamani-Psarrakou A, Yovos JG, Karamitsos D. Effect of various treatments on leptin, adiponectin, ghrelin and neuropeptide Y in patients with type 2 diabetes mellitus. Expert opinion on therapeutic targets. 2011;15(4):401-20.
29. Mirza S, Hossain M, Mathews C, Martinez P, Pino P, Gay JL, et al. Type 2-diabetes is associated with elevated levels of TNF-alpha, IL-6 and adiponectin and low levels of leptin in a population of Mexican Americans: a cross-sectional study. Cytokine. 2012;57(1):136-42.
30. Mantzoros CS, Moschos S, Avramopoulos I, Kaklamani V, Liolios A, Doulgerakis DE, et al. Leptin concentrations in relation to body mass index and the tumor necrosis factor-alpha system in humans. The Journal of clinical endocrinology and metabolism. 1997;82(10):3408-13.
31. Isoda K, Young JL, Zirlik A, MacFarlane LA, Tsuboi N, Gerdes N, et al. Metformin inhibits proinflammatory responses and nuclear factor-kappaB in human vascular wall cells. Arteriosclerosis, thrombosis, and vascular biology. 2006;26(3):611-7.
32. Akbar DH. Effect of metformin and sulfonylurea on C-reactive protein level in well-controlled type 2 diabetics with metabolic syndrome. Endocrine. 2003;20(3):215-8.
33. Solano ME, Sander V, Wald MR, Motta AB. Dehydroepiandrosterone and metformin regulate proliferation of murine T lymphocytes. Clinical and experimental immunology. 2008;153(2):289-96.