Systemic and white adipose tissue inflammation in obesity and insulin resistance

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4 Obesity is associated with species-specific composition of the leukocyte population in blood and adipose tissue

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Abstract

Aim: Obesity is associated with increased inflammation that is manifested in white adipose tissue (WAT) and the circulation. High fat diet-induced obese male C57Bl/6J mice are commonly used to study obesity related inflammation. However, it is unclear to what extent increased inflammation in WAT and the circulation are comparable between obese mice and humans.

Methods: Subcutaneous (sWAT) and omental (oWAT) adipose tissue specimens were obtained from obese women (BMI>40kg/m2). Subcutaneous, gonadal (gWAT) and mesenteric (mWAT) depots were obtained from obese male C57Bl/6J mice (body weight>40g). Adipocyte size and immune cell composition were determined for the different WAT depots. Blood samples were obtained from lean and obese women and male C57Bl/6J mice to determine the composition of the circulating leukocyte population and the activation status of the circulating leukocyte subsets.

Results: The composition of the leukocyte population in mouse WAT depots differed from all region-comparable human WAT depots in the obese state. Especially mouse gWAT contained a significantly higher fraction of macrophages and CLS as compared to human oWAT. In addition, the composition of the leukocyte population in blood differed remarkably between obese mice and obese humans. In comparison to obese humans, in obese mice the leukocyte population consisted of a large fraction of B cells and monocytes at the expense of granulocytes. Despite these differences in composition, the effect of obesity on the circulating immune cells showed similarities between humans and mice, including increased lymphocyte activation.

Conclusion: There are distinct differences between humans and mice regarding the composition of the leukocyte population in WAT and the circulation in obesity. However, the effect of obesity on the activity of the circulating lymphocyte subsets shows similarities. These data imply that caution should be taken when directly translating mouse findings to the human with respect to the relative role of specific immune cells in obesity related inflammation.

Introduction

Obesity is associated with white adipose tissue (WAT) inflammation (1). During the development of obesity, hypertrophic adipocytes release increased levels of fatty acids (FA) and pro-inflammatory cytokines, which are thought to attract immune cells into the WAT. Several immune cells, like T cells, B cells, macrophages, neutrophils and mast cells, have been shown to contribute to the development of obesity and related metabolic disorders (2-5). These immune cells undergo a phenotypic switch from an anti-inflammatory to a pro-inflammatory state. The number of pro-inflammatory M1 type macrophages and cytotoxic T cells increase, whereas the anti-inflammatory M2 type macrophages and regulatory T cells decrease in expanding WAT (6, 7). Obesity eventually leads to systemic inflammation, characterized by increased levels of circulating activated immune cells and higher levels of pro-inflammatory cytokines in the circulation (8-10). Pro-inflammatory immune cells in obese WAT and the circulation are both thought to contribute to the development of insulin resistance (IR) (11, 12).

Mouse models are widely used to gain mechanistic insight in human biology and disease. However, not all aspects of mouse biology directly reflect the human situation (13). WAT consist of various depots, and location and distribution of WAT differs between humans and mice. In general, WAT can be divided into subcutaneous WAT (sWAT) located underneath the skin, and visceral WAT (vWAT) around the abdominal organs. Human vWAT is generally subdivided into a mesenteric (in between the organs; mWAT) and an omental depot (in front of the major omentum; oWAT). Mouse vWAT consists of mesenteric WAT (in between the organs; mWAT) and gonadal WAT (around the testis/ovaries; gWAT). As it is not possible to perform longitudinal studies in humans, high fat diet-induced obese mouse models are commonly used to study obesity induced inflammation and related disorders. Therefore, it is important to determine the translational potential of (patho)physiological mechanisms from mouse to human. The effect of obesity on mouse WAT depot immune cell composition has previously been described by us and others (14, 15). Not only the composition of the immune cell pool, but also the effect of obesity differed remarkably for the different WAT depots in mice.

Here, we set out to directly compare the composition of the leukocyte population in different obese WAT depots and in the circulation between humans and mice. We have previously shown that obesity leads to increased activation of circulating immune cells in humans (10). However, the effect of obesity on the composition of the leukocyte population and the activation status of the leukocyte subsets in the circulation have not been studied in mice. Our study is the first to describe direct human-mouse comparisons in the composition of the leukocyte population in different WAT depots and the circulation, as well as the effect of obesity on the activation status of circulating leukocyte subsets in mice.

Materials and methods

Human subjects

The study group consisted of healthy lean (BMI: 21.7 ± 1.6 kg/m2; age: 50 ± 5 y; n=12), and morbidly obese women (BMI: 44.0 ± 3.4 kg/m2; age: 48 ± 6 y; n=26) with normal glucose tolerance. The women were part of a clinical trial of which the research methods and design have been described elsewhere (16). From both lean and obese individuals blood was drawn which was used for further analysis. A subgroup of the obese individuals (n=10) underwent bariatric surgery. Within 1h after opening the abdominal wall WAT specimens were taken from the epigastric region of the abdominal wall (sWAT) and from the major omentum (oWAT). These samples were used for further analysis. The study (ClinicalTrials.gov: NTC01167959) was approved by the Ethics Committee of Leiden University. All subjects gave informed consent to participate in the study.

Animals

Wild-type (WT) male mice (C57Bl/6J background) were purchased from Charles river (Maastricht, The Netherlands). Mice were housed under standard conditions with free access to water and food. To induce obesity, mice were fed a high fat diet (HFD; 45% energy derived from lard fat, D12451, Research Diet Services, Wijk bij Duurstede, The Netherlands) for different number of weeks. At the end of the diet intervention, mice were killed and blood was collected via orbital bleeding for further analysis. The mice were perfused with PBS to clear the organs from blood, and sWAT, gWAT, and mWAT depots were dissected for further analysis. This study consisted of lean and obese mice with a body weight of <30 g (26.7 ± 1.7 g; n=14) or >40 g (48.1 ± 3.9 g; n=18), respectively. WT mice from different experiments were combined for this study, as previously described (14). All experiments were approved by the animal ethics committee of Leiden University Medical Center.

Adipocyte, stromal vascular fraction and blood cell isolation

WAT depots from humans and mice were processed for adipocyte size determination as previously described (10). The residue of the WAT filtrate was used for the isolation of the stromal vascular fraction (SVF) as previously described (14).

Fresh heparinized blood from human and mouse was washed with PBS and erythrocytes were lysed using BD lysis solution (BD biosciences, CA, USA). Absolute numbers of leukocytes in human blood were determined at the laboratory for Clincal Chemistry at the Leiden University Medical Center, using a fully automated Hitachi 704/911 system (Krefeld, Germany). Absolute numbers of leukocytes in mouse blood were determined using an automated cell counter (TC10, Biorad, Berkeley, CA, USA). The remaining cells were fixed with 1% paraformaldehyde, stored at 4 °C and analyzed within one week. SVF and blood cells were measured by flow cytometry analysis to determine the composition of the immune cell pool.

Flow cytometry analysis

Human SVF and blood cells were stained with fluorescently labeled antibodies for CD45-PerCP, CD3-PE, CD4-PB, CD8-APC (Dako, Glostrup, Denmark), CD25-PeCy7, CD19-PE, CD38-PerCPCy5.5 and CD14-PeCy7 (all purchased from BD biosciences, CA, USA unless stated otherwise). Granulocytes were determined by selecting their distinct population in the forward and sideward scatter. Mouse SVF and blood cells were stained with fluorescently labeled antibodies for CD45.2-FITC (BioLegend), CD3-APC, CD4-Qdot605, CD8a-PercpCy5.5, CD25-PeCy7 CD19-PE, F4/80-PE, CD11B-PB and GR1-PeCy7 (all purchased from eBioscience, CA, USA or BioLegend, CA, USA). Cells were measured on a LSR II flow cytometer (BD Biosciences, CA, USA). Data were analyzed using FlowJo software (Treestar, OR, USA).

Immunohistochemistry of crown-like structures

The number of crown-like structures (CLS) per mm2 WAT was determined after immunostaining of CD68 in 8 human oWAT samples as described in (10) and in 14 mouse gWAT samples after staining of F4/80 as described in (14).

Statistics

Data are presented as mean ± SD. Statistical differences between groups were calculated with the student's t-test using GraphPad Prism version 6 (GraphPad software, CA, USA). $p<0.05$ was considered statistically significant.

Results

Comparison of WAT depots between obese humans and mice

To directly compare human and mouse WAT depots, biopsies were taken from sWAT and oWAT from obese women and compared with sWAT, gWAT and mWAT depots from obese mice. Adipocyte sizes in all WAT depots from human and mouse were comparable (Table 1). The absolute number of SVF cells per gram WAT was lower in the human WAT depots than in the mouse WAT depots (Table 1). Within the SVF, the percentage of leukocytes (CD45) in human WAT depots was comparable to those in mouse WAT depots (Table 1, Figure 1A). The percentage of T cells (CD3) in the SVF of human and mouse sWAT was comparable. Human oWAT had comparable percentages of T cells to mouse mWAT, but this was higher as compared to mouse gWAT, (Table 1, Figure 1A). The SVF of human sWAT contained relatively more T helper cells (CD4) than cytotoxic T cells (CD8) cells as compared to mouse sWAT, indicated by the higher CD4:CD8 ratio (Table 1). Human oWAT had a similar CD4:CD8 ratio as mouse gWAT and mWAT (Table 1). The percentage of B cells in the human WAT depots was lower than in all mouse WAT depots (Table 1, Figure 1A). The percentage of macrophages was particularly high in mouse gWAT and differed significantly from human oWAT. The other human and mouse depots had comparable percentages of macrophages (Table 1, Figure 1A). Thus, the composition of the human and mouse WAT depot immune cell population appears to be different. After multiple test correction the lower number of macrophages in human oWAT as compared to mouse gWAT remained significant. Also the number of CLS was significantly higher in human oWAT as compared to mouse gWAT (Figure 2).

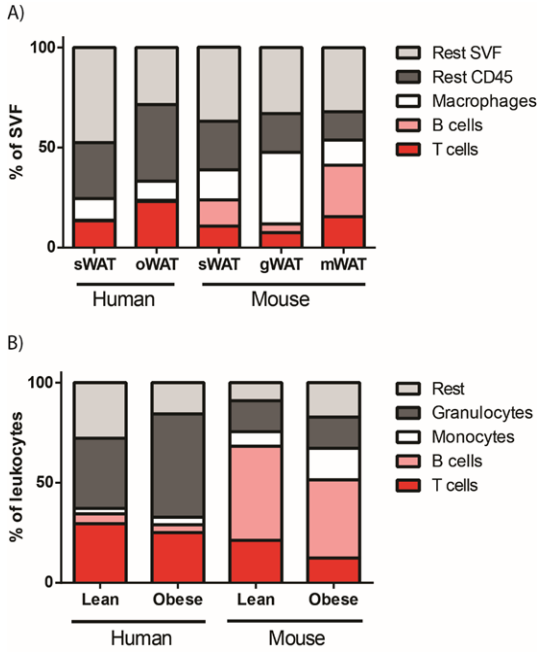

Figure 1. Schematic comparison of the composition of immune cell population of WAT depots and circulation from humans and mice. Composition of immune cell population of WAT as percentage of SVF is depicted for human (sWAT and oWAT) and mouse (sWAT, gWAT, and mWAT) depots (A). Different immune cell types are represented in this graph, T cells (red), B cells (pink), macrophages (white), rest leukocytes (leukocytes which are not T cell, B cell or macrophage; dark grey), and rest SVF (SVF cells which are not leukocytes; light grey). Blood immune cell composition as percentage of leukocytes is depicted for lean and obese human and mouse (B). Different immune cell types are represented in this graph, T cells (red), B cells (pink), monocytes (white), granulocytes (dark grey), and rest (leukocytes which are not T cells, B cells, monocytes or granulocytes; light grey).

Figure 2. Number of crown-like structures per area of adipose tissue section. CLS were determined by immunostaining of CD68 in human oWAT samples (n=8) or staining of F4/80 in mouse gWAT samples (n=14).

Table 1. Composition and comparison of WAT depots from obese human subjects and mice

	Human WAT		Mouse WAT*			Statistics		
	sWAT	oWAT	sWAT	gWAT	mWAT	T-test sWAT vs sWAT	T-test oWAT vs gWAT	T-test oWAT vs mWAT
Adipocyte size (µm)	114.6±15.8	115.6±14.1	112.0±2.5	124.4±8.0	113.9±5.5	0.7239	0.2111	0.7560
SVF no. per gr AT (*10^6)	0.27±0.10	0.24±0.86	0.60±0.14	1.46±0.52	1.83±1.42	**1.28E-8**	**3.19E-5**	**0.0181**
Leukocytes (%CD45 of SVF)	52.5±12.7	71.5±7.5	63.1±7.9	67.0±3.5	67.9±16.4	0.1250	0.2111	0.6908
T cells (%CD3 of SVF)	13.5±6.8	23.0±12.3	12.5±4.2	6.6±2.0	11.3±9.4	0.7560	**0.0179**	0.1086
T cell ratio (CD4:CD8)	2.55±1.70	1.35±0.59	1.00±0.24	1.15±0.47	1.77±0.56	0.0572	0.5591	0.2111
B cells (%CD19 of SVF)	0.23±0.21	0.66±0.62	13.0±10.2	2.1±1.2	19.3±21.2	**0.0210**	0.0572	0.0887
Macrophages (%CD14 or F4/80 of SVF)	10.9±8.0	9.5±6.1	6.5±1.8	37.5±3.9	17.9±10.5	0.2111	**2.45E-6**	0.2111

In bold, statistically significant p-values (p<0.05)
After Bonferroni multiple test correction p<0.0024 is considered statistically significant
*data partly published in van Beek *et al.* 2015

Comparison of circulating immune cells between humans and mice, in the lean and obese state

Blood from lean or obese humans and mice was analysed to compare the composition and activation status of the circulating leukocyte population between humans and mice. Absolute numbers of leukocytes per ml were higher in human blood as compared to mouse blood in the lean, but not in the obese state (Table 2). The percentage of T lymphocytes was lower in mouse blood as compared to human blood, only in the obese state (Table 2, Figure 1B). Within the circulating T cell population mice had a lower CD4:CD8 ratio, and thus relatively more cytotoxic T cells than humans. This was due to both lower percentages of T helper cells and higher percentages of cytotoxic T cells in mice (Table 2). Activation status (CD25+) of the circulating T helper cells was lower in lean humans compared to mice, and comparable during obesity (Table 2). Within the cytotoxic T cell population, the percentage of activated cells was higher in the circulation in mice compared to humans both in the lean and the obese state (Table 2). The percentage of B cells was approximately 10 times higher in blood from mice as compared to humans, both in the lean and the obese state (Table 2, Figure 1B). Within these B cells, humans had a higher percentage of activated cells as compared to mice in both the lean and obese state, however only significantly different after multiple test correction in obesity (Table 2). The percentage of monocytes was higher in mice, whereas granulocytes were higher in the human circulation in the lean and the obese state (Table 2, Figure 1B). Thus, the composition and activation status of the circulating leukocyte population was remarkably different between humans and mice, in both the lean and the obese state.

Table 2. Composition and comparison of blood from lean and obese human subjects and mice

	Human blood*		Mouse blood		Statistics			
	Lean	Obese	Lean	Obese	T-test lean human vs mouse	T-test obese human vs mouse	T-test human lean vs obese	T-test mouse lean vs obese
Absolute leukocyte no. per ml (*10^6)	6.26±1.35	6.59±1.40	2.97±1.46	8.40±6.53	**1.07E-5**	0.2245	0.5643	**0.0030**
T cells (%CD3 of leukocytes)	29.5±10.9	25.0±6.4	21.2±3.5	12.4±4.0	**0.0185**	**1.89E-8**	0.1531	**1.20E-6**
T cell ratio (CD4:CD8)	3.35±1.08	3.86±1.57	1.50±0.26	1.32±0.16	**7.18E-6**	**1.10E-7**	0.3681	**0.0248**
T helper cells (%CD4 of CD3)	72.4±6.9	73.7±7.6	52.8±4.0	48.7±4.0	**1.89E-8**	**6.66E-15**	0.6336	**0.0130**
Activation T helper cells (%CD25 of CD4)	3.37±2.72	10.3±10.1	8.23±1.94	8.68±1.98	**0.0016**	0.5643	0.0064	0.6336
Cytotoxic T cells (%CD8 of CD3)	23.0±5.0	21.3±6.5	35.6±3.6	37.2±2.3	**4.56E-07**	**1.21E-11**	0.4833	0.1838
Activation cytotoxic T cells (%CD25 of CD8)	0.27±0.24	1.59±2.08	13.7±4.8	24.2±5.9	**1.04E-07**	**1.54E-19**	**0.0074**	**8.58E-4**
B cells (%CD19 of leukocytes)	4.96±2.01	4.01±2.16	47.0±10.6	39.0±7.8	**3.00E-12**	**7.13E-23**	0.2476	**0.0246**
Activation B cells (%CD38 or CD25 of CD19)	49.3±21.6	70.9±17.7	32.4±7.8	42.2±4.2	**0.0204**	**2.05E-5**	**0.0052**	**0.0076**
Monocytes (%CD14 or CD11B of leukocytes)	2.71±1.32	3.73±1.44	7.28±3.98	15.8±11.2	**0.0016**	**1.07E-5**	0.0601	**0.0091**
Granulocytes (%GR1 of leukocytes)	35.0±12.0	51.5±13.4	15.5±10.9	15.5±13.6	**2.61E-04**	**6.26E-10**	**0.0018**	0.9934

In bold, statistically significant p-values (p<0.05)
After Bonferroni multiple test correction p<0.0012 is considered statistically significant
*data partly published in van Beek *et al.* 2014

Obesity induced changes in circulating immune cells in humans and mice

The effect of obesity on the composition and activation status of the circulating immune cell population was determined in humans and mice. Absolute numbers of circulating leukocytes were higher, in obese versus lean mice. This variable was similar between lean and obese humans (Table 2). The percentage of T lymphocytes decreased with obesity in the circulation of mice, whereas this remained similar in humans (Table 2, Figure 1B). Obesity led to a lower CD4:CD8 ratio in mice, primarily caused by a lower percentage of T helper cells within the T cell fraction (Table 2). The percentage of activated T helper cells was increased in the human circulation by obesity. Activation of circulating cytotoxic T cells was increased by obesity in both humans and mice, (Table 2). The percentage of B cells was lowered by obesity in mice (Table 2, Figure 1B). The percentage of activated B cells was increased by obesity in the circulation of both humans and mice (Table 2). Obesity led to increased percentages of monocytes

in the circulation of both humans and mice (Table 2, Figure 1B). The percentage of granulocytes was increased by obesity only in the human circulation (Table 2, Figure 1B). After correction for multiple testing the increased number of T cells and activation state of cytotoxic T cells and B cells in mice and the increased number of granulocytes in humans remained significant.

Discussion

Mouse models are widely used to study mechanisms underlying human diseases. Although there is significant overlap in gene regulation of important biological processes between mice and humans, fine regulation and activity of numerous genes and proteins of the immune system and metabolic processes vary between both species (13, 17-20). Also the innate and adaptive immune system have been shown to differ between mice and humans (21). In the current study, we set out to determine whether and to what extent WAT depots and the circulation from mice are comparable to humans with respect to the composition of the immune cell population in obesity. Our data show important inter-species differences in the composition of the immune cell pool of both WAT depots and the circulation. These differences should be taken into account when using mouse models to study mechanisms relevant for human pathology. Furthermore, we determined the effect of obesity on the composition of the immune cell pool and the activation status of the leukocyte subtypes in the circulation of mice and compared this to humans. Our data shows that obesity leads to increased lymphocyte activation in the circulation of both humans and mice.

Mouse gWAT is considered to be most similar to oWAT in humans. However, we found a much higher fraction of macrophages and more CLS in obese mouse gWAT than in human oWAT. Mouse gWAT is characterized by a high macrophage influx and formation of CLS during the development of obesity (14, 15, 22). We have previously shown that gWAT primarily expands in the initial phase of body weight gain after which it stops expanding, while sWAT and vWAT remain growing (14). This difference in expansion may explain the relative high percentage of macrophages that we observed in mouse gWAT. In contrast to the mice, which were still in the process of developing obesity; the humans had already been morbidly obese for at least five years and therefore they appeared to be in a constant state of obesity. This difference in state of obesity between the mice and humans may in part explain the difference in number of macrophages between human oWAT and mouse gWAT. The difference in the composition of the immune cell population between human and mouse WAT may also be explained by differences in the process of obesity development. Although HFD-induced obesity appears to be most comparable to the human situation, it still is a relative harsh way to induce obesity in mice. A diet containing 45-60% energy derived from fat is fed to the mice and they usually double in weight within 10 weeks. For humans, this process of obesity development generally takes decades. Therefore, it is imaginable that HFD-induced obesity in mice leads to a higher inflammatory state as compared to the development of obesity in humans.

Studies comparing the composition of blood or tissue from humans and mice are scarce. Recently, Ip et al. reviewed the role of lymphocyte subsets in metabolic disease in humans and mice (23).

However, direct comparison of circulating and WAT depot immune cell composition in relation to obesity between humans and mice has not been described. In the lean state, humans have generally more granulocytes and less lymphocytes in the circulation as compared to mice (21). Here we show that the mouse blood contains relatively more B cells and monocytes, and fewer granulocytes than human blood, both in the lean and the obese state. We have previously shown that obesity leads to increased systemic inflammation in humans, with increased activation of lymphocytes and higher pro-inflammatory cytokine levels in the circulation (10). Trottier et al showed HFD induced elevations in white blood cell counts in mice, primarily caused by higher numbers of lymphocytes in blood (24). Our data indicate similarities between humans and mice regarding the effect of obesity on activation of circulating immune cells. We show in particular an increased activation of cytotoxic T cells and to a lesser extent an increased activation of B cells in the circulation of both humans and mice. Thus, obesity seems to induce lymphocyte activation in both humans and mice.

It should be mentioned that there is a good chance that some of our significant differences between mice and human may be false positives because many significant differences disappeared after stringent multiple test corrections. This was due to the high variability in the data and in particularly in the human data. However, an unfortunate byproduct of correcting for multiple testing is that you may increase the number of false negatives which are biologically relevant effects that are not detected as statistically significant.

In this study, we analysed WAT and blood from male C57Bl/6J mice. It is known that male C57Bl/6J mice are more prone to develop HFD-induced obesity and associated metabolic derangement compared to female C57Bl/6J mice (25, 26). Therefore, when studying obesity related disorders, male mice are generally used. Recently, it has been shown that these sex differences in mice are caused by enhanced pro-inflammatory responses to HFD-induced obesity in males (26). Humans show gender differences with respect to obesity and metabolic disorders as well (27, 28), and several reports describe sex differences in immune responses in the circulation (29, 30). However, the effect of gender differences on WAT immune cell composition remains to be investigated.

Some limitations of this study have to be addressed. During this study, we focussed on the most prevalent immune cell types that are known to contribute to obesity induced inflammation, thereby we may have overlooked other potentially important immune cell subtypes. To eliminate blood cells from the WAT depots, mice were thoroughly perfused during our experiment. Obviously, this is not possible for the WAT biopsies from human, and therefore human WAT can contain circulating immune cells that might affect the reported composition of the immune cells in the WAT depots. From lean humans, we unfortunately could not obtain WAT tissue and therefore we cannot assess the effect of obesity on the immune cells in WAT in humans, or compare this to the effect of obesity on the immune cells in mouse WAT. However, as obesity leads to systemic inflammation, circulating immune cells are thought to reflect the inflammatory status of the WAT depots (31, 32). Cytokine levels in blood are a valuable measure for systemic inflammation in humans. However, in mice several general cytokines, like TNF-α and IL-6, are hardly detectable in the circulation via standard ELISA-based measurements.

Our data shows that obese human WAT depots differ from mouse WAT depots regarding immune cell composition, with mouse gWAT mainly containing a higher percentage macrophages compared to human oWAT. The composition of circulating immune cells is remarkably different between humans and mice, showing differences in each immune cell subtype studied. Obesity leads to increased percentages of activated lymphocytes in both humans and mice. Thus, we can conclude that there are significant inter-species differences regarding WAT and circulating immune cell composition, however, the effect of obesity on the activation of circulating immune cells shows similarities. Nevertheless, caution should be taken when directly translating mouse findings regarding the effect of obesity on inflammation to the human.

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