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Lianne van Beek

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Systemic and adipose tissue inflammation in obesity and insulin resistance

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General introduction

1

Introduction

Obesity and metabolic disorders

Obesity is defined as abnormal or excessive fat accumulation and presents a risk to health. It is a major risk factor for the development of various chronic diseases, like diabetes mellitus, cardiovascular disease and several cancers (1), and is one of the leading preventable causes of morbidity and mortality (2-4). As obesity is reaching epidemic proportions and the prevalence is still increasing not only in western societies but also in low- and middle-income countries, it is a cause for worldwide concern (5).

When energy intake exceeds energy expenditure this will lead to weight gain and eventually to obesity. Excess energy will be stored as triglycerides (TG) primarily in the white adipose tissue (WAT), leading to WAT expansion. One TG molecule is composed of one glycerol and three fatty acid (FA) molecules. TG are common lipids in our diet and the main energy source for the body. FA derived from TG can be used for ATP production by the muscle and for generation of heat by brown adipose tissue (BAT) (6, 7). However, if TG are not being used for energy production or properly stored in WAT, plasma lipid levels may increase resulting in hyperlipidemia. TG can also be stored in organs other than WAT, like liver or muscle which is known as ectopic fat storage. Ectopic fat storage can contribute to the development of metabolic disorders, which is discussed later on.

Obesity is the major cause of the development of the metabolic syndrome, which is characterized by the co-occurrence of several cardio-metabolic risk factors, including central obesity, insulin resistance, hypertension, and dyslipidemia (8). The metabolic syndrome represents a major risk for the development of type 2 diabetes and cardiovascular disease. However, about 20 percent of the obese population seem to remain relatively insulin sensitive and metabolically healthy. The reason why these people are protected from the development of metabolic disorders has not been extensively characterized yet.

White adipose tissue

For a long time it was thought that WAT did little else than storing and releasing energy and to function as cushioning and insulation of the body. It is now known that WAT also is an endocrine and inflammatory organ (9). Adipocytes (or fat cells) release hormones like leptin and adiponectin that regulate satiety and metabolic processes, as well as cytokines which are all known as adipokines (10). Recently, it was found that adipocytes also have infection-protective properties via the production of antimicrobial peptides (11). Furthermore, adipocytes show similarities to immune cells; in addition to the capacity to produce all kinds of cytokines, they have antigen-presenting cell (APC) properties and express MHCII molecules on their cell surface (12, 13).

WAT is distributed in different depots throughout the body. A subcutaneous depot (sWAT) is located underneath the skin, and visceral depots (vWAT) are situated around the abdominal organs. vWAT is known to be more metabolically active and pro-inflammatory, and to exert more negative effects on health as compared with sWAT (14-16). WAT is composed of adipocytes and a number of different cell types which are compositely known as the stromal vascular fraction (SVF). The SVF

contains vascular endothelial cells, fibroblasts, pre-adipocytes, and several types of immune cells (17). Adipocytes mature from pre-adipocytes that originate from the mesenchymal cell lineage (18).

The effect of obesity on white adipose tissue

During the development of obesity, WAT expands by increase in size (hypertrophy) and/or number (hyperplasia) of adipocytes (18, 19). A typical human adipocyte has a size of 0.1 mm in diameter, however, adipocytes can expand to more than twice that size during obesity. Generally, hypertrophy occurs prior to hyperplasia to increase the fat storage capacity of WAT during the development of obesity (20). In humans, adipocyte hyperplasia is a matter of some debate. According to Arner et al. (21), the number of adipocytes is determined during childhood and remains stable during life. This is supported by others that show hypertrophy rather than hyperplasia during weight gain in humans (22). A recent study did, however, show increased numbers of newly generated adipocytes in sWAT of healthy volunteers after overfeeding (23). Also in some individuals with morbidly obesity, hyperplasia of the adipocytes has been demonstrated once hypertrophy of the adipocytes was limited (24).

During the development of obesity, hypertrophic adipocytes release increased levels of FA and adipokines that both have immune modulatory activities (25, 26). Pro-inflammatory adipokines, including leptin, TNFα, and IL6, stimulate the influx of pro-inflammatory immune cells into the obese WAT. Obesity-induced WAT inflammation is associated with metabolic dysfunction and is hypothesized to contribute to the development of insulin resistance, as discussed later on in the section “Impact of inflammation on insulin signalling”.

WAT expansion requires tissue remodelling, which includes extracellular matrix breakdown and resynthesis as well as angiogenesis to maintain nutrient and oxygen supply (27). It has been shown that the inflammatory response induced by expanding adipocytes is essential during this process, as these signals drive healthy adipose tissue expansion and remodelling (28). However, when expansion of adipocytes is not associated with appropriate remodelling and angiogenesis, oxygen may become deficient in WAT, leading to hypoxia. Adipose tissue hypoxia leads to adipocyte stress and cell death, characterized by dysregulation of the production of cytokines, as well as altered FA fluxes (29) which contributes to the development of WAT dysfunction in obesity (30). However, WAT hyperoxia during obesity has also been associated with adipose tissue dysfunction and insulin resistance (31). Obese subjects seem to have higher oxygen tension despite lower adipose tissue blood flow, which could be explained by a lower oxygen consumption of obese adipose tissue.

Obesity-induced inflammation

The body induces an immune response to eliminate pathogens or damaged cells. Two general types of immune responses are recognized: the innate and the adaptive immune response. The innate immune response is characterized by a relatively non-specific and rapid response of the body to fight infections, and is in evolutionary terms the oldest. It is known as a first line of defence mechanism, and the cellular component includes macrophages, granulocytes (neutrophils, eosinophils, and basophils),

mast cells, dendritic cells, and natural killer cells. The evolutionary more recent adaptive immune response is antigen-specific and includes T and B lymphocytes that express variable T-cell receptors and produce antibodies, respectively. Antibodies undergo a process called affinity maturation to increase the specificity of the antigen recognition. This process takes time and upon first exposure, the adaptive immune response is relatively slow. However, T and B lymphocytes create immunological memory after initial response to a pathogen, which ensures a rapid response upon repeated exposure.

WAT contains several types of immune cells that in the lean state are mainly considered as anti-inflammatory immune cells (Figure 1). These cells are presumably involved in immune surveillance and adipose tissue remodelling (32). During the development of obesity, the expanding adipocytes release adipokines, like leptin, MCP1, TNFα, and IL6, which attract and activate pro-inflammatory immune cells into the WAT (Figure 1). The immune cells themselves also release pro-inflammatory cytokines and chemokines, which lead to additional infiltration of immune cells into WAT during WAT expansion (33). WAT inflammation eventually causes chronic low-grade systemic inflammation, characterized by increased levels of cytokines and other inflammatory markers in the circulation which are thought to contribute to the development of insulin resistance in peripheral organs (34). The immune cells playing an important role in obesity-induced inflammation and studied in this thesis are discussed below.

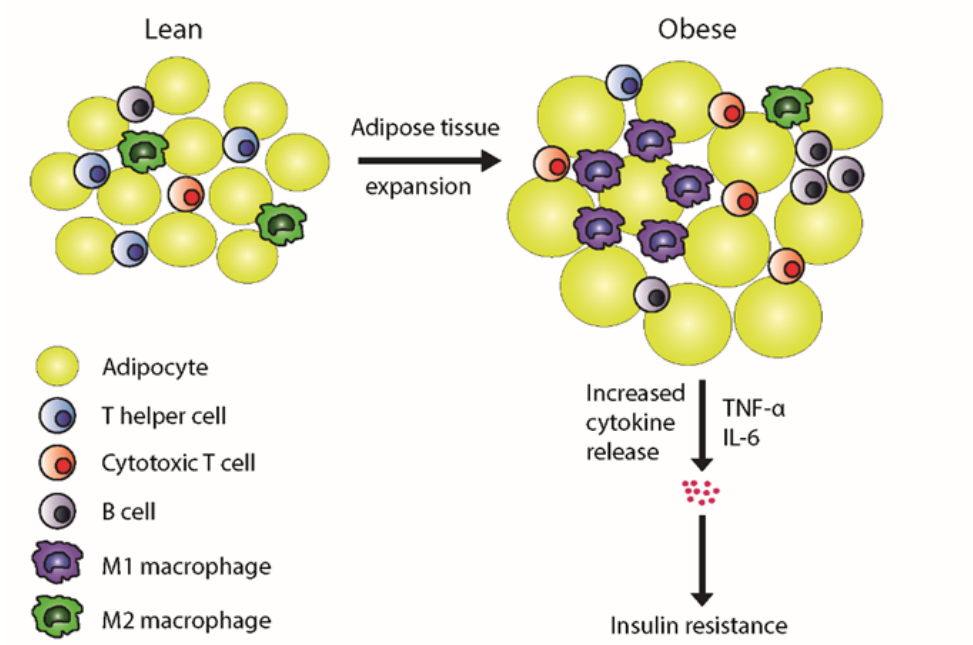

Figure 1. Schematic overview of adipose tissue inflammation during the lean and obese state. Lean adipose tissue mainly contains anti-inflammatory immune cells. During the development of obesity adipocytes expand and pro-inflammatory immune cells infiltrate into the adipose tissue. Adipocytes and immune cells in the obese adipose tissue release increased levels of pro-inflammatory cytokines, which are thought to contribute to the development of insulin resistance. Adapted from Kalupahana et al (33).

Role of monocytes and macrophages

Monocytes are circulating immune cells that are able to migrate into tissue in response to inflammatory signals. These inflammatory signals include increased expression of chemokines, like monocyte chemotactic protein 1 (MCP1) which is specifically known to recruit monocytes to the site of inflammation (35). Monocytes differentiate into macrophages or dendritic cells once they enter the tissue. Macrophages are phagocytic cells, specialized in the removal of pathogens, dead cells and cellular debris by engulfment and digestion (36). In the adipose tissue this can be recognized by the presence of crown-like structures (CLS) that exist of macrophages surrounding dying and dead adipocytes (37). After digestion of pathogenic material, the macrophage presents pathogen-derived antigens via its MHC molecules to corresponding T cells to facilitate further effective pathogen clearance and to initiate immune memory. Tissue resident macrophages, that are presumably involved in immune surveillance, are termed alternatively activated macrophages (M2 type) and have an anti-inflammatory phenotype, characterized by release of anti-inflammatory cytokines such as IL10 and TGFβ (38, 39). Pro-inflammatory macrophages are termed classically activated or M1 type macrophages and secrete pro-inflammatory cytokines like IL1β, IL6, IL12, and TNFα (38, 39).

Numbers of monocytes are increased in the circulation of obese human subjects and diet-induced obese mice compared to lean (40, 41). Furthermore, cytokine levels in the circulation of obese subjects are increased as compared to lean, including IL6 and TNFα which are known to be secreted, amongst others, by monocytes and macrophages (42). IL6 and TNFα are both pro-inflammatory cytokines, indicative for systemic inflammation and may contribute to the development of metabolic disorders (43).

Obese WAT is known to contain higher numbers of macrophages and CLS as compared to lean WAT (44). The presence of CLS in the adipose tissue has been linked with adipose tissue dysfunction and the development of metabolic disorders (37, 45). Recently, it has been shown that in addition to monocyte recruitment, macrophages are also able to proliferate locally in WAT to increase the number of macrophages during obesity (46). Lean WAT primarily contains M2 types macrophages, whereas obesity induces a phenotypic switch leading to increased numbers of pro-inflammatory M1 types macrophages (47). Macrophages thus do seem to play an important role in the development of obesity induced inflammation.

Role of T cells

T cells are lymphocytes characterized by the expression of the T cell receptor on their cell surface. Several subtypes of T cells are recognized, including T helper and cytotoxic T cells, characterized by expression of the cell surface markers CD4 and CD8, respectively. The main function of T helper cells is to assist other immune cells during immune responses. Antigen presenting cells (APCs), such as macrophages, can present antigens via their MHCII molecules to T helper cells. In this way T helper cells can become activated, which triggers them to produce cytokines that regulate immunological processes including B cell maturation, monocyte recruitment, and cytotoxic T cell activation (48).

There are different subtypes of T helper cells, including Th1, Th2, Th17, and regulatory T cells. Th1 and Th17 T cells are generally considered pro-inflammatory, whereas Th2 and regulatory T cell are considered as anti-inflammatory cell types (49). Cytotoxic T cells are also known as killer cells, as they can directly destroy for example infected cells by releasing cytotoxins that induce apoptosis of the target cell. The infected cells present antigens via their MHCI molecules, which are expressed by nearly all nucleated cells. The presented antigens are recognized by the antigen specific T cell receptor on the cytotoxic T cell. After recognition, the antigen-specific T cells undergo clonal expansion to eliminate the antigen-positive target cells. In addition, memory T cells are formed in order to induce a quick and efficient immune response after re-exposure to the same pathogen.

T cells originate from precursors in the bone marrow which mature and differentiate in the thymus before they end up in the circulation. Thymus size, as well as numbers of thymocytes are increased in diet-induced obese mice (40). Also circulating T cell numbers are elevated by obesity, which is primarily caused by an increase of T helper cells (50, 51). The activation status of T cells in the circulation is also increased with obesity (52). Although the type and activation status of circulating lymphocyte subsets in relation with obesity have been studied extensively in humans (41, 50, 52), lymphocyte subsets in relation with obesity in the mouse systemic circulation have been poorly characterized (53).

Numbers of T cells are increased in obese WAT of both humans and mice (54, 55). Obese WAT is characterized by increased numbers of pro-inflammatory Th1 and cytotoxic T cells, whereas the number of regulatory T cells is decreased (55). Both Th1 and cytotoxic T cells have been associated with increased insulin resistance (56, 57). T cell infiltration into the WAT seems to be a primary event in WAT inflammation (58). Several studies have shown that Th1 and cytotoxic T cells help recruit macrophages into the expanding WAT and stimulate macrophage polarization towards the pro-inflammatory M1 subtype (56, 57, 59). Thereby, this clearly implicates T cell mediated inflammation in the pro-inflammatory phenotype of obesity.

Role of B cells and immunoglobulins

The primary function of B cells is to produce antibodies against specific antigens. Furthermore, they can function as antigen presenting cells and release cytokines that regulate immune responses (60). Similar to T cells, B cells can form an immune memory pool after first exposure, to enable a quicker and stronger response after re-exposure to the same antigen. After antigen exposure, plasma B cells are formed that produce large amounts of antibodies, also termed immunoglobulins (Ig). An antibody consists of a variable region containing a specific antigen binding site and a constant Fc-region to communicate with and activate other immune components (61), the Fc-region determines the antibody isotype. Upon binding of antigens, the different antibody isotypes bind to isotype-specific Fc-receptors (e.g. IgG binds to Fcγ-receptors), thereby inducing specific immune responses. Fc-receptors are expressed by a variety of cells, including macrophages, dendritic cells, B cells and mast cells which can be activated by antibody-antigen immune complexes (62). Binding to Fc-receptors can induce phagocytosis of the immune complex, cytokine production, and cell death of the target cell (61, 63).

Furthermore, immune complexes are able to bind to C1q, the recognition component of the classical complement pathway. Binding of C1q activates the complement system and initiates a cascade of reactions that finally cleaves the central complement component C3, inducing phagocytosis and/or lysis of the pathogen (64). There are distinct differences between the human and mouse Fc-receptor biology (65). The human IgG Fc-receptor family consists of six receptors, whereas in mice only four Fcγ-receptors have been identified. Human and mouse Fcγ-receptors both include several activating receptors and one inhibitory receptor. Two of the human and all mouse activating Fcγ-receptors contain a γ-subunit, which is necessary for signalling and cell surface expression of the receptors. For the IgG subclasses there are also differences between humans and mice. Human IgG nomenclature is given by order of abundance in plasma (IgG1-4), which does not account for mice (IgG1,2A/B,3). Moreover, the human and mouse IgG subtypes differ in function and receptor affinity (65).

Total leukocyte counts as well as B cell numbers are increased in the circulation of obese women compared to non-obese women (41). Circulating B cells show a pro-inflammatory cytokine profile (increased IL6 and TNFα, and decreased IL10 secretion) in diabetic patients, as well as in spleens from obese mice (66, 67). This is thought to promote pro-inflammatory T cell functioning and to regulate inflammation in T2DM (67). Obese children have elevated total plasma IgG levels as compared with lean children, which is associated with a less favourable metabolic phenotype (68). In the mouse circulation IgG3 is primarily present, however IgG2c is the only subtype that is increased by HFD intervention in the circulation and in WAT (69).

Shortly after high fat diet (HFD) feeding in mice, numbers of B cells increase in WAT (55), the accumulation of B cells in the obese WAT contributes to the development of insulin resistance by the production of pathogenic IgG antibodies (Figure 2) (69, 70). Transfer of IgG from obese mice to HFD-fed B-null mice induced rapid local and systemic changes in the inflammatory cytokine production and a phenotypical conversion of the WAT macrophages to a pro-inflammatory M1 phenotype (69). Obesity related antigens, against which B cells produce antibodies have not been identified yet. However, as IgG antibodies were found to be located in CLS, it is possible that dead adipocytes are a source of the antigens (70). B cells and their antibodies may thus be important regulators during the development of obesity related insulin resistance.

Impact of inflammation on insulin signalling

Insulin is produced by beta cells in the pancreas and regulates postprandial glucose metabolism, via inducing glucose uptake from the circulation by muscle and adipose tissue, and by inhibition of the glucose production by the liver (71). Obesity can lead to the development of insulin resistance, a condition where organs and tissues like muscle, liver, and adipose tissue do not respond properly to insulin anymore and higher levels of insulin than normal are required to maintain glucose levels. Insulin resistance can lead to disturbed insulin mediated glucose uptake by the muscle and adipose tissue (72). Furthermore, hepatic glucose production may not be efficiently repressed by insulin (72). When the condition proceeds, more and more insulin is needed eventually leading to pancreatic

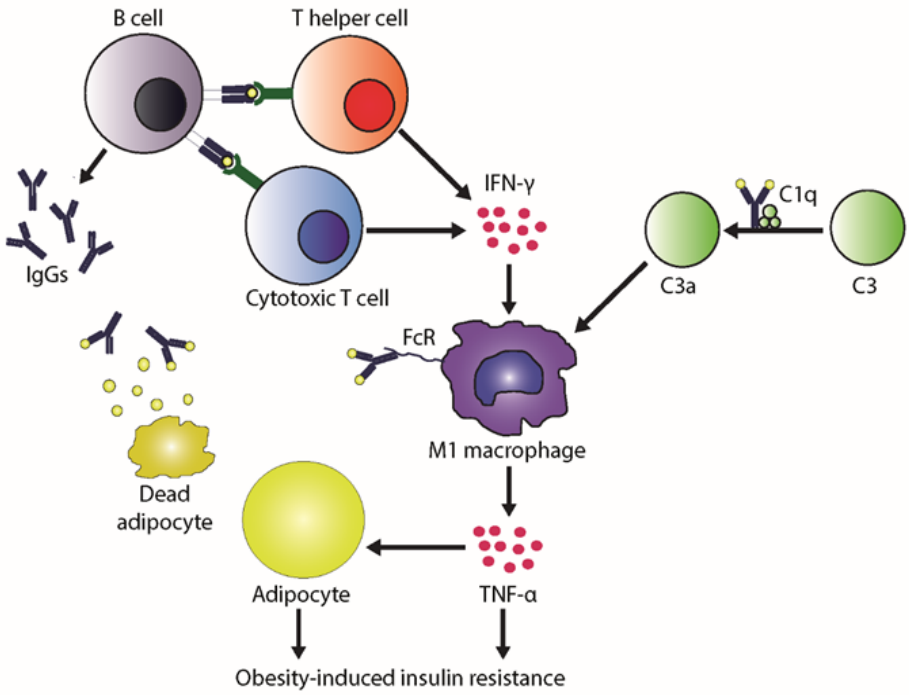

Figure 2. Schematic overview of the role of B cells and IgG antibodies in obesity-induced adipose tissue inflammation and related insulin resistance. Obesity leads to B cell activation possibly by antigens from dead adipocytes. B cells mediate MHC-dependent antigen presentation to T helper and cytotoxic T cells, which leads to an immune response. Activated B cells produce antigen specific IgG antibodies which form immune complexes. These immune complexes are able to activate immune cells via Fc-receptors and to induce complement activation via binding to C1q. This leads to immune responses including pro-inflammatory cytokine release, which contributes to the development of obesity-induced insulin resistance. Adapted from Mallat et al (70).

beta cell exhaustion and failure of the pancreatic beta cells to secrete the required levels of insulin. Pancreatic beta cell failure is a direct cause for type 2 diabetes mellitus.

As discussed in previous sections, obesity-induced adipose tissue and systemic inflammation are thought to contribute to the development of insulin resistance. Pro-inflammatory mediators like IL1β, IL6, and TNFα are secreted by immune cells in the adipose tissue during obesity (73). These pro-inflammatory cytokines are able to directly interfere with the insulin signalling pathway, thereby inducing insulin resistance. Obesity-induced inflammation is thought to activate the Jun N-terminal kinase (JNK) and IκB kinase-β (IKKβ)/nuclear factor-κB (NF- κB) pathways in muscle, liver, and fat cells (74, 75). Pro-inflammatory cytokines can activate these pathways via classical receptor-mediated mechanisms (e.g. TNF- and IL1-receptors). Other mechanism that activate these pathways are toll-like receptor (TLR) ligand binding and cellular stress factors including reactive oxygen species (ROS) and endoplasmic reticulum (ER) stress. JNK has been shown to induce insulin resistance via serine phosphorylation of insulin receptor substrate-1 (IRS-1) (76, 77). IKKβ induces transcriptional activation

of NF-κB that leads to increased expression of inflammatory markers and mediators which lead to the development of insulin resistance (78). The IKKβ inhibitor salicylate promotes insulin sensitivity and improves glucose tolerance in obese mice and diabetic patients (79, 80). Furthermore, JNK and IKKβ knock-out mouse models are protected against the development of HFD-induced insulin resistance (76, 79).

Pro-inflammatory cytokines have direct effects on adipocytes. TNFα inhibits GLUT4 mediated glucose uptake, attenuates PPARγ and lipoprotein lipase (LPL) activity and thus affects FA esterification. Furthermore, TNFα increases cAMP that leads to hormone-sensitive lipase (HSL) activation (75). Thereby, obesity-induced inflammation increases lipolysis and decreases TG synthesis in adipocytes. These actions result in increased circulating FFA levels that may induce ectopic fat storage in muscle and liver (81). Impaired insulin responsiveness of skeletal muscle is a precursor of the development of T2DM. Ectopic lipids decrease the expression of genes involved in mitochondrial functioning, such as PPARγ co-activator-1 (PGC-1). Additionally, endogenous lipids are thought to activate TLRs during obesity, which is supported by the finding that saturated FA can activate TLR2 and TLR4 and induce pro-inflammatory responses (82-84). Thus, obesity-induced inflammation likely contributes to the development of insulin resistance of muscle, liver, and adipose tissue through several pathways. This may therefore be promising targets to treat obesity-induced insulin resistance.

Outline of the thesis

Obesity induced adipose tissue inflammation is thought to play a key role in the development of insulin resistance and other metabolic disorders. If specific inflammatory pathways are causal, they present promising targets in the treatment of metabolic disorders. However, this requires extensive knowledge on the triggers for adipose tissue inflammation and subsequent inflammatory pathways and their effect on metabolic functioning. The research described in this thesis aims to gain more insight in the development of obesity-associated adipose tissue and systemic inflammation and the contribution thereof to metabolic disorders.

In **chapter 2** we studied systemic as well as adipose tissue inflammation in a human cohort of lean women, obese women with normal glucose tolerance, and obese women with type 2 diabetes mellitus. We determined to what extent differences in metabolic health are associated with differences in inflammatory phenotype and found increased systemic and WAT inflammation in obese women with T2DM compared to obese women with normal glucose tolerance. In **chapter 3** we determined adipose tissue depot specific differences in expandability and immune cell influx during the development of obesity in mice. We characterized adipocyte size and functionality of different adipose tissue depots, as well as extent of inflammation as a function of body weight. We observed significant differences in WAT depot expandability and immune cell composition. Furthermore, we found that gonadal WAT seems to primarily expand during the initial development of obesity in mice, after which the expansion tapered off and CLS formation, liver steatosis, and insulin resistance progressed. **Chapter 4** describes the composition of immune cells in the circulation and WAT of obese humans and mice. A comparison

of the WAT depots indicated major differences of the composition of immune cells between obese humans and mice. The composition of immune cells in the circulation was also significantly different between humans and mice, however the effect of obesity on circulating immune cells shows similarities.

B cells and their immunoglobulins have been shown to contribute to the development of obesity related insulin resistance (69, 70). Immunoglobulins can induce immune responses by immune cell activation via Fc-receptors, or via complement activation. **Chapter 5** describes the role of the FcRγ-chain in the development of HFD-induced obesity and related metabolic disorders. We studied FcRγ -/- mice, which lack the signal transducing γ-chain of the Fc-receptors, leading to non-functional FcγRI, III, and IV and FcεRI, and therefore have diminished IgG and IgE antibody mediated cellular responses. Mice that lack the FcRγ-chain are protected against HFD-induced obesity and related disorders. To further identify the effector pathway by which obesity-induced IgG antibodies contribute to the development of insulin resistance, we studied FcγR1234-/-, FcγR2b-/-, and complement C3-/- mice during HFD-induced obesity in **chapter 6**. We showed that FcγR or C3 deficiency does not result in decreased WAT inflammation or insulin resistance. This suggests that if obesity-induced IgG antibodies play a role in insulin resistance, this is not limited by deletion of FcγR or complement mediated pathways. **Chapter 7** provides an overall summary and discussion of the results described in this thesis.

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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, $r=0.28$, $p<0.05$).

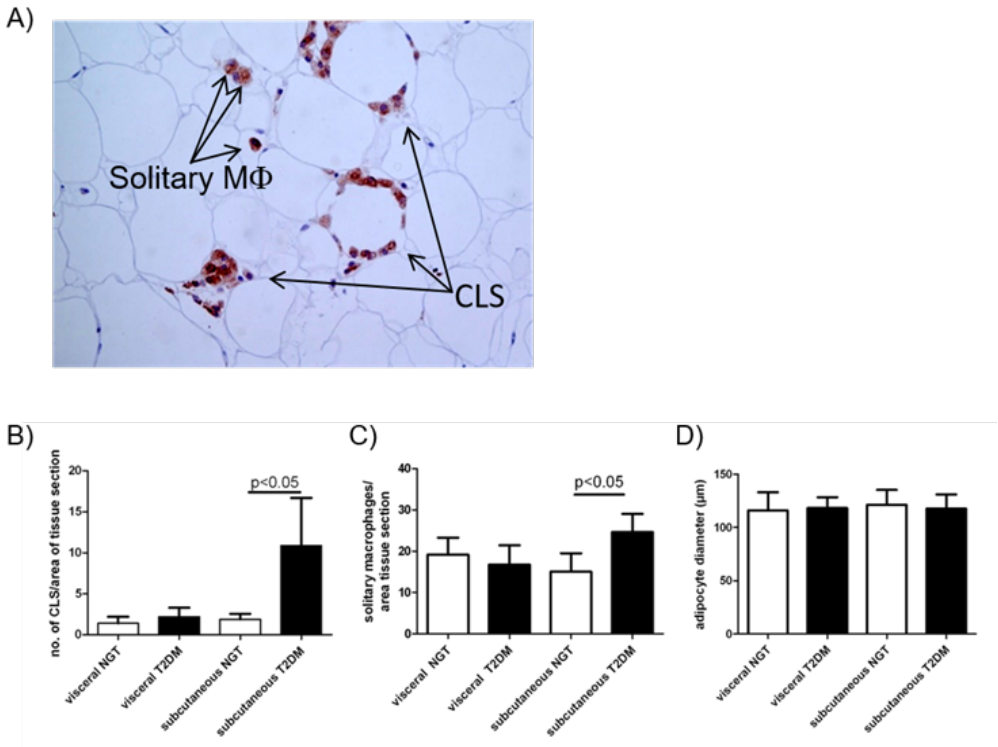

Figure 1. Quantification of macrophages and fat cell size determination in visceral and subcutaneous adipose tissue of obese women with NGT or T2DM. A) CD68 staining in adipose tissue showing solitary macrophages (MΦ**)** and crown like structures (CLS). B) Number of CLS and C) solitary or single macrophages 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.

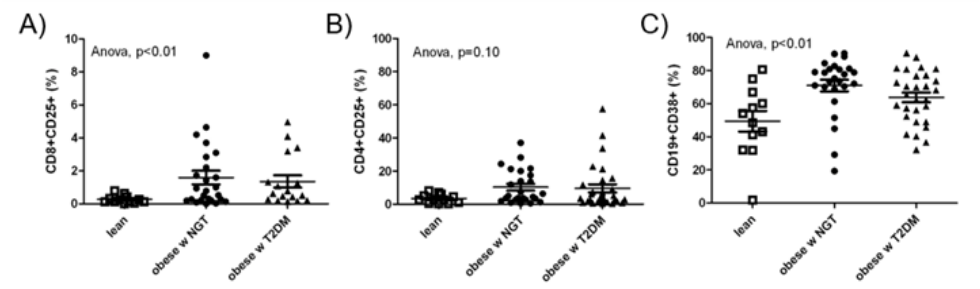

Figure 2. A-B) **Expression of CD25** in CD8 and CD4 T cells (as % of CD8 or CD4 cells) and C) **Expression of CD38** in CD19 B cells (as % of CD19 cells) in lean and obese women with NGT or T2DM.

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).

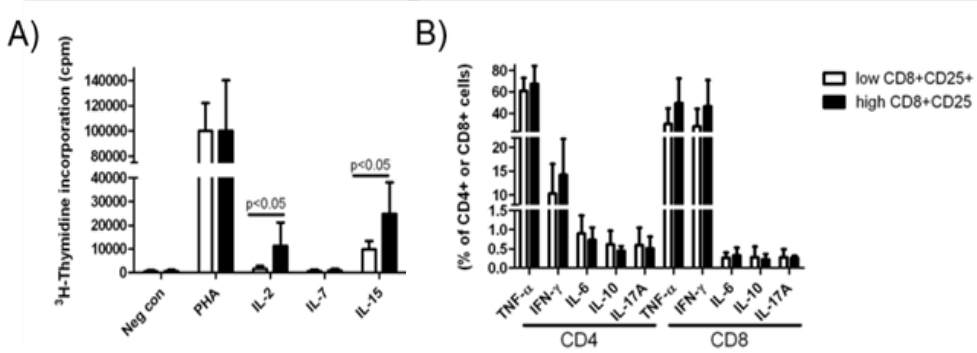

Figure 3. Proliferation capacity and intracellular cytokine staining of PBMCs from obese women with low or high peripheral CD8+CD25+. A) Proliferation of T cells after either PHA, IL-2, IL-7 or IL-15 stimulation. B) Percentage CD4+ or CD8+ cells that are positive for TNF-α, IFN γ, IL-6, IL-10 or IL-17A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

The limited storage capacity of gonadal adipose tissue directs the development of metabolic disorders in male C57Bl/6J mice

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Abstract

Aim: White adipose tissue (WAT) consists of various depots with different adipocyte functionality and immune cell composition. Knowledge about WAT depot-specific differences in expandability and immune cell influx during the development of obesity is limited, therefore we aimed to characterize different WAT depots during the development of obesity in mice.

Methods: Gonadal (gWAT), subcutaneous (sWAT) and mesenteric WAT (mWAT) were isolated from male C57Bl/6J mice with different body weights (approximately 25-60 g), analysed, and linear and non-linear regression models were used to describe the extent of WAT depot expandability and immune cell composition as a function of body weight.

Results: Whereas mouse sWAT and mWAT remained expanding with body weight, gWAT expanded mainly during the initial phase of body weight gain, after which the expansion diminished around a body weight of 40 grams. From this point on, gWAT crown-like structure formation, liver steatosis and insulin resistance occurred. Mouse WAT depots showed major differences in immune cell composition; gWAT mainly consisted of macrophages, whereas sWAT and mWAT contained primarily lymphocytes.

Conclusion: Marked inter-depot differences exist regarding WAT immune cell composition and expandability. The limited storage capacity of gWAT seems to direct the development of metabolic disorders in male C57Bl/6J mice.

Introduction

White adipose tissue (WAT) is the main energy storage organ, which is distributed over various depots. Regional distribution and inflammatory status of WAT are strongly associated with the development of metabolic disorders. Excessive abdominal fat, or central obesity, is known as a strong risk factor for type 2 diabetes mellitus and cardiovascular disease (1, 2). WAT can be divided into subcutaneous WAT (sWAT) and visceral WAT (vWAT), located underneath the skin and around the abdominal organs, respectively. Mouse vWAT is generally subdivided into mesenteric WAT (in between the organs; mWAT) and gonadal WAT (around the testes; gWAT). While WAT was originally considered as an organ with homogeneous function, vWAT is now thought to exert more adverse effects on health as compared to sWAT (2-4). These pathophysiological differences in WAT depots are linked to the metabolic and inflammatory status of the tissue.

Due to excessive fat accumulation in WAT during obesity, adipocytes become stressed and release increased amounts of fatty acids and pro-inflammatory adipokines and chemokines. These inflammatory signals induce immune cell infiltration and dysfunction of the obese WAT (5-7). Macrophage accumulation or more specifically the presence of crown-like structures (CLS) in the WAT is associated with adipocyte death caused by cellular lipid overload (8, 9). Furthermore, T and B lymphocytes are increased in WAT during obesity and do contribute to the development of metabolic disorders (10, 11). Pro-inflammatory cytokines produced by both adipocytes and infiltrated immune cells directly interfere with the insulin signalling pathway, thereby affecting insulin sensitivity both locally and systemically, leading to insulin resistance (IR) and type 2 diabetes (12, 13). vWAT as compared to sWAT secretes more fatty acids and pro-inflammatory cytokines and has a higher infiltration of cytotoxic T cells and macrophages during obesity (14-16).

Most of the human studies on WAT inflammation compare WAT between lean and obese individuals and considered only one WAT depot. The majority of mouse studies use male C57Bl/6J mice as a model to induce obesity by a high fat diet and assess only the gWAT whereas the sWAT and mWAT are being neglected (17). As different WAT depots have different function and cellular composition, it is of importance to determine the functional and immunological phenotypes of the various WAT depots. Moreover, longitudinal studies following the development of obesity are sparse and as a consequence the inflammatory response of the different WAT depots during body weight gain has until now being poorly characterized. Therefore, the aim of the current study was to phenotype the different WAT depots and to determine regional differences with regard to WAT expandability and inflammation in male C57Bl/6J mice during the development of high fat diet (HFD) induced obesity. In addition, we set out to develop a set of linear and non-linear regression models to describe organ weights and WAT (immune cell) composition as a function of body weight.

Materials and methods

Animals

Experiments were performed with six different batches male C57Bl/6J mice (Charles River, Maastricht, The Netherlands). The batches differed in duration (4-34 weeks) and type of HFD (45% or 60% energy derived from lard fat; D12451 or D12492, Research Diet Services, Wijk bij Duurstede, The Netherlands) (ESM Table 1). Body weight was measured and lean and fat mass was assessed by MRI based body composition analysis (Echo MRI, Echo Medical Systems, USA). At the end of the diet intervention, mice were sacrificed, perfused, and organs were dissected for further analysis. All experiments were approved by the animal ethics committee of Leiden University Medical Center.

Adipocyte and stromal vascular cell isolation

gWAT (one side), sWAT (posterior, one side) and mWAT depots of the mice were dissected and kept in PBS after diet intervention. Tissues were processed for adipocyte size determination as previously described (18). Adipocyte number per fat pad was calculated from the fat pad mass and adipocyte size. The residue of the WAT filtrate was used for the isolation of stromal vascular fraction (SVF) to analyse immune cell composition using flow cytometry. After centrifugation (350 x g, 10 minutes) the supernatant was discarded and the pellet was treated with erythrocyte lysis buffer after which the cells were counted using an automated cell counter (TC10, Biorad, CA, USA). The SVF was fixed using 0.5% paraformaldehyde, stored in FACS buffer (PBS, 0.02% sodium azide, 0.5% FCS) in the dark at 4°C and analysed within one week.

Additional analyses

Plasma, liver TG, adipocytes lipolysis, histology, and flow cytometry analysis are performed as described in the ESM methods.

Statistics

Data are presented as single data points, or mean ± SD. Statistical differences between groups were calculated with the student's t-test using GraphPad Prism version 6 (GraphPad software, CA, USA). Correlation analyses were performed by making correlation plots of body weight versus the various parameters measured in this study. We modelled the association between each variable and body weight using regression assuming either a linear ($y=b*x+c$; $a=1$) or non-linear power function. The non-linear power functions could either have y-intercept ($y=b*x^a+c$; $a>1$, exponential form), or x-intercept ($y=b*(x-c)^a$; $a<1$, curve tapering off); x=body weight, y=lean, fat or individual organ mass. For each analysis the p-value zero slope (p) indicated if the slope was significantly different from zero (b=0, horizontal line). In addition, superiority of the non-linear (power) function over the linear model was determined by testing the hypothesis $a=1$ using the extra sum-of-squares F test; the corresponding p-value was termed p-value linear indicated by p*. A Spearman rank correlation coefficient (r) was determined for every association. $P<0.05$ was considered statistically significant, unless stated otherwise after Bonferroni multiple test correction, significant values in the tables are shown in bold.

Results

HFD-induced changes in body composition and organ weight.

C57Bl/6J mice were subjected to different diet interventions, for a variable number of weeks and with a varying fat percentage in the diet to be able to study mice with a broad range of body weight, ranging from lean to severely obese (26.3-59.3 g, n=54). The fat percentage of lean mice (<30 g) consisted of about 80% lean mass and 20% fat mass. Mice exposed to HFD increased both lean and fat mass, although fat mass increased relatively more (Figure 1A,B; ESM Table 2). Obese mice consisted of up to 50% fat mass. Figure 1C shows that the liver weight increased non-linearly with a power >1 when correlated to body weight, with a substantial increased liver weight from approximately 40 grams on. This was mainly caused by an increase of fat in the liver (ESM Figure 1A). Also heart weight had a non-linear correlation with body weight (Figure 1D). Spleen weight correlated linearly with body weight (Figure 1E), even as brown adipose tissue (BAT) which showed a very strong linear positive correlation with body weight (Figure 1F). BAT lipid droplet content correlated positively both with body weight (ESM Figure 1B) and BAT weight ($r=0.64$, $p=0.0001$, data not shown). ESM Table 2 shows the equations of the curves of the correlations between the individual organs and body weight after best fit comparison statistics. Plasma glucose and insulin as well as plasma lipid levels were measured and correlated with body weight. Glucose increased at the start of body weight gain, after which it tapered off (Figure 2A). As glucose levels are regulated by insulin, the flattening of the glucose curve can be linked to increasing insulin levels (Figure 2B). Plasma total cholesterol correlated positively with body weight ($r=0.72$, $p<0.0001$), whereas other plasma lipids (triacylglycerol (TG) and non-esterified fatty acid) were not correlating with body weight (data not shown).

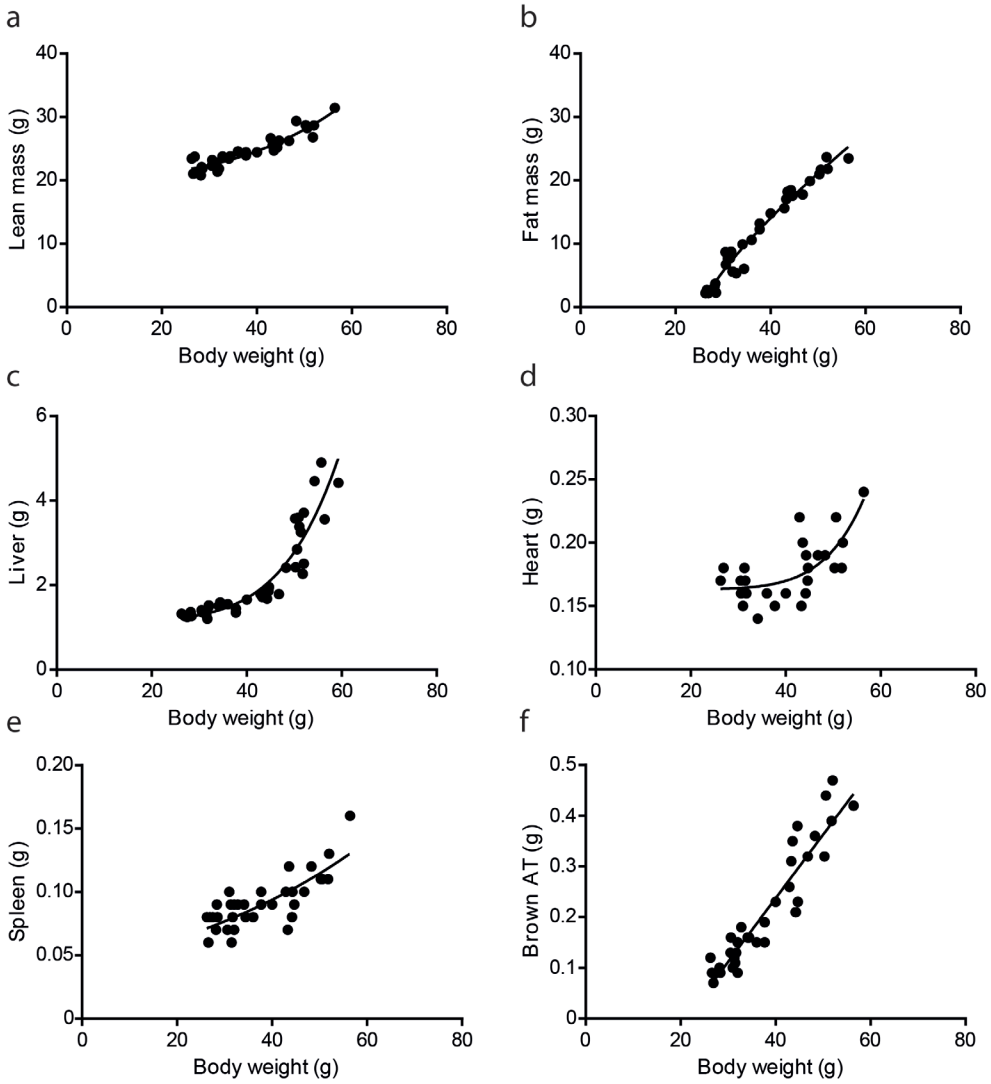

Figure 1. HFD-induced changes in body composition and organ weight in correlation to body weight. Correlations are shown between lean mass (A), fat mass (B) and different organ weights; liver (C), heart (D), spleen (E), and interscapular BAT (F) with body weight of male C57Bl/6J mice (ranging from approximately 25-60 grams). Correlations were determined by fitting a linear model or non-linear power function, 95% confidence interval is shown as grey bands, see ESM Table 2 for equations, correlations and p-values.

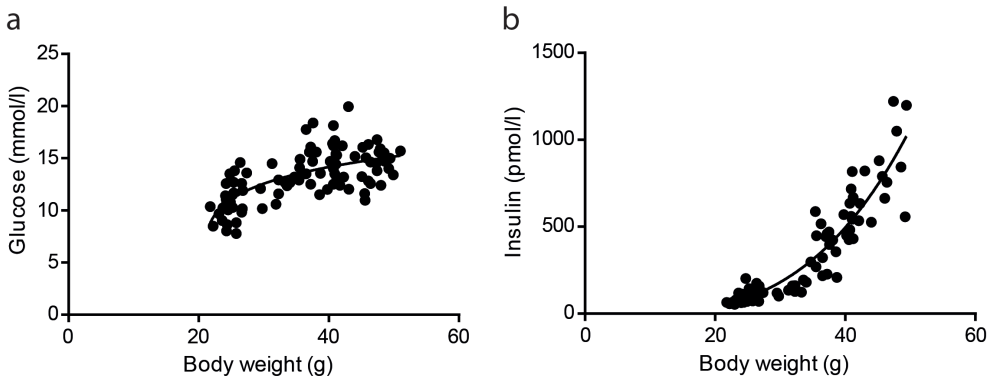

Figure 2. HFD-induced changes in glucose and insulin plasma levels in correlation to body weight. The correlation between glucose (A; r=0.67, p<0.0001, p*=0.0044) and insulin (B; r=0.93, p<0.0001, p*<0.0001) plasma levels with body weight of male C57Bl/6J mice. Correlations were determined by fitting a linear model or non-linear power function, 95% confidence interval is shown as grey bands. A significant value of p provides evidence of a non-zero slope in the linear model; a significant value of p* provides evidence that the association in non-linear

Expandability of mouse WAT depots.

Fat pad weight of the various WAT depots reflects expandability during HFD exposure. The gWAT depot expanded mostly during the initial phase of weight gain compared to both sWAT and mWAT. With progressing weight gain, the gWAT growth curve tapered off, whereas sWAT and mWAT remained growing with body weight (Figure 3A,C,E; ESM Table 2). This is also illustrated by ESM Figure 2, where mice were divided into groups based on body weight to determine the adipocyte size distribution. Whereas for both sWAT and mWAT the adipocyte size distribution curve shifted towards larger adipocytes with higher body weight, gWAT adipocytes remained comparable in size from approximately 40 grams onwards. Interestingly, the gonadal adipocytes were larger compared to adipocytes of sWAT and mWAT, for both lean and obese mice (Figure 3B,D,F; ESM Table 3A,B). The potency of insulin to inhibit lipolysis in gonadal adipocytes was tested ex vivo and revealed a negative correlation with body weight (Figure 4A), as well as with adipocyte size (r=-0.42, p=0.0043, data not shown). WAT growth is accomplished by hypertrophy (increase in size) or hyperplasia (increase in number) of the adipocytes. For all three WAT depots, there was a significant correlation between body weight and adipocyte size (Figure 3B,D,F; Table 1) whereas adipocyte number did not correlate with body weight (Table 1). However, when correlated with WAT depot weight, adipocyte number did show a slight positive correlation for gWAT and mWAT (data not shown). These data indicate that WAT expansion occurred predominantly by adipocyte hypertrophy and somewhat by hyperplasia in gWAT and mWAT, whereas sWAT only expanded by adipocyte hypertrophy.

Table 1. WAT depot composition correlated to body weight of mice on a HFD

	Correlation to body weight - Statistics					
	a	b	c	r	P-value zero slope	P-value linear
Gonadal WAT						
Adipocyte size (µm; n=54)	0.1482	78.63	25.46	0.8112	1.40E-19***	2.92E-07***
Adipocyte no/fat pad (n=46)				0.1822	3.773	-
SVF nr/fat pad (n=54)	1	40,829	-672,214	0.6768	1.20E-05***	5.282
Leukocytes (% CD45 of SVF; n=44)	1	0.5060	42.98	0.4131	0.1197	-
T lymphocytes (% CD3 of SVF; n=44)	1	0.2878	0.1631	0.3518	0.0315*	-
T lymphocyte ratio (CD4:CD8; n=51)	1	-0.1689	10.71	-0.6287	7.28E-05***	0.8226
B lymphocytes (% CD19 of SVF; n=35)	1	0.1398	-1.206	0.3631	0.0963	-
Macrophages (% F4/80 of SVF; n=50)				-0.1962	3.834	-
Macrophage ratio (M1:M2; n=45)	1	0.01534	- 0.3574	0.7710	7.92E-08***	8.699
Subcutaneous WAT						
Adipocyte size (µm; n=54)	0.2473	49.44	24.39	0.8886	3.26E-20***	4.33E-04***
Adipocyte no/fat pad (n=46)				-0.03627	7.093	-
SVF nr/fat pad (n=54)				0.2907	1.252	-
Leukocytes (% CD45 of SVF; n=50)	1	0.6146	32.41	0.4680	0.0036**	4.694
T lymphocytes (% CD3 of SVF; n=40)				0.1068	8.776	-
T lymphocyte ratio (CD4:CD8; n=50)	1	-0.03013	2.784	-0.4064	0.1818	-
B lymphocytes (% CD19 of SVF; n=43)				-0.07501	4.874	-
Macrophages (% F4/80 of SVF; n=29)				-0.1822	5.057	-
Macrophage ratio (M1:M2; n=34)	1	0.01025	- 0.2255	0.6262	1.95E-06***	0.5508
Mesenteric WAT						
Adipocyte size (µm; n=54)	0.3524	34.09	22.17	0.9528	3.17E-29***	3.23E-04***
Adipocyte no/fat pad (n=46)				-0.06658	5.114	-
SVF nr/fat pad (n=54)				0.1914	2.070	-
Leukocytes (% CD45 of SVF; n=49)	1	-0.6190	97.37	-0.3613	0.0729	-
T lymphocytes (% CD3 of SVF; n=48)	1	-0.7400	54.93	-0.6355	7.07E-06***	0.5445
T lymphocyte ratio (CD4:CD8; n=48)				0.06193	3.512	-
B lymphocytes (% CD19 of SVF; n=30)	1	-0.9100	72.53	-0.3696	0.0261	-
Macrophages (% F4/80 of SVF; n=23)	1	0.9423	-22.89	0.5198	0.0153*	0.1161
Macrophage ratio (M1:M2; n=29)	1	0.01035	- 0.2701	0.6768	6.93E-04***	0.2763

P<0.0019 is considered statistically significant after Bonferroni multiple test correction.

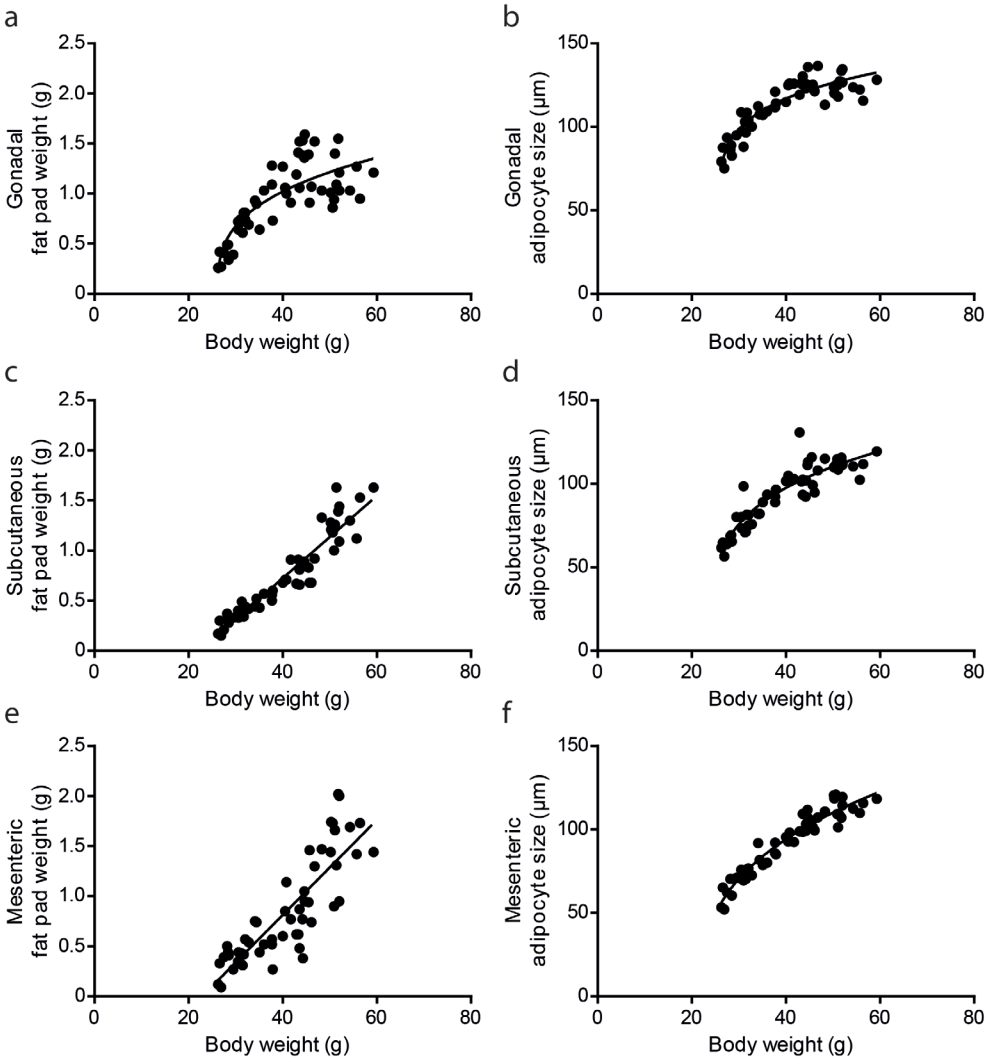

Figure 3. HFD-induced changes in fat pad weight and adipocyte size in correlation to body weight. Fat pad weight (A, C, E) and adipocyte size (B, D, F) of gWAT, sWAT and mWAT in correlation to body weight of male C57Bl/6J mice. For gWAT and sWAT one fad pad is used for representation. Correlations were determined by fitting a linear model or non-linear power function, 95% confidence interval is shown as grey bands, see Table 1 and ESM Table 2 for equations, correlations and p-values.

HFD-induced changes in immune cell composition in mouse WAT depots.

WAT depots were processed to isolate the SVF, which contains immune cells as well as pre-adipocytes and endothelial cells. The absolute SVF cell number was determined and represented per fat pad. The SVF cell count of gWAT correlated positively with body weight, whereas the SVF cell counts of sWAT and mWAT did not correlate with body weight (Table 1). Absolute leukocyte numbers (CD45+ cells) per fat pad showed a linear correlation with body weight for both gWAT and sWAT, whereas leukocyte

numbers in mWAT showed no correlation with body weight (ESM Figure 3A, 4A, 5A). Absolute T cell numbers of gWAT and sWAT, but not mWAT correlated positively with body weight (ESM Figure 3B, 4B, 5B). Within the T cell population, the ratio between T helper cells and cytotoxic T cells (CD4+ and CD8+ cells, respectively) was determined. In both gWAT and sWAT the CD4:CD8 ratio showed a negative correlation with bodyweight, which indicates a relative larger increase in cytotoxic T cells compared to T helper cells (Table 1). Absolute B cell numbers (CD19+ cells) showed a positive correlation with body weight for gWAT (ESM Figure 4D).

Absolute macrophage numbers (F4/80+ cells) of all three WAT depots correlated positively with body weight (ESM Figure 3C, 4C, 5C). Interestingly, absolute macrophage numbers in gWAT and sWAT showed a non-linear correlation with body weight with a power >1, while the correlation in mWAT was linear. WAT macrophages can form CLS, which is shown in Figure 4B by F4/80 staining of gWAT. CLS increased non-linearly with increasing body weight with a power >1 in the gWAT depot (Figure 4C). Within the F4/80+ cell population, M1 and M2 macrophages were distinguished using CD11B and CD11C markers (M1: CD11B+CD11C+; M2: CD11B+CD11C-). Figure 4D shows the correlation of M1 and M2 macrophages as percentage of F4/80 cells from the gWAT depot (representative for the other two depots, data not shown) to body weight. Within all AT depots, M1 macrophages were positively correlated and M2 macrophages were negatively correlated with body weight. The M1:M2 ratio also showed a strong positive correlation with body weight within all WAT depots (Table 1). This indicates relatively more M1 macrophages in WAT with a higher body weight. Thus, HFD induces immune cell compositional changes in all three WAT depots, with an increase in immune cell numbers in mainly gWAT and sWAT.

Immune cell composition of distinct WAT depots from lean and obese mice.

gWAT, sWAT and mWAT depots from either lean or obese mice (mean body weight 31.0±2.9 g, n=10 and 50.1±3.6 g, n=8, respectively) were analysed and compared with each other to determine differences in immune cell composition between the adipose tissue regions. In lean mice, approximately 60% of the SVF from gWAT and sWAT consisted of leukocytes (57.5±9.9% and 62.7±6.2%, respectively), and in mWAT this percentage was even higher (78.3±12.1%) (ESM Table 3A). In obese mice, there were no differences in leukocyte percentages in the SVF between the different WAT depots (approximately 65%, ESM Table 3B). T cells were present in all three WAT depots. Interestingly, in mWAT from lean mice the percentage of T cells in the SVF was significantly higher as compared to gWAT and sWAT (ESM Table 3A). The CD4:CD8 ratio in lean sWAT and mWAT was between 1 and 2, which indicates slightly more CD4 cells over CD8 cells. However, lean gWAT contained even more CD4 than CD8 cells as the ratio was around 5 (ESM Table 3A). In obese mice, there were no differences in T cell percentages between the WAT depots (ESM Table 3B). There were major differences in B cell content between the depots ranging from hardly any B cells in gWAT to approximately 35% of the SVF in mWAT of lean mice and approximately 20% of the SVF in mWAT of obese mice (ESM Table 3A,B). gWAT predominantly contained macrophages (approximately 30% of SVF both in lean and obese mice), while less than 10%

of the SVF from sWAT consisted of macrophages in both lean and obese mice. These data indicate that there are large immune cell composition differences between different WAT depots from both lean and obese mice.

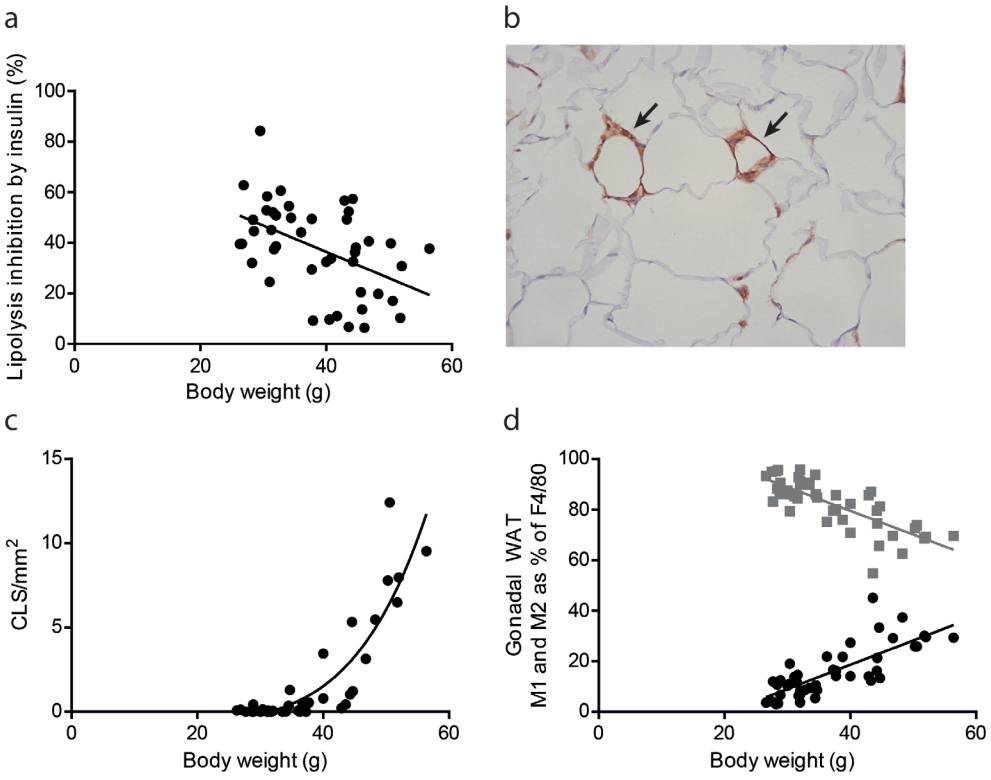

Figure 4. Insulin responsiveness of adipocytes and macrophage phenotype in gWAT in correlation to body weight. Percentage inhibition of lipolysis by insulin of gonadal adipocytes correlated with body weight (A; r=-0.47, p=0.0010). Insulin responsiveness of the adipocytes was determined by measuring the response of the adipocytes to 8-bromo-cAMP stimulated lipolysis and the percentage inhibition thereof by insulin. F4/80 stained macrophages in gWAT (B), CLS are indicated by an arrow. CLS counts per mm^2 WAT correlated with body weight of male C57Bl/6J mice (C; r=0.71, p<0.0001, p*<0.0001). Macrophage type 1 (CD11B+CD11C+; black dots; r=0.77, p<0.0001) and 2 (CD11B+CD11C-; grey dots; r=-0.76, p<0.0001) as percentage of F4/80+ cells in SVF of gWAT by flow cytometry (D) correlated to body weight of male C57Bl/6J mice. Correlations were determined by fitting a linear model or non-linear power function, 95% confidence interval is shown as grey bands. A significant value of p provides evidence of a non-zero slope in the linear model; a significant value of p* provides evidence that the association in non-linear.

Discussion

In the current study we determined intra-depot differences in WAT immune cell composition in relation to WAT expandability. Mouse WAT depots showed major differences in expandability and immune cell infiltration during the development of obesity. Furthermore, a body weight of approximately 40 grams emerged as a critical tipping point from where on metabolic dysfunction occurs, at least in male C57Bl/6J mice. In this paper, we also provide a set of parameterized linear and non-linear functions which can be used by researchers in the field of obesity to assess the extent of WAT depot expansion and inflammation in HFD fed male C57Bl/6J mice as a function of body weight.

It has extensively been shown that distinct WAT depots from both mice and humans feature different metabolic functions. This is due to intrinsic differences in adipocyte characteristics but has also been attributed to differences in immune cell composition in the various depots (19-21). Here, we have performed a direct comparison of the different WAT depots in male C57Bl/6J mice and focussed on the expandability and immune cell composition simultaneously during the development of obesity, which has until now being poorly characterized. Our data confirm great variability in immune cell composition between WAT depots. The characteristics of the different mouse WAT depots already differed in the lean state, and each depot responded differently to body weight gain with respect to immune cell composition as well as expandability. Mouse gWAT expanded mostly during the initial phase of body weight gain, and increased less after a body weight of around 40 grams. Although sWAT and mWAT did not primary expand as fast as gWAT, they both remained expanding with a body weight after 40 grams. This implies that gWAT is the primary storage depot that grows initially in HFD-induced obesity, followed by sWAT and mWAT. This is also reflected by the larger gWAT adipocytes during both the lean and obese state, which has similarly been identified by Sachmann-Sala et al. albeit only in lean mice (22).

Around a body weight of 40 grams where the gWAT growth curve tapered off, the liver started to grow significantly, which is mainly caused by an increase in fat content. Apparently, the gWAT adipocytes were saturated and could not grow any larger to store additional fat. As a consequence, the excess fat which could not be stored in the gWAT depot led to ectopic fat storage in the liver (23). Moreover, around this point of body weight gain, the number of CLS started to increase in the gWAT. As CLS are found around stressed and dying adipocytes (9), the rise in numbers of CLS around 40 grams of body weight appear associated with increased adipocyte death. Also, insulin levels increased substantially from this point on, indicating the development of insulin resistance. Our data therefore imply that approximately 40 grams body weight of male C57Bl/6J mice is an important tipping point from where on WAT and systemic metabolic dysfunction occur concomitantly. In this study, data has been exclusively obtained from male C57Bl/6J mice in combination with HFD to induce obesity. Whether females, other mouse strains / models or humans also have such a threshold BMI upon which WAT inflammation and metabolic dysfunction rapidly increase, remains to be investigated.

Our data are in agreement with the study of Strissel et al., that also showed that at a certain body weight in mice gWAT stops expanding due to increased adipocyte death, whereas liver starts

accumulating fat (24). Strikingly, while our data showed a constant gWAT weight and continuous increase of CLS, they showed a reduction of both gWAT weight and CLS formation after a body weight of 40 grams. This was accompanied by a reduction in adipocyte size and increased adipocyte numbers, which they attributed to newly differentiated adipocytes. One explanation for their findings might be the fact that they fed their mice a 60% HFD for 20 weeks, which may be a more extreme intervention than the interventions used in our study. There are several other studies elucidating regional differences regarding WAT growth (25-27), although these studies do not extensively link WAT expandability to immune cell infiltration and metabolic characterization. This study is the first that focusses on all these processes simultaneously.

Adipocyte size of all three WAT depots increased during body weight gain. Adipocyte number per fat pad remained comparable in all depots when correlated to body weight, whereas in gWAT and mWAT adipocyte numbers increased slightly during the HFD intervention when correlated with fat pad weight. Our observations are in line with previous mouse studies that showed expansion by hypertrophy only in sWAT, whereas mWAT expanded by both hypertrophy and hyperplasia (25, 27). Adipocytes in gWAT were larger as compared to sWAT and mWAT in mice. Large adipocytes are thought to release more pro-inflammatory cytokines and chemokines which attract circulating monocytes into the WAT that in turn differentiate into macrophages (5, 28). Indeed, the SVF of the gWAT contained a higher fraction of macrophages compared to sWAT and mWAT. Also, the absolute number of macrophages per WAT depot was higher in the gWAT than in the other WAT depots.

Absolute leukocyte numbers in mouse mWAT were much higher compared to sWAT and gWAT. mWAT surrounds the intestine, which represents the first line of defence against intestinal pathogens and could therefore explain the large number of leukocytes. However, mWAT is also known to contain a large amount of lymphoid tissue including lymph nodes and milky spots (29). Although we took great care in removing all visible lymph nodes from the mWAT before the immune cell characterization, we cannot exclude the possibility that we missed some lymph nodes. As lymph nodes contain numerous leukocytes, this could also explain the high number of leukocytes present in mouse mWAT. Another issue affecting analyses of WAT inflammation, might be contamination of the SVF with immune cells from the circulation. However, our mice were perfused before removal of the WAT depots.

Numerous pathophysiological processes are known to be associated with the development of IR. In this study, we focussed on WAT expandability and inflammation as a measure for WAT dysfunction. However, inadequate angiogenesis and related hypoxia are known as early determinants for WAT dysfunction as well (30, 31), and can induce WAT fibrosis which has also been associated with IR (32). Although beyond the scope of the current study, it is interesting to determine the association between these pathologies, WAT expansion and inflammation. Macrophages are highly abundant in WAT, with specifically M1 macrophages accumulating during obesity and contributing to IR. Our data showed a phenotypic switch from M2 to M1 type macrophages during obesity, which has previously also been shown by Lumeng et al. (17). CLS numbers increased as well with body weight, which are primarily formed by M1 macrophages (33). M1 macrophages are known to accumulate lipids and form foam

cells. Lipid accumulation in macrophages has previously directly been related to the expansion of WAT (34). Whether lipid loaded macrophages are a consequence of the limited expansion of WAT remains to be investigated.

BAT is known to be a prominent player in body weight control, as it burns TG to produce heat (35, 36). Here we show that BAT weight is strongly correlated to body weight. In general, high BAT weight is associated with inactive BAT, as TG is being stored instead of being used for heat production (37). This is supported by an increased lipid droplet content in BAT with higher body weight. Our observed correlation between BAT weight and body weight can be explained by the thermal insulation function of WAT. The increased WAT in obesity is enough to keep the animal warm and heat production by BAT activity is reduced. Thus, body weight is an important confounder when studying BAT activity.

We conclude that mouse WAT depots vary considerably in expandability and immune cell composition during HFD induced body weight gain. With a body weight threshold of approximately 40 grams in mice, gWAT seems to have reached its maximum expansion capacity and at this point WAT dysfunction and concomitant systemic metabolic dysfunction will commence.

Acknowledgements

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ESM methods

Plasma parameters

At different time points during the HFD intervention blood was drawn from 6 hour fasted mice via the tail vein into paraoxon (Sigma, St. Louis, MO) coated capillary tubes to prevent ongoing *in vitro* lipolysis. After centrifugation, plasma was collected and glucose, insulin, triacylglycerol (TG), total cholesterol, and non-esterified fatty acid were determined using commercially available kits (Instruchemie, Delfzijl, The Netherlands; Crystal Chem Inc., IL, USA; 11488872 and 236691, Roche Molecular Biochemicals, Indianapolis; NEFA-C, Wako chemicals GmbH, Neuss, Germany, respectively).

Liver TG analysis

Lipids were extracted from livers according to a modified protocol from Bligh and Dyer (1). Briefly, small liver pieces were homogenized in ice-cold methanol. Lipids were extracted by addition of 1800 µl ice-cold chloroform/methanol (3:1) to 45 µl homogenate. The chloroform/methanol phase was dried and dissolved in 2% (vol./vol.) Triton X-100. Hepatic TG concentrations were measured using TG kit (11488872, Roche Molecular Biochemicals, Indianapolis). Liver TG were expressed per mg protein, which was determined using the BCA protein assay kit (Pierce Biotechnologie, Rockford, USA).

Adipocyte lipolysis assay

The potency of insulin to inhibit lipolysis in gonadal adipocytes was determined by incubating isolated adipocytes (±10,000 cells/ml) for 2 h at 37°C, with DMEM/F12 with 2% (vol./vol.) BSA and 8-bromo-cAMP (10^{-3} mol/l; Sigma, St. Louis, MO) in combination with or without insulin (10^{-9} mol/l). Glycerol concentrations were determined as a measure for lipolysis, using a free glycerol kit (Sigma, St. Louis, MO) and the hydrogen peroxide sensitive fluorescence dye Amplex Ultra Red, as previously described by Clark et al. (2).

Histology

Formalin fixed and paraffin embedded sections of gWAT and intrascapular BAT were used for histological analysis. An F4/80+ antibody (1:250) (Leiden University Medical Center, Leiden, The Netherlands) was used to stain macrophages in gWAT. Vectastain ABC (Vector laboratories, CA, US) was used for visualization of the antibody complex according to manufacturer's instructions. Haematoxylin staining of the gWAT and BAT sections was done using a standard protocol. The area of intracellular lipid vacuoles in BAT was quantified using Image J (NIH, US).

Flow cytometry analysis

Mouse SVF cells were stained with fluorescently labelled antibodies for CD45.2-FITC (BioLegend), CD3-APC, CD4-Qdot605, CD8a-PercpCy5.5, CD19-PE, F4/80-PE, CD11B-PB, CD11C-APCCy7 (all purchased from eBioscience, CA, USA or BioLegend, CA, USA). Cells were measured on a LSR II flow cytometer (BD Biosciences). Data were analysed using FlowJo software (Treestar, OR, USA).

Reference list

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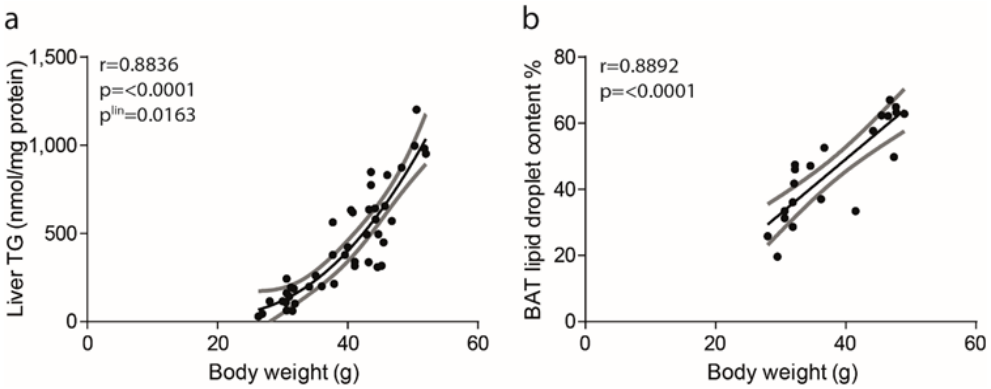
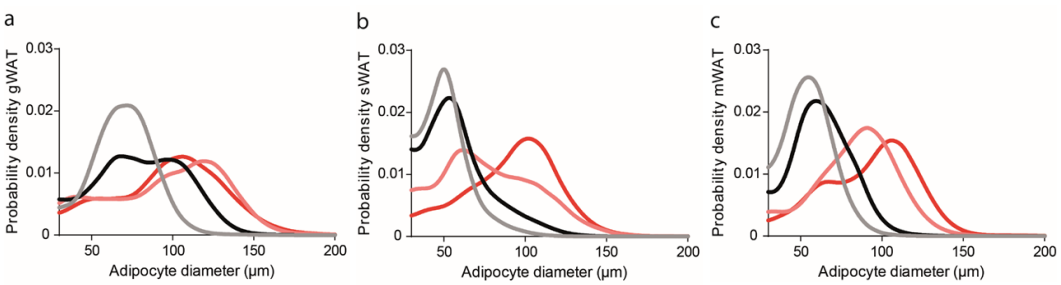
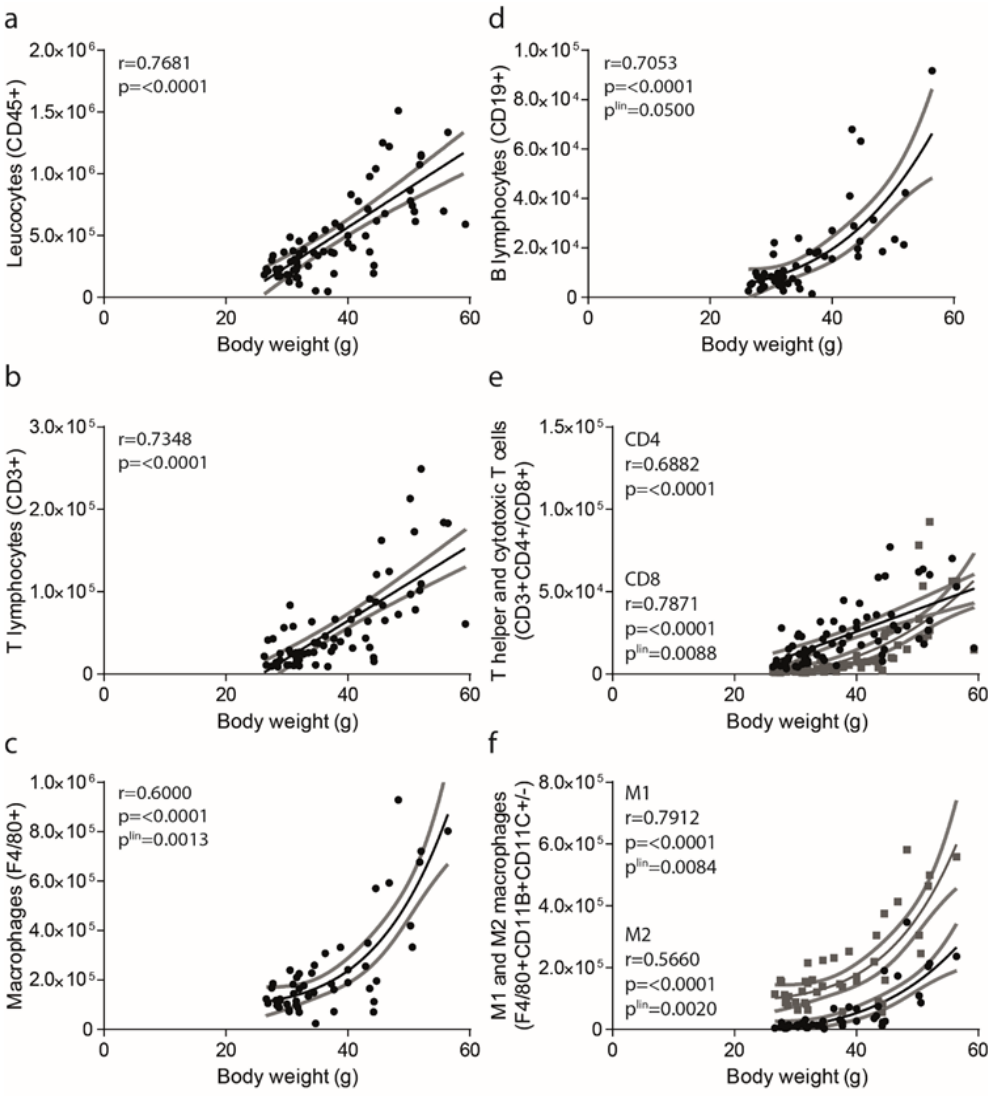
ESM figures and tables



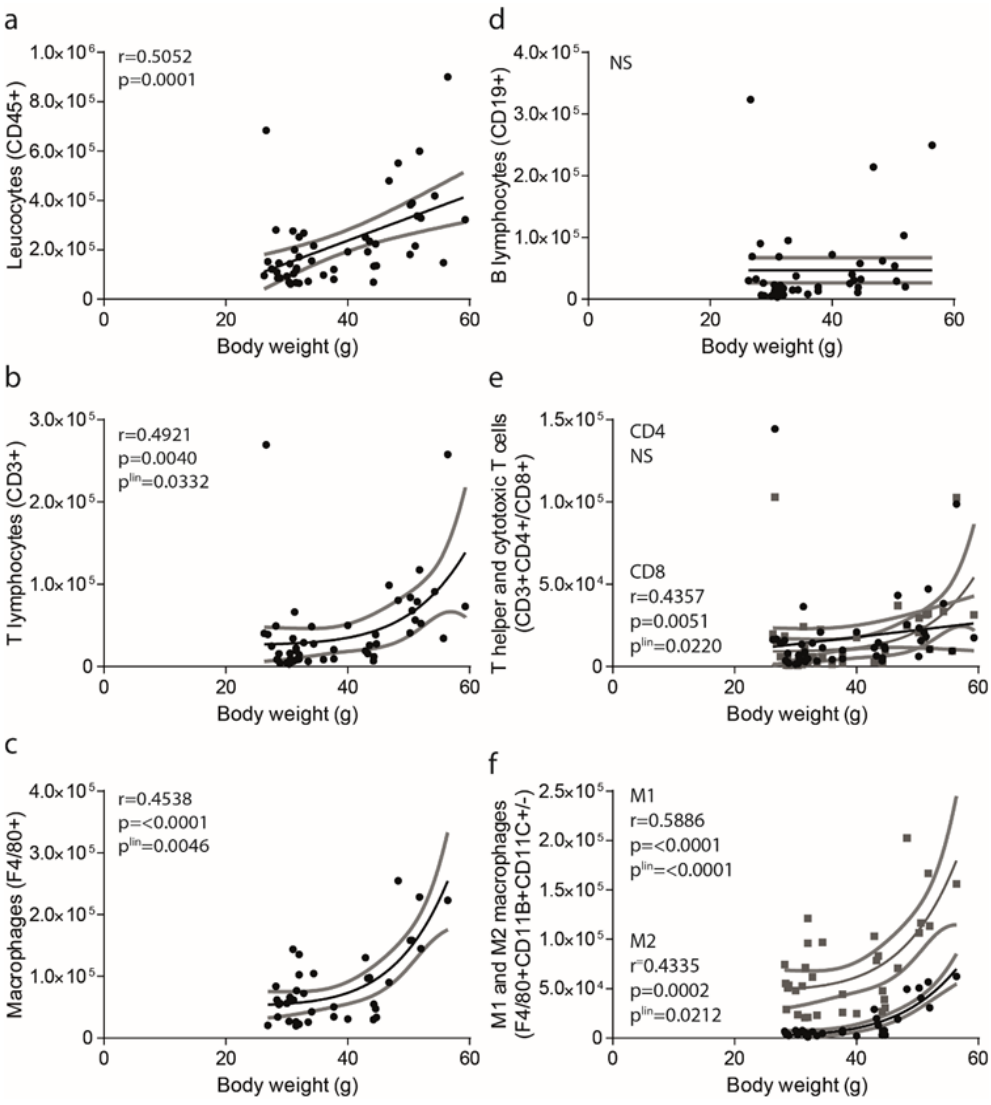
ESM Figure 1. Liver TG and BAT lipid droplet content. Liver TG content (A) and BAT lipid droplet content (B) are depicted per body weight. Associations were modelled using either a linear or non-linear function, 95% confidence interval is shown as grey bands. A significant value of *p* provides evidence of a non-zero slope in the linear model; a significant value of p^{lin} provides evidence that the association is non-linear.



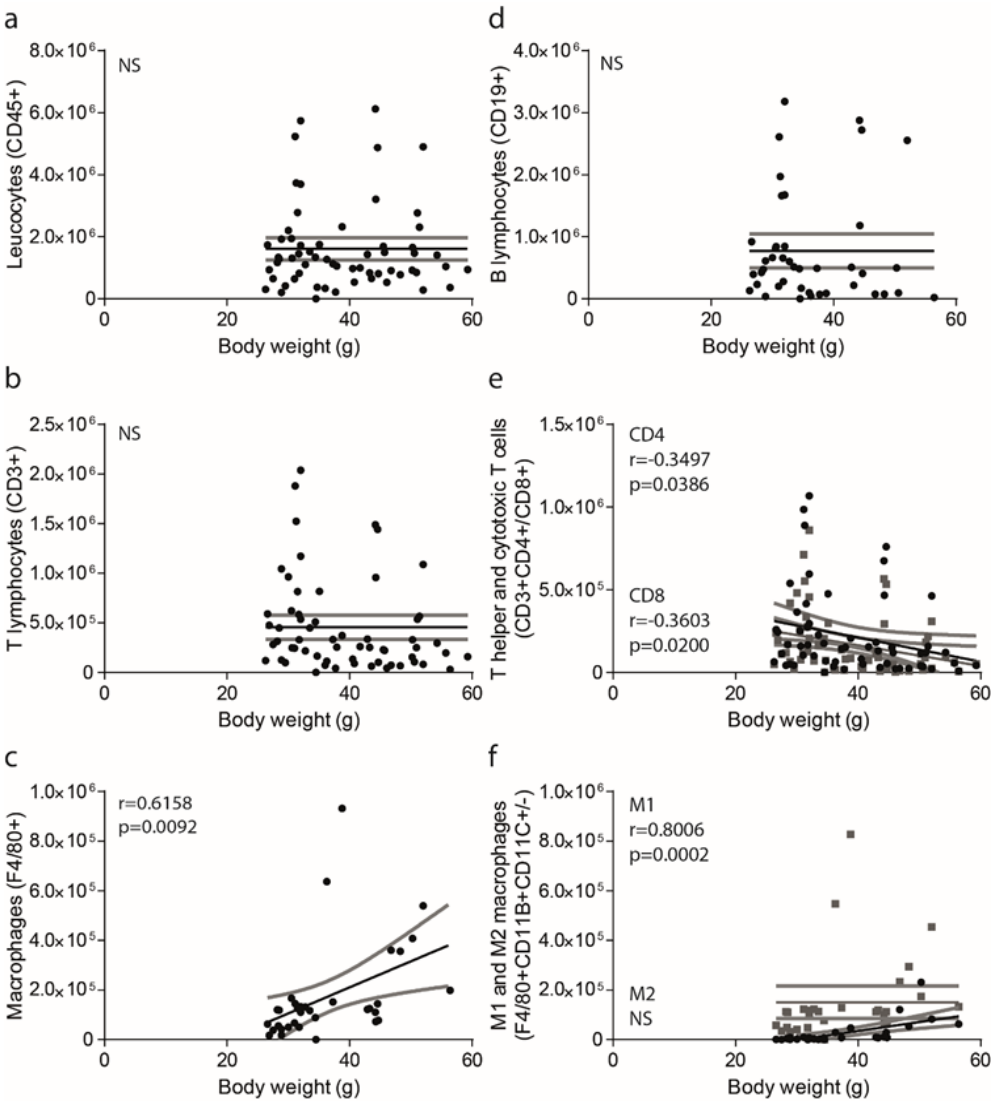
ESM Figure 2. Adipocyte size distributions with different body weights depicted per WAT depot. Mice were subdivided into different body weight groups; grey <30 grams, black 30-40 grams, pink 40-50 grams, red >50 grams. The mean adipocyte size distribution per group was depicted for the different WAT depots; gWAT (A), sWAT (B) and mWAT (C).



ESM Figure 3. Absolute immune cell numbers per fat pad of gonadal WAT correlated to body weight. Absolute numbers of leucocytes (A), T lymphocytes (B), macrophages (C), B lymphocytes (D), T helper (black dots) and cytotoxic T lymphocytes (grey squares) (E), and M1 (black dots) and M2 (grey squares) types of macrophages (F) are depicted per body weight. Associations were modelled using either a linear or non-linear function, 95% confidence interval is shown as grey bands. A significant value of *p* provides evidence of a non-zero slope in the linear model; a significant value of p^{lin} provides evidence that the association is non-linear.



ESM Figure 4. Absolute immune cell numbers per fat pad of subcutaneous WAT correlated to body weight. Absolute numbers of leucocytes (A), T lymphocytes (B), macrophages (C), B lymphocytes (D), T helper (black dots) and cytotoxic T lymphocytes (grey squares) (E), and M1 (black dots) and M2 (grey squares) types of macrophages (F) are depicted per body weight. Associations were modelled using either a linear or non-linear function, 95% confidence interval is shown as grey bands. A significant value of *p* provides evidence of a non-zero slope in the linear model; a significant value of p^{lin} provides evidence that the association is non-linear.



ESM Figure 5. Absolute immune cell numbers per fat pad of mesenteric WAT correlated to body weight. Absolute numbers of leucocytes (A), T lymphocytes (B), macrophages (C), B lymphocytes (D), T helper (black dots) and cytotoxic T lymphocytes (grey squares) (E), and M1 (black dots) and M2 (grey squares) types of macrophages (F) are depicted per body weight. Associations were modelled using either a linear or non-linear function, 95% confidence interval is shown as grey bands. A significant value of *p* provides evidence of a non-zero slope in the linear model; a significant value of p^{lin} provides evidence that the association is non-linear.

ESM Table 1. Batches of mice with different type and duration of diet intervention

Batch number	Weeks on diet	Type of diet	Number of animals (n)
1	4	45% HFD	10
2	8	45% HFD	10
3	11	45% HFD	9
4	34	45% HFD	8
5	5	60% HFD	9
6	15	60% HFD	8

ESM Table 2. Body composition and organ weight correlated to body weight of mice on a HFD

	Correlation to body weight - Statistics					
	a	**b**	**c**	***r***	***P*-value[a] zero slope**	***P*-value[a] linear**
Lean mass (g; n=36)	2.757	1.536e-004	20.60	0.9059	2.95E-15***	0.1206
Fat mass (g; n=36)	0.781	1.738	25.48	0.9517	7.75E-24***	0.1782
Liver weight (g; n=43)	5.123	3.160e-009	1.180	0.9424	3.94E-17***	3.12E-06***
Heart weight (g; n=26)	6.893	6.024e-014	0.1633	0.5734	2.70E-03**	0.1728
Spleen weight (g; n=35)	1	1.786e-003	0.0237	0.7271	5.07E-07***	1.000
BAT weight (g; 36)	1	0.01254	0.2645	0.9289	6.24E-16***	1.000
Gonadal FP weight[b] (g; n=54)	0.320	0.4411	26.21	0.7254	3.18E-11***	7.73E-04***
Subcutaneous FP weight[b] (g; n=54)	1	0.04113	-0.9205	0.9607	4.20E-26***	0.1620
Mesenteric FP weight (g; n=54)	1	0.0474	-1.084	0.8765	2.36E-20***	0.7785

Linear correlation: a=1; equation: $y=b \times x+c$

Non-linear power function with y-intercept: a>1; equation: $y=b \times x^a+c$

Non-linear power function with x-intercept: a<1; equation: $y=b \times (x-c)^a$

x=body weight; y=lean, fat or individual organ mass.

[a]*p*-value after bonferroni multiple test correction; **p*<0.05, ***p*<0.01, ****p*<0.001

[b]for gonadal and subcutaneous fat pad (FP) weight one of the two pads was used for calculations

ESM Table 3. Composition and comparison of WAT depots from lean mice

	Mouse WAT			Statistics[a]		
	gWAT (n=10)	sWAT (n=10)	mWAT (n=10)	T-test gWAT *vs* sWAT	T-test gWAT *vs* mWAT	T-test sWAT *vs* mWAT
Adipocyte size (µm)	97.8±13.2	77.0±13.1	71.3±11.8	0.0184*	0.0016**	1.000
Adipocyte no/ FP (*10^6)	2.03±0.70	2.81±0.98	2.69±0.59	0.4440	0.2912	1.000
SVF nr/FP (*10^6)	0.50±0.16	0.23±0.11	1.96±1.29	0.0024**	0.0192*	0.0040**
Leucocytes (% CD45 of SVF)	62.7±6.2	57.5±9.9	78.3±12.1	1.000	0.0184*	0.0088**
T lymphocytes (% CD3 of SVF)	7.5±2.2	14.2±8.9	27.5±10.2	0.2768	9.44E-05***	0.0744
T lymphocyte ratio (CD4:CD8)	5.1±2.3	1.54±0.66	1.29±0.36	0.0024**	8.00E-04***	1.000
B lymphocytes (% CD19 of SVF)	1.6±0.6	13.5±8.7	36.5±13.3	0.0032**	1.70E-06***	0.0040**
Macrophages (% F4/80 of SVF)	29.8±3.0	6.4±2.2	4.7±2.6	5.14E-06***	2.62E-05***	1.000

[a]*p*-value after bonferroni multiple test correction; *$p<0.05$, **$p<0.01$, ***$p<0.001$

ESM Table 4. Composition and comparison of WAT depots from obese mice

	Mouse WAT			Statistics[a]		
	gWAT (n=8)	sWAT (n=8)	mWAT (n=8)	T-test gWAT *vs* sWAT	T-test gWAT *vs* mWAT	T-test sWAT *vs* mWAT
Adipocyte size (µm)	124.4±8.0	112.0±2.5	113.9±5.5	0.0072**	0.0696	1.000
Adipocyte no/FP (*10^6)	2.10±0.36	3.02±0.47	3.41±0.84	0.0048**	0.0096**	1.000
SVF nr/FP (*10^6)	1.67±0.45	0.75±0.26	2.79±1.78	0.0016**	0.8328	0.0504
Leucocytes (% CD45 of SVF)	63.1±7.9	67.0±3.5	67.9±16.4	1.000	1.000	1.000
T lymphocytes (% CD3 of SVF)	6.6±2.0	12.5±4.2	11.3±9.4	0.0368*	1.000	1.000
T lymphocyte ratio (CD4:CD8)	1.15±0.47	1.00±0.24	1.77±0.56	1.000	0.3376	0.0280*
B lymphocytes (% CD19 of SVF)	2.1±1.2	13.0±10.2	19.3±21.2	0.1224	0.4192	1.000
Macrophages (% F4/80 of SVF)	37.5±3.9	6.5±1.8	17.9±10.5	7.69E-11***	0.0032**	0.0792

[a]*p*-value after bonferroni multiple test correction; *$p<0.05$, **$p<0.01$, ***$p<0.001$

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

Lianne van Beek, Annemieke Visser, Mirjam A Lips, Hanno Pijl, Frits Koning, Ko Willems van Dijk, Vanessa van Harmelen

In preparation

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).

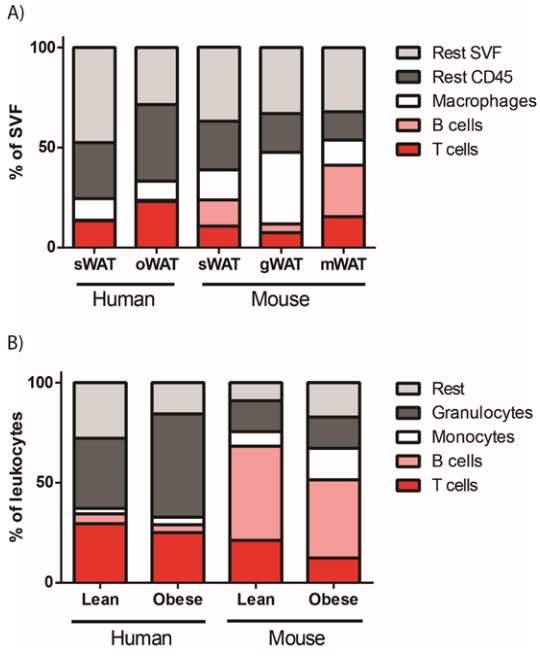


Figure 1. Schematic comparison of the composition of immune cell population of WAT depots and circulation from humans and mice. Composition of immune cell population of WAT as percentage of SVF is depicted for human (sWAT and oWAT) and mouse (sWAT, gWAT, and mWAT) depots (A). Different immune cell types 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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5

FcRγ-chain deficiency reduces the development of diet-induced obesity

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Abstract

Objective: Pathogenic immunoglobulins (Ig) are produced during the development of obesity and contribute to the development of insulin resistance (IR). However, the mechanisms by which these antibodies affect IR are largely unknown. We investigated whether Fc-receptors contribute to the development of diet-induced obesity and IR by studying FcRγ-/- mice that lack the γ-subunit necessary for signalling and cell surface expression of FcγR and FcεRI.

Methods: FcRγ-/- and WT mice were fed a high fat diet (HFD) to induce obesity. At 4 and 11 weeks, body weight and insulin sensitivity were measured, and adipose tissue (AT) inflammation was determined. Furthermore, intestinal triglyceride uptake and plasma triglyceride clearance were determined, and gut microbiota composition was analysed.

Results: FcRγ-/- mice gained less weight after 11 weeks of HFD. They had reduced adiposity, adipose tissue inflammation, and IR. Interestingly, FcRγ-/- mice had higher lean mass compared to WT mice, which was associated with increased energy expenditure. Intestinal TG absorption was increased whereas plasma TG clearance was not affected in FcRγ-/- mice. Gut microbial composition differed significantly and might therefore have added to the observed phenotype.

Conclusion: FcRγ-chain deficiency reduces the development of diet-induced obesity, as well as associated AT inflammation and IR at 11 weeks of HFD.

Introduction

Obesity and its related metabolic disorders, such as insulin resistance (IR) and type 2 diabetes mellitus (T2DM), have been associated with low-grade systemic inflammation, which is thought to be initiated by white adipose tissue (WAT) inflammation (1-4). Expanding WAT leads to infiltration of 'classically' activated (M1 type) macrophages, which are known to express pro-inflammatory cytokines and to form crown-like structures (CLS) around hypertrophic and dying adipocytes (5-7). In addition, T lymphocytes are known to accumulate in the expanding WAT, specifically cytotoxic T lymphocytes and TH1 lymphocytes which contribute to the pro-inflammatory environment (8-10). Pro-inflammatory cytokines, produced by infiltrated immune cells as well as by stressed adipocytes, contribute to the development of IR by directly affecting the insulin signalling pathway (11).

B lymphocytes have also been shown to play a role in the development of obesity related WAT inflammation and the development of IR. Duffaut et al. (12) reported an early accumulation of B lymphocytes in WAT when mice were subjected to a high fat diet (HFD). In Pima Indians, who are genetically predisposed to T2DM, immunoglobulin (Ig) gamma levels positively correlated with body mass index and predicted T2DM (13). These studies suggest that antibody-mediated immune responses are involved in the development of IR. Interestingly, Winer et al. (14) demonstrated that B lymphocyte deficient mice are protected from obesity related IR, and that adoptive transfer of IgG antibodies from obese WT mice into obese B lymphocyte deficient mice induced IR. In humans, blood lipid levels are associated with the increased expression of high-affinity IgE receptor FcεRI, which is strongly associated with increased serum IgE levels (15). These results indicate that also IgE might play a role in metabolic disorders. However, the mechanisms by which antibodies affect IR are largely unknown.

In this study, we have used FcRγ-chain deficient (FcRγ-/-) mice to investigate whether Fc-receptors play a role in the development of diet-induced obesity, WAT inflammation, and IR. FcRγ-/- mice lack the signal transducing γ-chain of the Fc-receptors, leading to non-functional FcγRI, III, and IV and FcεRI, and therefore diminished IgG and IgE antibody mediated cellular responses. Here we show that mice deficient for the FcRγ-chain have decreased diet-induced obesity, WAT inflammation and IR.

Materials and methods

Animals

The generation of FcRγ-/- mice on a C57Bl/6J background has been described previously (16). FcRγ-/- mice were bred at the Leiden University Medical Center, Leiden, The Netherlands. Wild-type (WT) control mice (C57Bl/6J background) were purchased from Charles River (Maastricht, The Netherlands). Mice used in experiments were males, housed under standard conditions with free access to water and food, randomization was not applicable. Mice were fed a HFD (45% energy derived from lard fat; D12451, Research Diet Services, Wijk bij Duurstede, The Netherlands) for 4 and 11 weeks to induce obesity (n=8-10 per group, which is determined as adequate power from power calculations and previous experiments; age=approx. 10 weeks). Body weight was measured regularly during the diet

intervention. In addition, lean and fat mass were regularly determined by MRI-based body composition analysis (Echo MRI, Echo Medical Systems, USA). All experiments were performed blinded if applicable and were approved by the animal ethics committee of Leiden University Medical Center.

Analysis

Determination of adipocyte size, adipocyte lipolysis and preadipocyte differentiation capacity were performed as described in the Supporting Information. Furthermore, qPCR, immunohistochemistry, plasma parameter, hyperinsulinemic-euglycemic clamp, indirect calorimetry, instinal TG absorption, postprandial response, in vivo clearance of TG-rich emulsion particles, fecal TG content, and cecal 16s rRNA sequencing analysis were performed as described in the Supporting Information Methods section. Primer sequences are listed in Table S1.

Statistical analysis

Data are presented as means ± SD. Statistical differences were calculated using the unpaired two-tailed Student's T-test (GraphPad Prism 6, CA, USA). For the microbiota sequencing data a non-parametric T-test was used, and the data was corrected for multiple testing using false discovery rate (FDR) method. $p<0.05$ was considered statistically significant.

Results

FcRγ-/- mice gain less weight after HFD-induced obesity, have smaller adipocytes but preadipocytes with higher adipogenic capacity

At the onset of the HFD, body weight was similar between WT and FcRγ-/- mice (Fig. 1A). However, after six weeks of HFD, body weight of FcRγ-/- mice became significantly lower compared to WT mice (Fig. 1A). After 11 weeks of HFD the mice were sacrificed. Body weight gain (-38%, $p<0.01$; Supplemental Fig. 1A) and gWAT weight (Fig. 1B) were lower, and gWAT adipocyte size (-26%, $p<0.001$; Supplemental Fig. 1B) was significantly smaller in the FcRγ-/- mice. To further investigate the reduced AT depot weight and adipocyte size, it was tested whether adipocytes from FcRγ-/- mice showed differences in adipogenesis. This was done by determining adipogenic capacity of gonadal preadipocytes ex vivo after 11 weeks of HFD. FcRγ-/- mice showed a significantly higher adipogenic capacity as compared to WT mice (Fig. 1C). Thus, after 11 weeks of HFD FcRγ-/- mice had reduced adiposity, whereas preadipocyte adipogenic capacity was increased.

FcRγ-/- mice have lower adipose tissue inflammation after HFD-induced obesity

After 11 weeks of HFD, gene expression of inflammatory markers in gWAT was lower in FcRγ-/- mice compared to WT mice. Expression of F4/80 (-47%; $p<0.05$) and Cd68 (-68%; $p<0.001$), two macrophage markers, as well as the chemokine Mcp1 (monocyte chemotactic protein 1) (-80%; $p<0.001$), and cytokines Tnfa (-59%; $p<0.05$), Il1b (-50%; $p<0.001$), Il6 (-42%; $p<0.01$) and Il10 (-58%; $p<0.01$) were lower in gWAT of FcRγ-/- compared to WT mice (Fig. 1D). Immunohistochemical characterization of

F4/80 showed diminished macrophage infiltration and CLS formation in gWAT of FcRγ-/- mice (Fig. 1E). These data indicate diminished gWAT inflammation in FcRγ-/- mice after 11 weeks of HFD.

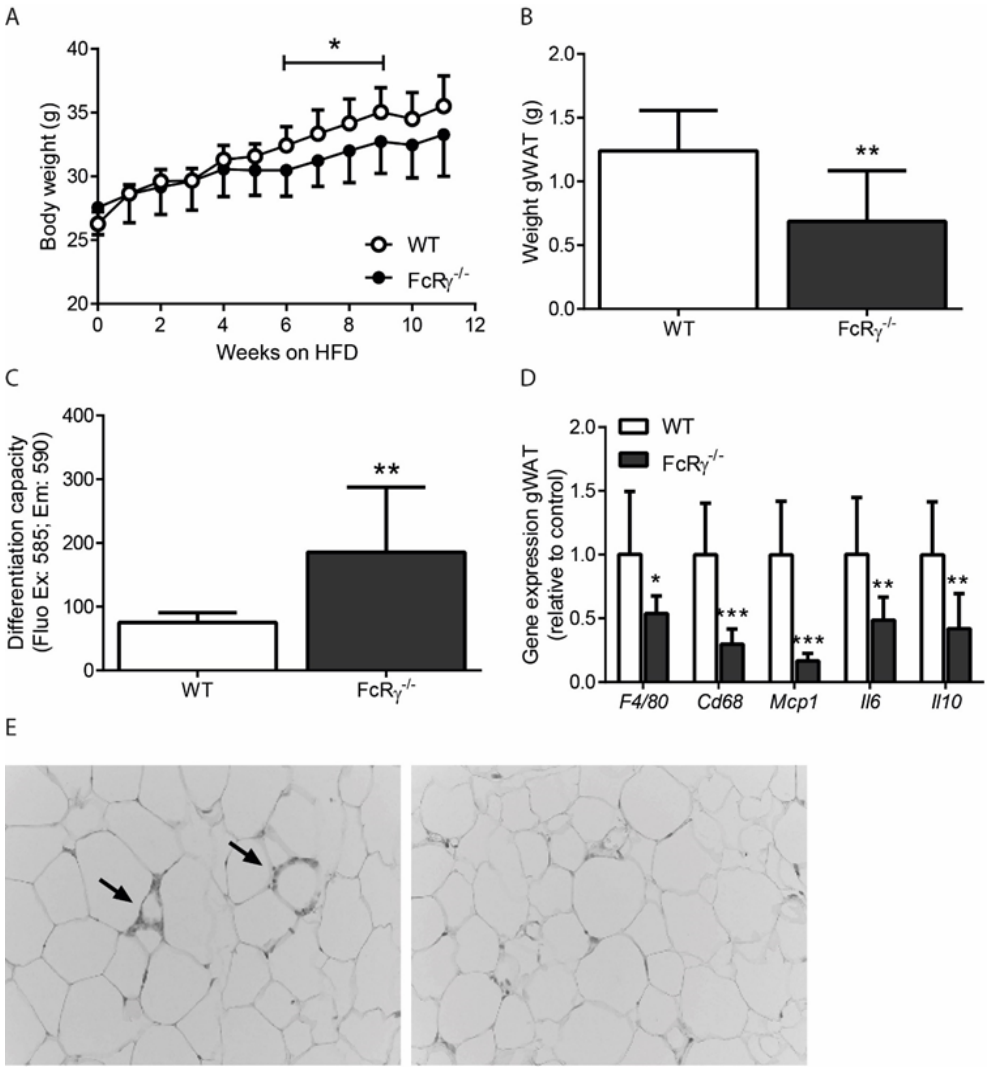

Figure 1. FcRγ$^{-/-}$ mice gain less weight after HFD-induced obesity, have smaller adipocytes but preadipocytes with higher adipogenic capacity, and lower adipose tissue inflammation. Wild-type (WT) and FcRγ$^{-/-}$ mice were fed a HFD for 11 weeks. Body weight was measured weekly (A). After 11 weeks of HFD gonadal WAT weight was determined (B) as well as gonadal preadipocyte adipogenic capacity (C). The expression of inflammatory genes was determined in gonadal WAT from WT and FcRγ$^{-/-}$ mice (D). F4/80 staining (E) of gonadal WAT from WT (left picture) and FcRγ$^{-/-}$ mice (right picture), a x20 magnification is used, crown-like structures are indicated by an arrow. Data presented as mean ± SD (n=9-10 per group); *p<0.05, **p<0.01, ***p<0.001.

FcRγ-/- mice have decreased HFD-induced insulin resistance

Plasma glucose and insulin levels of overnight fasted FcRγ-/- and WT mice were measured after 11 weeks of HFD. Both glucose (Fig. 2A) and insulin levels (Fig. 2B) were significantly lower in FcRγ-/- mice compared to WT mice, which suggests decreased IR in the FcRγ-/- mice. A hyperinsulinemic euglycemic clamp analysis revealed increased glucose disposal rate in FcRγ-/- mice during the hyperinsulinemic period (Fig. 2C), indicating decreased peripheral IR compared to WT mice. Hepatic insulin sensitivity, as well as insulin mediated suppression of hepatic glucose production was similar in both groups (Fig. 2D). Thus, FcRγ-/- mice have diminished IR compared to WT mice after 11 weeks of HFD.

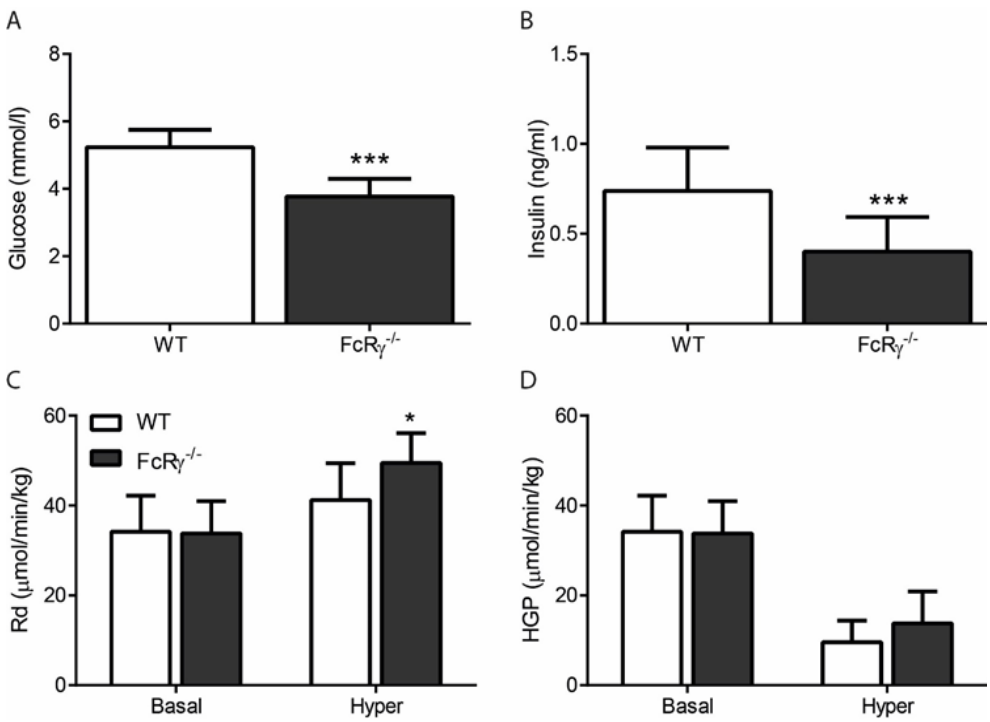

Figure 2. FcRγ$^{-/-}$ mice have decreased HFD-induced insulin resistance. After 11 weeks of HFD, blood was drawn after an overnight fast and plasma glucose (A) and insulin (B) levels were measured in both WT and FcRγ$^{-/-}$ mice. A hyperinsulinemic-euglycemic clamp analysis determined the body weight-specific rate of disappearance (Rd) of glucose (C) and the hepatic glucose production (HGP) rate (D) during basal and hyper state in WT and FcRγ$^{-/-}$ mice.

FcRγ-/- mice have increased lean mass, food intake and energy expenditure on chow diet

Prior to the HFD-intervention, FcRγ-/- and WT mice had a comparable fat mass, however the lean mass of FcRγ-/- mice was significantly higher compared to WT mice (Fig. 3A), as determined by MRI-based body composition analysis. The heart weight of FcRγ-/- mice was significantly higher (Fig. 3B), which suggests that the higher lean mass was caused by a higher muscle mass in the FcRγ-/- mice. FcRγ-/- mice showed increased food intake during the nocturnal period (night; high physical activity) on chow diet, whereas food intake after 5 week HFD-intervention did not differ (Fig. 3C). Furthermore,

nocturnal energy expenditure was higher for the FcRγ-/- mice on chow diet, with no differences on HFD (Fig. 3D). Nocturnal physical activity was slightly increased on both chow as HFD for the FcRγ-/- mice, however not significantly different (Supplemental Fig. 1C). FAox rate did not differ between both groups (Fig. 3E), while CHox was significantly higher in the nocturnal period during chow and HFD-intervention in FcRγ-/- mice (Fig. 3F).

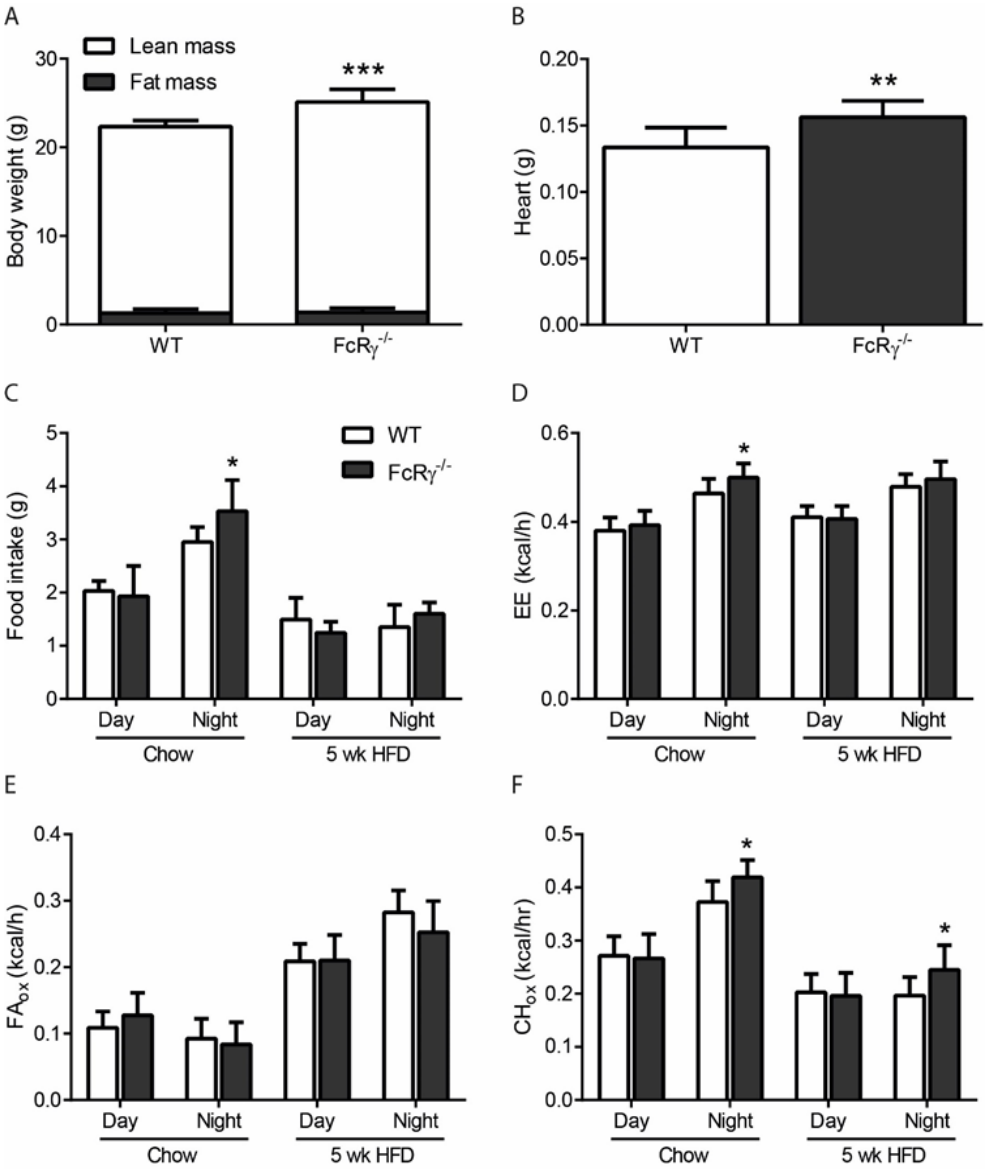

Figure 3. FcRγ$^{-/-}$ mice have increased lean mass, food intake and energy expenditure on chow diet. Body composition (A) was determined for both WT and FcRγ$^{-/-}$ mice on chow diet. Heart weight was measured after 4 weeks of HFD (B). Food intake (C) as well as energy expenditure (EE; D), fatty acid oxidation (FA_{ox}; E), and carbohydrate oxidation (CH_{ox}; F) on chow and after 5 weeks of HFD were determined using indirect calorimetry measurement. Data presented as mean ± SD (n=8 per group); *p<0.05, **p<0.01, ***p<0.001.

With comparable adiposity, FcRγ-/- mice have similar adipocyte size and functionality, and adipose tissue inflammation as WT mice

To determine whether FcRγ-/- mice are characterized by an intrinsic difference in WAT inflammation, independent of WAT weight, mice were subjected to a short 4 week HFD-intervention. As expected from Fig. 1A, both WT and FcRγ-/- mice had gained similar body weight at this time point. gWAT depot (Fig. 4A), as well as mWAT and sWAT depots (data not shown) were comparable in weight between both groups. The adipocyte size of gWAT, mWAT and sWAT were similar between FcRγ-/- and WT mice (Supplemental Fig. 1D). Gonadal adipocytes ex vivo showed comparable basal lipolysis, 8b-cAMP-stimulated lipolysis and insulin-mediated inhibition of 8b-cAMP stimulated lipolysis between the groups (Fig. 4B). Inflammatory gene expression levels in gWAT were comparable between FcRγ-/- and WT mice (Fig. 4C). TG and FFA plasma levels were comparable, whereas TC was reduced after 4 weeks of HFD in FcRγ-/- mice compared to WT mice (Fig. 4D). Furthermore, plasma glucose (Fig. 4E) and insulin levels (Fig. 4F) were comparable at this stage, indicating no differences in insulin sensitivity. Thus, with comparable adiposity, adipose tissue inflammation and insulin sensitivity were comparable for WT and FcRγ-/- mice.

FcRγ-/- mice have increased intestinal absorption and comparable clearance capacity

After a 4 week HFD-intervention, with comparable body weight and adiposity, intestinal absorption and excretion was measured. The fractional intestinal fat absorption, determined by a non-absorbable food additive (SPB), was significantly increased in the FcRγ-/- mice (Fig. 5A). Increased intestinal TG absorption was confirmed by increased TG plasma levels after olive oil gavage in FcRγ-/- mice (Fig. 5B). TG plasma clearance capacity, determined using [3H]TO-labelled TG-rich VLDL-like emulsion particles, was identical between WT and FcRγ-/- mice (Fig. 5C). Fecal excretion of TG was reduced as fecal glycerol levels were lower for FcRγ-/- mice (Fig. 5D). These data indicate that FcRγ-/- mice have increased intestinal TG absorption, whereas plasma clearance did not differ and fecal excretion was lower. Thus, increased TG clearance capacity, for instance via brown adipose tissue activation, is not causal for the reduced weight gain in the FcRγ-/- mice.

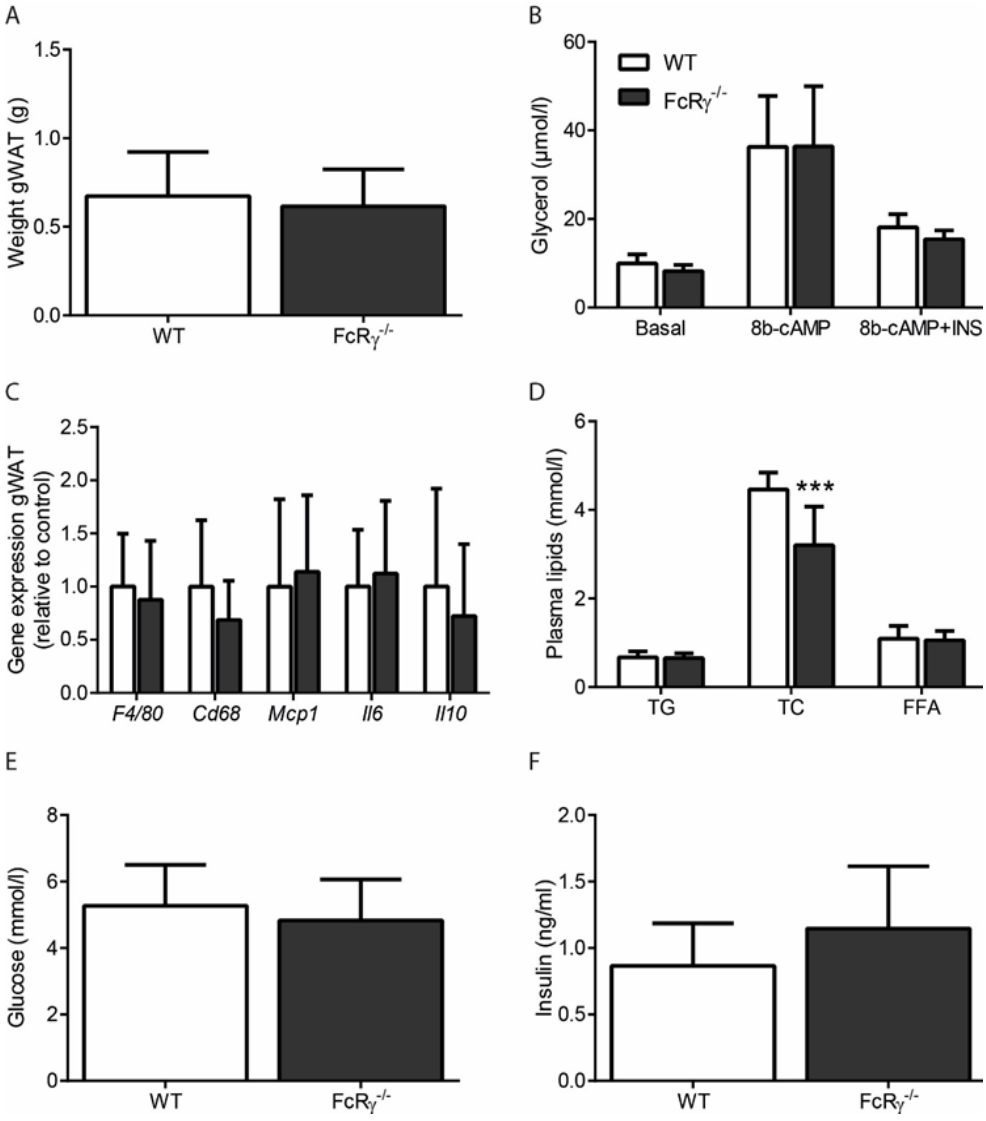

Figure 4. With comparable adiposity, FcRγ$^{-/-}$ mice have similar adipocyte size and functionality, and adipose tissue inflammation as WT mice. WT and FcRγ$^{-/-}$ mice were fed a HFD for 4 weeks. Gonadal WAT weight (A) and basal lipolysis, 8b-cAMP stimulated lipolysis and insulin inhibition of 8b-cAMP stimulated lipolysis of gonadal adipocytes (B) were determined. Inflammatory gene expression was determined in gonadal WAT from WT and FcRγ$^{-/-}$ mice (C). After an overnight fast, blood was drawn and plasma lipid levels (D) as well as glucose (E) and insulin (F) were measured. Data presented as mean ± SD (n=10 per group); ***p<0.001.

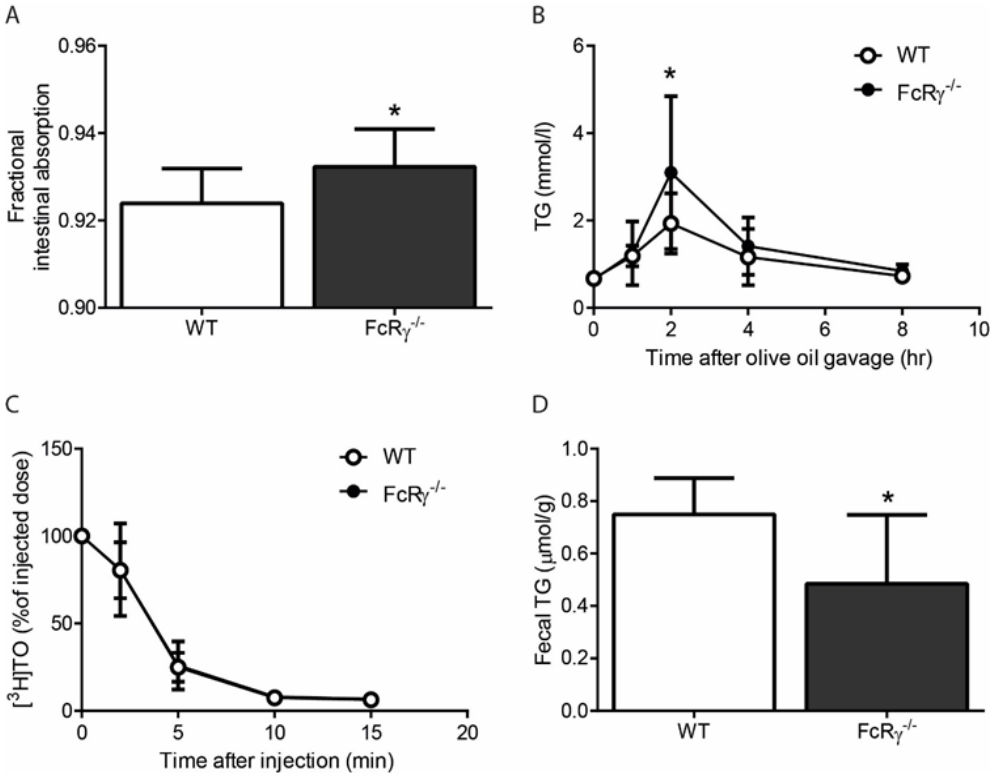

Figure 5. FcRγ$^{-/-}$ mice have increased intestinal absorption and comparable clearance capacity. After 4 weeks of HFD, intestinal fat absorption was determined by using a non-absorbable food additive (A), and by a post prandial response of triglycerides (TG) after a 4 hour fasting period (B). After a 4 hour fast, the in vivo clearance of glycerol tri[^{3}H]oleate-labeled ([^{3}H]TO)-labeled TG-rich emulsion particles was determined in WT and FcRγ$^{-/-}$ mice (C). Fecal TG was determined in feces collected from WT and FcRγ$^{-/-}$ mice (D). Data presented as mean ± SD (n=9-10 per group); *p<0.05.

FcRγ-/- mice have a different microbiota

As gut microbiota is an important player in the development of obesity and related disorders, the microbial composition of cecal content was determined using 16S rRNA gene sequence analysis of WT and FcRγ-/- mice after 4 weeks of HFD. Taxonomy-based analysis of the sequence data showed that, at phylum level, the mouse microbiota was dominated by Firmicutes (Fig. 6A, Table 1). When comparing the microbial composition of WT and FcRγ-/- mice, a distinct difference in Verrucomicrobia could be seen, WT microbiota consisted for 12% of Verrucomicrobia whereas this phylum completely lacked in the microbiota of the FcRγ-/- mice (Fig. 6A, Table 1). Furthermore, the abundance of Deferribacteres was higher in the FcRγ-/- mice compared to WT mice, but not significantly different after multiple comparison correction (19.9% versus 11.8% respectively, p<0.077, Table 1). Microbial composition was similarly determined on genus level and compared for WT and FcRγ-/- mice. The absence of Verrucomicrobia on phylum level in the FcRγ-/- mice was totally explained by absence of Akkermansia

on genus level (Table 1). Additionally, Oscillospira and Prevotella were both significantly lower in the microbiota of FcRγ-/- mice compared to WT mice, whereas Mucispirillum was higher, albeit that significance was lost after multiple test correction (Table 1). Alpha-diversity metrics of observed species indicated that the microbiota of FcRγ-/- mice was less diverse as compared to WT mice (Fig. 6B). Beta-diversity illustrates that the microbiota from WT and FcRγ-/- mice are two separate microbial communities (Fig. 6C). Thus, there are clear microbial composition differences on both phylum and genus level in the ceca of WT and FcRγ-/- mice.

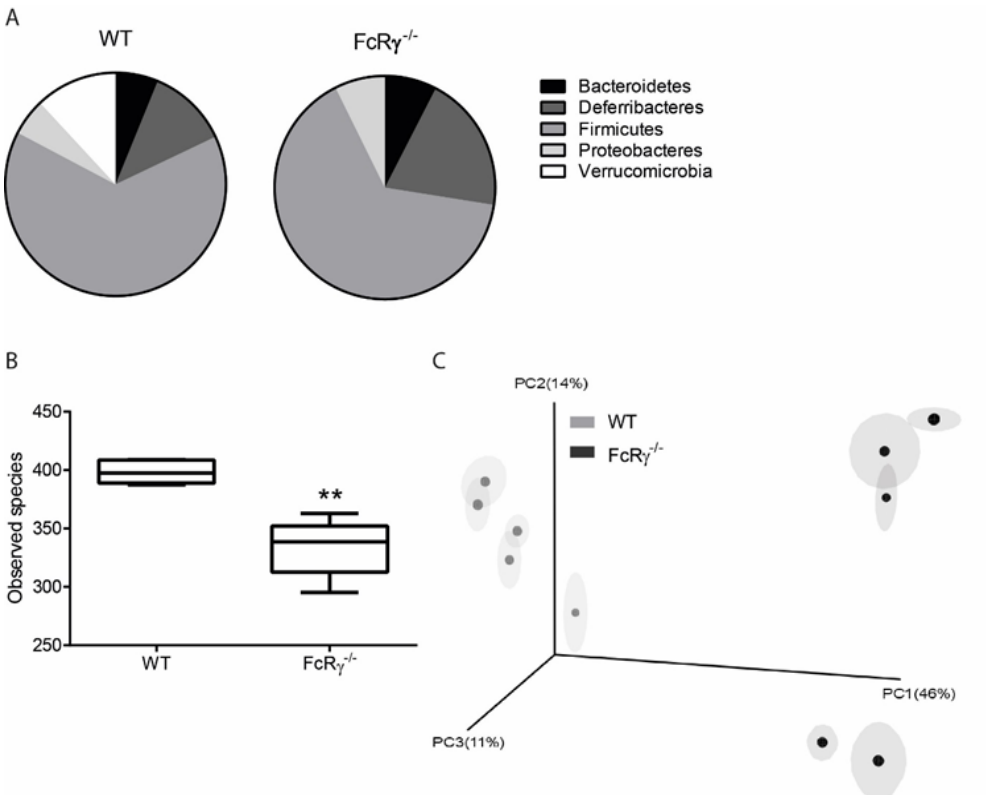

Figure 6. FcRγ$^{-/-}$ mice have a different microbiota. After 4 weeks of HFD, cecal content was isolated from WT and FcRγ$^{-/-}$ mice. 16S rRNA sequence analysis was performed and microbial composition was determined on phylum level for both WT and FcRγ$^{-/-}$ mice (A). Mean percentage OTU's of different bacterial phyla are shown in the pie-chart, including *Bacteroidetes* (black), *Deferribacteres* (dark-grey), *Firmicutes* (grey), *Proteobacteres* (light-grey), and *Verrucomicrobia* (white). Alpha-diversity metrics observed species (B) even as beta-diversity (C) of the microbiota was determined for WT and FcRγ$^{-/-}$ mice. Data presented as mean ± SD (n=5 per group); $^{**}p<0.01$.

Table 1. Gut microbial composition of WT and FcRγ$^{-/-}$ mice.

	WT	**FcRγ$^{-/-}$**	***p*-value**
Phylum			
Bacteroidetes	6.2±2.8	7.6±5.1	0.753
Deferribacteres	11.8±5.6	19.9±4.3	0.077
Firmicutes	64.7±11.8	65.1±4.4	0.940
Proteobacteres	5.4±2.8	7.4±2.6	0.380
Verrucomicrobia	12.0±16.9	0	0.035*
Genus			
Akkermansia	12.0±16.9	0	0.045*
Allobaculum	6.9±8.5	1.8±1.1	0.306
Bacteroides	0.5±0.3	3.2±3.6	0.268
Bilophila	5.4±2.8	7.4±2.6	0.345
Dehalobacterium	1.9±0.3	1.5±0.2	0.079
Mucispirillum	11.8±5.6	19.9±4.3	0.065
Oscillospira	9.0±3.7	3.5±2.0	0.049*
Prevotella	1.4±0.8	0.1±0.1	0.030*

Values are mean percentage read numbers ± SD, n=5 per group
p-values are FDR corrected; *$p<0.05$

Discussion

This study is, to our knowledge, the first to investigate the role of the FcRγ-chain in the development of diet-induced obesity and IR. We showed that after a HFD for 11 weeks, FcRγ-/- mice gained less weight and developed less WAT inflammation and peripheral IR compared to WT controls. Our results thus suggest that FcRγ-chain mediated pathways play a role in the development of diet-induced obesity.

Obesity related antibodies have previously been shown to contribute to the development of IR. Our study intended to investigate whether obesity related antibodies affect IR via Fc-receptors by using the FcRγ-/- mouse model. This model has previously successfully been used to study the role of Fc-receptor mediated antibody effector pathways in other pathologies, such as collagen induced arthritis (17). As the FcRγ-chain is crucial for full activation of IgG and IgE antibody effector pathways, our results may indicate that Fc-receptor mediated antibody effector pathways play a role in the development of diet-induced obesity. However, the fact that the FcRγ-/- mice unexpectedly showed a higher lean mass independent of the HFD intervention makes it difficult to easily draw such conclusions. On a regular chow diet the FcRγ-/- mice showed a higher food intake, energy expenditure and a trend towards higher physical activity during the night period, which may be explained by the higher lean mass which has a higher energy turnover. On a HFD, the FcRγ-/- mice showed similar food intake and energy expenditure, though the FcRγ-/- mice showed a negative energy balance compared to the WT mice. Taking into account that the lean mass was higher of the FcRγ-/- mice, relatively more energy was needed for energy turnover in the lean mass and less energy was left for energy storage for the FcRγ-/- mice on a HFD. Indeed, total energy expenditure after 5 weeks of HFD tended to be higher in the FcRγ-/- mice. The increased lean mass in the FcRγ-/- mice thus may have contributed to the reduced diet-induced obesity in these mice. Therefore, from our experiments with the FcRγ-/- mice, we cannot conclude that obesity related antibodies act on Fc-receptors and modulate the development of diet-induced obesity and related IR.

The reason for the increased lean mass in FcRγ-/- mice remains to be investigated. The increase in lean mass in FcRγ-/- mice may be due to an increased muscle mass, as heart weight was increased. The transcription factor GATA4 is known to regulate genes involved in embryonic heart and muscle development (18, 19). Besides, GATA4 is found to be expressed in B lymphocytes and to regulate the expression of the inhibitory FcγRIIB (20), which is the only FcγR still present in the FcRγ-/- mice. GATA4 might play an important role in FcRγ-/- mice as regulator of lean mass development. Furthermore, we cannot exclude the possibility that the increased lean mass phenotype was caused by non Fc-receptor driven mechanisms. In addition to its association with Fc-receptors, the FcRγ-chain has been associated with several receptors involved in innate immunity including NKR-P1C, PIR-A, and IL3R. NKR-P1C is expressed on natural killer cells and mediates cytotoxicity against tumour cells (21). The paired Ig like receptor-A (PIR-A) is present on monocytes and macrophages, dendritic cells, mast cells, and certain B lymphocyte subsets expressing natural antibodies, and is able to activate these cells upon interaction with major histocompatibility complex (MHC) class 1 molecules (22). FcRγ-chain deficiency might affect the production of natural antibodies that are primarily produced by B1 cells. A compensatory increase

in IgM levels might happen, which has previously been shown to occur in mice deficient for secreted IgM that led to increased IgG levels (23). Some natural IgMs are inversely associated with cholesterol levels and are known to mediate atheroprotection as reviewed by Tsiantoulas et al. (24). This could underlie the lower plasma cholesterol levels that we detect in the FcRγ-/- mice. Additionally, the FcRγ-chain is a constitutive component of IL-3R and is necessary for IL-3-induced production of IL-4 by basophils (25). Thus, in addition to a signalling role in Fc-receptor mediated effector mechanisms, the FcRγ-chain plays also an important role in other signalling pathways, which could have contributed to the metabolic phenotype of the FcRγ-/- mice that we observed.

Another important contributor to energy metabolism in an organism is the intestinal microbiota (26).These microbiota harvest energy for their own use from the diet of the host, but they also produce short-chain fatty acids (SCFA) from indigestible fibres that are converted to an energy source for the host in this way (27). Moreover, they have been implicated in the synthesis of gut peptides by intestinal cells such as GLP-1 and PYY that regulate host energy homeostasis. Since intestinal microbiota are predominantly anaerobic, the energy extracted from the diet and used by the microbiota are difficult to measure by the indirect calorimetry-equipment which we used. It is possible that the microbiota from the FcRγ-/- mice differ in their energy expenditure and the efficiency to extract energy from the diet and to make this available for the host. Analysis of the 16S rRNA gene sequences of the cecum content indeed showed pronounced differences in cecal microbial composition between FcRγ-/- and WT mice. Prevotella and Oscillospira were significantly lower in FcRγ-/- mice compared to WT mice, which are both known as fibre degrading bacteria (28-31). Less of these bacteria imply reduced carbohydrate degradation capacity and thus reduced energy harvesting efficiency. This difference could play a role in the reduced bodyweight gain in HFD-fed FcRγ-/- mice. Furthermore, the FcRγ-/- mice lacked the total population of Verrucomicrobia, which was substituted by Deferribacteres. Both phyla are associated with a beneficial metabolic phenotype in humans and mice in some studies, but not in all (32-36). It is possible that a different ratio of Verrucomicrobia and Deferribacteres within the microbiota affects energy metabolism of the host, however, this requires further investigation. To elucidate the effect of microbial changes in FcRγ-/- mice, potential follow up studies regarding gut microbiota, including co-housing experiments, seem to be highly interesting.

The differences in body weight gain on a HFD between the WT and FcRγ-/- mice cannot be explained by a reduced adipogenic capacity of the adipocytes, as preadipocytes ex vivo actually showed a higher adipogenic capacity in FcRγ-/- mice. Also insulin responsiveness of adipocytes with comparable size from both WT and FcRγ-/- mice was similar. Thus, although Fc-receptors are expressed on adipocytes, at least in humans (37), it seems that there is no direct effect of FcRγ-chain deficiency on adipocyte functionality that explains the diminished body weight gain of the FcRγ-/- mice. Furthermore, intestinal fatty acid absorption or TG excretion from the intestine cannot explain the reduced body weight gain, as the FcRγ-/- mice showed an even higher fatty acid absorption and lower TG excretion from the gut. Recently, it was found that in the gut in the absence of IgA and the presence of microbiota, the intestinal epithelium down-regulates its metabolic functions and up-

regulates its immune functions, which ultimately results in lipid malabsorption. Moreover, duodenal samples of immunodeficient humans display a down-regulation of metabolic genes and an up-regulation of genes involved in immunity in intestinal epithelium, which was also regulated by GATA4 (38). As the FcRγ-/- mice represent an immune-compromised model, one would have expected that these mice have a reduced lipid absorption. Remarkably, we found the opposite. We do not know, however, whether IgA is involved in the observed phenotype of the FcRγ-/- mice, since in mice the Fc-receptor for IgA (FcαR) has never been identified. Humans express a γ-chain associated high affinity receptor for IgA, FcαRI (CD89), on myeloid cells such as macrophages, neutrophils and eosinophils (39). Interestingly, the FcεRI has also been found on human intestinal epithelial cells (40). This FcRγ-chain dependent receptor binds IgE to carry out a host defense function during parasitic infections. Lacking the γ-chain might affect intestinal epithelial function, which could contribute to increased intestinal lipid absorption which we found in the FcRγ-/- mice.

Although the FcRγ-/- mice showed reduced AT inflammation and IR after 11 weeks of HFD, we cannot conclude that there are direct effects of the FcRγ-chain mediated pathways on these processes. Adiposity was greatly reduced, and adiposity per se is an important determinant of AT inflammation and IR. After 4 weeks of HFD, when the FcRγ-/- mice did not show differences in body weight or AT depot weight yet, we did not find any differences in AT inflammation. This indicates that the effects of FcRγ-chain deficiency on AT inflammation after 11 weeks of HFD were at least partly body weight driven. However, it is also possible that the involvement of the FcRγ-chain in AT inflammation is a late event and that 4 weeks of HFD might not have been enough to induce AT inflammation with related antibodies that can activate Fc-receptor pathways. This is in agreement with previous work that showed antibody pathogenicity to occur as a late event after the initiation of HFD-induced obesity (14).

In conclusion, FcRγ-chain deficiency reduces the development of diet-induced obesity. The decrease in adiposity of FcRγ-/- mice after 11 weeks of HFD may well explain the reduced WAT inflammation and decreased IR observed at this time point. FcRγ-chain deficiency is associated with an increase in lean mass and a significantly different gut microbial composition. This can explain at least partially the reduced development of diet-induced obesity in FcRγ-/- mice.

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Supporting Information

Methods

Determination of adipocyte size, adipocyte lipolysis and preadipocyte differentiation capacity

Adipose tissue from the gonadal (gWAT, unilateral), subcutaneous (sWAT, unilateral) and mesenteric (mWAT) region were removed from the mice after 4 and 11 weeks of HFD and kept in PBS. The tissues were minced, digested (1 h at 37°C in 0.5 g/l collagenase (Type 1) in DMEM/F12 with 20 g/l of dialyzed bovine serum albumin (BSA, fraction V, Sigma, ST Louis, USA)), and filtered through a nylon mesh (236 μm pore). Adipocytes were obtained from the surface of the filtrate, and washed several times with PBS. Cell size and volume of mature adipocytes was determined from micrographs (approx. 1,000 cells per WAT sample) using image analysis software that was developed in house in MATLAB (MathWorks, Natick, MA). The adipocyte number per fat pad was calculated from the fat pad mass and adipocyte size. In addition, the anti-lipolytic effect of insulin on gonadal adipocytes was determined. Adipocytes were incubated for 2 h at 37°C in medium (DMEM/F12 with 2% BSA), in combination with or without 8-bromoadenosine 3'-5'-cyclic monophosphate (8b-cAMP) (10^{-3} M) (Sigma, Uithoorn, The Netherlands) and with or without insulin (10^{-9} M). Glycerol concentrations (as a measure of lipolysis) were determined using a free glycerol kit (Sigma, Uithoorn, The Netherlands) with the inclusion of the hydrogen peroxide sensitive fluorescence dye Amplex Ultra Red, as described by Clark et al (1).

For the isolation of preadipocytes, the residue of the adipose tissue filtrate was centrifuged (10 min, 200 g, RT) and treated with erythrocyte lysis buffer. The cells were washed twice, re-suspended in DMEM/F12 medium, supplemented with 10% fetal calf serum (FCS) and 100 μg/ml penicillin-streptomycin, inoculated into 96-well plates (12 wells/adipose tissue region) at a density of 40,000 cells/ml, and kept at 37°C in 5% CO_2. The cells were expanded until confluency after which adipocyte differentiation was induced using an adipogenic medium containing DMEM/F12 with 0.1 μM dexamethasone, 25 μM 3-isobutyl-1-methylxanthine, 17 nM insulin, 10% FCS and 100 μg/ml penicillin-streptomycin. After 3 days, medium was changed to maintenance medium containing 17 nM insulin and 10% FCS. After 5 more days the cells were lipid filled and intra cellular lipid accumulation was determined using a fluorometer after incubating the cells with Nile Red.

RNA isolation and qPCR analysis

RNA was isolated from gWAT using the Nucleospin RNA kit (Macherey-Nagel, Düren, Germany) according to the instructions of the manufacturer, except for the addition of an extra centrifugation step (10 min, 300 g) to discard lipids from the lysate. Quality of the mRNA was confirmed by lab-on-a-chip technology (Bio-analyzer, Agilent). Total mRNA (600 ng) was reverse transcribed with iScript cDNA synthesis kit (Bio-Rad) and purified with Nucleospin Extract II kit (Macherey-Nagel, Düren, Germany). RT- PCR was carried out on the IQ5 PCR machine (Biorad, CA, USA) using the Sensimix SYBR Green RT-PCR mix (Quantace, London, UK). mRNA levels were normalized to mRNA levels of housekeeping genes; glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), cyclophilin (*Cyclo*) or ribosomal protein large P0 (*Rplp0*). Primer sequences are listed in Table S1.

Immunohistochemistry

Immunohistochemistry was performed on formalin fixed and paraffin embedded sections of gWAT. For detection of macrophages, an F4/80+ antibody (1:150) (MCA497, AbD Serotec, Puchheim, Germany) was used. Visualization of the complex was done using diaminobenzidene for 10 minutes. Negative and positive controls were included. Haematoxylin and Eosin (HE) stainings of sections were done using a standard protocol.

Plasma parameters

After 4 and 11 weeks of HFD intervention, blood was drawn from overnight fasted mice via the tail vein into paraoxon (Sigma, St. Louis, MO) coated capillary tubes to prevent ongoing *in vitro* lipolysis (2). After centrifugation, plasma was collected and triglyceride (TG), total cholesterol (TC), free fatty acid (FA), glucose, and insulin levels were determined using commercially available kits (11488872 and 236691, Roche Molecular Biochemicals, Indianapolis; NEFA-C Wako Chemicals GmbH, Neuss, Germany; Instruchemie, Delfzijl, The Netherlands and Crystal Chem Inc., IL, USA, respectively).

Hyperinsulinemic-euglycemic clamp analysis

After 11 weeks of HFD, hyperinsulinemic-euglycemic clamps were performed as described earlier (3). Briefly, after an overnight fast, animals were anesthetized and an infusion needle was placed in the tail vein. Basal glucose parameters were determined during a 60-min period, by infusion of D-[3-^{3}H] glucose to achieve steady-state levels. A bolus of insulin (3 mU) was given and a hyperinsulinemic euglycemic clamp was started with a continuous infusion of insulin (5 mU/h) D-[3-^{3}H]glucose and a variable infusion of 12.5% D-glucose (in PBS) to maintain blood glucose levels at euglycemic levels. Blood samples were taken every 5-10 min from the tip of the tail to monitor plasma glucose levels (Accu-Check, Roche, Almere, The Netherlands). Seventy, eighty, and ninety minutes (steady-state) after the start of the clamp, blood samples (70 µl) were taken for determination of plasma glucose, insulin, and FFA levels. Turnover rates of glucose (µmol/min/kg) were calculated during the basal period and in steady-state clamp conditions as the rate of tracer infusion (dpm/min) divided by the plasma-specific activity of ^{3}H-glucose (dpm/µmol). All metabolic parameters were expressed per bodyweight. The hepatic glucose production (HGP) was calculated from the rate of disappearance (Rd) and glucose infusion rate (GIR) by the following equation: Rd=HGP+GIR. The Rd was measured from Steele's equation in steady state using the tracer infusion rate (Vin) and plasma-specific activity (SA) of ^{3}H-glucose (dpm/µmol) by the following formula: Rd=Vin/SA.

Indirect calorimetry

During week 0 and 5 of HFD feeding, mice were subjected to individual indirect calorimetry measurements using metabolic cages (LabMaster System, TSE Systems, Bad Homburg, Germany). After one day of acclimatization, water and food intake were monitored, and oxygen consumption (VO_2) and carbon dioxide production (VCO_2) were measured during 3 consecutive days. Carbohydrate

(CH_{ox}) and fat (FA_{ox}) oxidation rates were calculated from VO_2 and VCO_2 as previously described (4). Total energy expenditure (EE) was calculated from the sum of CH and FA oxidation. Activity was monitored as 2-dimensional infrared beam breaks.

Intestinal TG absorption

Sucrose polybehenate (SPB; Mitsubishi-Kagaku Food Corporation, Tokyo, Japan) was added to the diet (5 gram % of the fat content) and was used as a non-invasive method for the measurement of intestinal fat absorption, as previously described (5). Fat absorption was calculated from the ratios behenic acid (non-absorbable) and absorbable stearic acid in the diet and feces, measured by gas chromatography.

Postprandial response

After 4 weeks of HFD, postprandial TG handling was investigated. Mice were fasted 4 h prior to the start of the experiment. Mice received an intragastric load of 200 µl olive oil (Carbonell extra virgin, Cordoba, Spain). Prior to the bolus and 1, 2, 4, and 8 h after bolus administration blood samples were obtained via tail vein bleeding for determination of plasma TG as described above.

In vivo clearance of TG-rich emulsion particles

VLDL-like TG-rich emulsion particles (80 nm) labelled with glycerol tri[^{3}H]oleate ([^{3}H]TO) were prepared as described previously (6). After a 4 h fast, mice were injected intravenously with 200 µl of emulsion particles (1 mg TG/mouse), to study the *in vivo* clearance of the VLDL-like particles. At 2, 5, 10 and 15 min after particle injection blood was drawn from the tail vein and plasma [^{3}H]TO was counted. Plasma volumes were calculated as 0.04706 * body weight (g), as determined from ^{125}I-BSA clearance studies as described previously (7).

Fecal TG content

Fecal TG content was determined in feces collected during week 6 of HFD feeding. TG was extracted according to a modified protocol from Bligh and Dyer (8). In short, 100 mg of crushed feces was homogenized in methanol: chloroform (2:1). 600 µl chloroform was added for TG extraction and samples were centrifuged. This procedure was repeated twice to maximize TG extraction. After centrifugation, the chloroform layer was collected and chloroform was evaporated. The remaining TG pellet was dissolved in 2% TritonX100 (Sigma). TG content was determined using a commercially available kit (11488872, Roche Molecular Biochemicals, Indianapolis).

Cecal DNA isolation and sequencing

DNA was extracted from cecal content of WT and FcRγ$^{-/-}$ mice (n=5 per group) according to manufacturer's instructions (12830-50, MO BIO laboratories, CA, USA). The V4 region of the 16s rRNA gene was amplified using sample-specific barcodes adapted to universal 520F (5'-AYTGGGYDTAAAGNG-3') and 802R ((5'-TACNVGGGTATCTAATCC-3') primers (9). Samples were sequenced on Illumina MiSeq

platform, at the Leiden Genome Technology Center (Leiden, The Netherlands).

16s rDNA data processing

Raw sequence data quality was assessed using FastQC, version: 0.11.2 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Subsequently, adapter cutting was performed using cutadapt, version: 1.4.2 (10) and reads shorter than 50 base pairs were discarded. Low quality reads were removed with Sickle, version: 1.33 (https://github.com/najoshi/sickle). For visualising the taxonomic composition of the cecal microbiota, and further alpha and beta diversity analysis, QIIME, version: 1.8.0 (11) was used. In short, closed reference UTO picking with 97% sequence similarity against GreenGenes 13.8 reference database was done. Observed species alpha-diversity metrics rarefied at 115,000 sequence per sample was calculated and Jackknifed beta-diversity of unweighted UniFrac distances with 10 jackknife replicates was measured at the same rarefaction depth.

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Figures and Tables

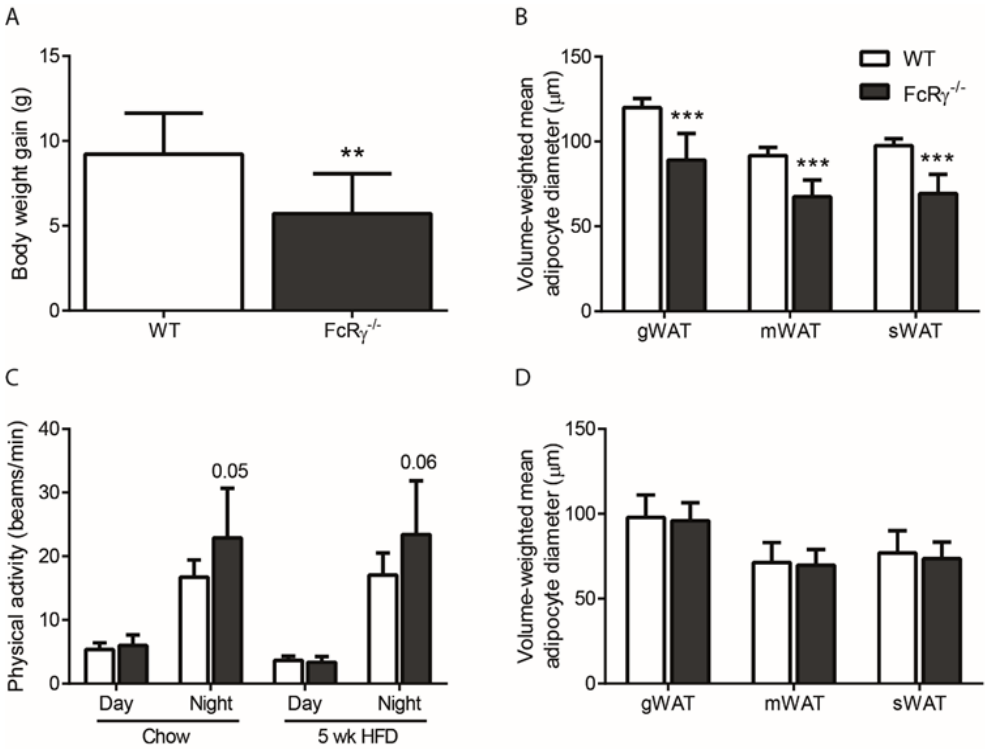

Figure S1. Body weight gain, adipocyte size and physical activity of WT and FcRγ$^{-/-}$ mice. Wild-type (WT) and FcRγ$^{-/-}$ mice were fed a HFD for 11 weeks. Body weight gain during the HFD-intervention was determined (A) and adipocyte size for gWAT, mWAT and sWAT after the intervention (B) for WT and FcRγ$^{-/-}$ mice. Physical activity was determined during chow diet and after 5 weeks of HFD for WT and FcRγ$^{-/-}$ mice (C). After 4 weeks of HFD adipocyte size of gWAT, mWAT and sWAT was determined for WT and FcRγ$^{-/-}$ mice (D). Data presented as mean ± SD; *p<0.05, **p<0.01, ***p<0.001.

Table S1. Primers used for quantitative real-time PCR analysis.

Gene	Forward primer	Reverse primer
Acox1	TATGGGATCAGCCAGAAAGG	ACAGAGCCAAGGGTCACATG
Cd68	ATCCCCACCTGTCTCTCTCA	TTGCATTTCCACAGCAGAAG
Cpt1a	GAGACTTCCAACGCATGACA	ATGGGTTGGGGTGATGTAGA
Cyclo	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG
F4/80	CTTTGGCTATGGGCTTCCAGTC	GCAAGGAGGACAGAGTTTATCGTG
Gapdh	GAGACTTCCAACGCATGACA	ATGGGTTGGGGTGATGTAGA
Il1b	TTGACGGACCCCAAAAGATG	AGAAGGTGCTCATGTCCTCAT
Il6	ACCACGGCCTTCCCTACTTC	CTCATTTCCACGATTTCCCAG
Il10	GACAACATACTGCTAACCGACTC	ATCACTCTTCACCTGCTCCACT
Mcp1	GCATCTGCCCTAAGGTCTTCA	TTCACTGTCACACTGGTCACTCCTA
Rplp0	GGACCCGAGAAGACCTCCTT	GCACATCACTCAGAATTTCAATGG
Tnfa	GATCGGTCCCCAAAGGGATG	CACTTGGTGGTTTGCTACGAC

6 The role of IgG antibodies in obesity-associated pathology: deletion of Fc-receptor or complement pathway does not diminish adipose tissue inflammation or insulin resistance

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In preparation

Abstract

Introduction: During the development of obesity pathogenic IgG antibodies are produced, which are thought to contribute to the development of insulin resistance. Downstream effects of these antibodies are mediated via IgG Fc-receptors (FcR) that are present on various immune effector cells, and/or via complement activation. To investigate if either of these mechanisms are limiting in the development of insulin resistance, we studied mice which lack all four FcγRs (FcγR1234-/-), the inhibitory FcγR2b (FcγR2b-/-), or the central component of the complement system C3 (C3-/-).

Methods: FcγR1234-/-, FcγR2-/-, C3-/- and control mice were fed a high fat lard diet (HFD) for 15 weeks to induce obesity. Body weight and insulin sensitivity were analyzed and gonadal white adipose tissue (gWAT) was characterized for adipocyte functionality and extent of inflammation.

Results: After 15 weeks of HFD the FcγR1234-/-, FcγR2b-/- and C3-/- mice had body weight and composition comparable to control. The FcγR1234-/-, FcγR2b-/- and C3-/- mice showed similar adipose tissue inflammation, as gene expression of pro-inflammatory cytokines and immune cell composition in gWAT were comparable to control mice. FcγR1234-/-, FcγR2b-/- and C3-/- mice showed comparable adipocyte specific insulin responsiveness, and similar or deteriorated whole body glucose tolerance compared to control mice.

Conclusion: FcγR or C3 deficiency does not result in decreased adipose tissue inflammation or insulin resistance. These data suggest that if obesity-induced IgG antibodies play a role in insulin resistance, this is not dependent of FcγR or complement mediated pathways.

Introduction

Obesity is associated with adipose tissue (AT) inflammation. The expanding fat mass releases increased levels of fatty acids (FA) and pro-inflammatory (adipo)cytokines which are key mediators in inducing systemic insulin resistance (1). The pro-inflammatory mediators originate from hypertrophic adipocytes as well as from pro-inflammatory immune cells that infiltrate the expanding AT. Several immune cell types from both the innate and adaptive immune system have been identified in AT. These cells include macrophages, T and B lymphocytes, neutrophils and mast cells, and are observed both in obese and lean individuals (2-5).

Obesity seems to induce a phenotypic switch from an anti-inflammatory state to a pro-inflammatory state (6). The number of pro-inflammatory M1 macrophages and cytotoxic T cells have been shown to be increased in AT during obesity, which goes along with a decrease in the number of anti-inflammatory T helper cells and regulatory T cells (7). B cells have also been shown to play an important role in obesity and related disorders. We have previously shown an increased activity of circulating B cells in obese as compared to age matched lean females (8). Accumulation of B cells in the AT leads to increased production of pro-inflammatory cytokines, T cell activation and IgG antibody release, which all contribute to the development of insulin resistance (9). Furthermore, B cells have been shown to promote insulin resistance by producing pathogenic IgG antibodies (10). Transfer of IgG from mice with diet-induced obesity (DIO) to DIO B-null mice induced rapid local and systemic changes in the inflammatory cytokine production and a phenotypical conversion of the macrophages in the visceral adipose tissue to a pro-inflammatory M1 phenotype (10).

How obesity associated IgG antibodies induce these inflammatory effects is still unclear. Immune complexes can bind to Fc-receptors (FcR) present on several immune cells, and can activate complement to induce specific immune responses (11, 12). The activation of antibody effector pathways may be an early event in the initiation of adipose tissue inflammation. The current study aims to investigate the effector pathway by which obesity related IgG antibodies contribute to the development of AT inflammation and IR.

We studied FcγR1234-/- mice which lack all four FcγRs, FcγR2b-/- mice which lack the inhibitory FcγR, and complement C3-/- mice which lack the central component of the complement system. We hypothesized that the FcγR1234-/- and C3-/- mice would have a beneficial metabolic phenotype compared to WT mice on a HFD, when the obesity associated IgG antibodies contribute to IR via one of these pathways. The FcγRb-/- mice, which have an increased IgG antibody response, may show aggravated HFD-induced metabolic disorders. However, we found decreased glucose tolerance in the FcγR1234-/- mice, and no effect in the C3-/- mice. Thus, if anything, our data would indicate a deteriorated instead of a beneficial metabolic phenotype.

Materials and methods

Animals

The generation of the FcγR1234-/- mice will be published elsewhere; FcγR2b-/- mice have been described before (13). Complement C3-/- mice (14) were kindly provided by Mike Carroll (Harvard Medical School, Boston, MA). All mice were bred at the Leiden University Medical Center (Leiden, The Netherlands). WT mice (C57Bl/6J background) were purchased from Charles River (Maastricht, The Netherlands) and were used as control group for the FcγR1234-/- and complement C3-/- mice, whereas FcγR2bflox mice were used as control for the FcγR2b-/- mice (bred in-house). Mice used in these experiments were males, housed under standard conditions with free access to water and food. They were fed a HFD (45/60 energy% derived from lard fat; D12451 or D12492, Research Diet Services, Wijk bij Duurstede, The Netherlands) for 15 weeks to induce obesity starting around 12 weeks of age. Body weight was measured weekly and lean and fat mass was assessed by MRI-based body composition analysis (Echo MRI, Echo Medical Systems, Houston, TX, USA). Mice were matched on lean mass prior to HFD intervention. Indirect calorimetry measurements using metabolic cages (LabMaster System, TSE Systems, Bad Homburg, Germany) were performed as previously described (15). After 15 weeks of HFD-intervention, mice were killed, perfused and organs were dissected for further analysis. All experiments were approved by the animal ethics committee of Leiden University Medical Center.

Adipocyte and stromal vascular cell isolation

Gonadal (gWAT), mesenteric (mWAT) and subcutaneous (sWAT) white adipose tissue was dissected and kept in PBS after 15 weeks of HFD. The tissue was minced, digested for 1 h at 37°C (0.5g/l collagenase (Type 1) in DMEM/F12 (pH 7.4) with 20 g/l of dialyzed bovine serum albumin (BSA, fraction V, Sigma, ST Louis, USA)), and filtered through a nylon mesh (236 µm pore). Adipocytes were isolated from the surface of the filtrate and washed several times with PBS, to determine adipocyte size via direct microscopy and the anti-lipolytic effect of insulin on gonadal adipocytes as previously described (16). The residue of the adipose tissue filtrate was used for the isolation of stromal vascular cells. After centrifugation (350 x g, 10 min) the supernatant was discarded and the pellet was treated with erythrocyte lysis buffer after which the cells were counted using an automated cell counter (TC10, Biorad). The stromal vascular cells were fixed using 0.5 % paraformaldehyde, stored in FACS buffer (PBS, 0.02% natrium azide, 0.5% FCS) in the dark at 4°C and analyzed using flow cytometry within one week.

Flow cytometry analysis

Stromal vascular cells were analyzed using flow cytometry. Cells were stained with fluorescently labeled antibodies for CD45.2-FITC (104; BioLegend), CD3-APC (145-2c11; eBioscience), CD19-PE (1D3; eBioscience), F4/80-PE (BM8; eBioscience). Cells were measured on a LSR II flow cytometer (BD Biosciences, CA, USA). Data were analyzed using FlowJo software (Treestar, OR, USA).

RNA isolation and qPCR analysis

RNA was isolated from gWAT using the Nucleospin RNA kit (Macherey-Nagel, Düren, Germany) according to the instructions of the manufacturer, except for an extra centrifugation (350 x g, 10 minutes) step which was added to discard the lipids from the lysate. Quality of the mRNA was confirmed using lab-on-a-chip technology (Bioanalyzer, Agilent). 1µg of total mRNA was reverse transcribed with iScript cDNA synthesis kit (Bio-Rad) and purified with Nucleospin Extract II kit (Macherey-Nagel). RT-PCR with gene-specific primers was carried out on the CFX96 PCR machine (Bio-Rad), using 2x SYBR Green Mastermix (Bio-Rad). Ribosomal protein large P0 (RPLP0) and cyclophilin (cyclo) were used as housekeeping genes. Primer sequences are listed in Table 1.

Table 1. Primers used for quantitative real-time PCR analysis.

Gene	Forward primer	Reverse primer
Cd68	ACTTCGGGCCATGTTTCTCT	GGGGCTGGTAGGTTGATTGT
Cyclo	ACTGAATGGCTGGATGGCAA	TGTCCACAGTCGGAAATGGT
F4/80	CTTTGGCTATGGGCTTCCAGTC	GCAAGGAGGACAGAGTTTATCGTG
Il6	ACCACGGCCTTCCCTACTTC	CTCATTTCCACGATTTCCCAG
Il10	GACAACATACTGCTAACCGACTC	ATCACTCTTCACCTGCTCCACT
Mcp1	CACTCACCTGCTGCTACTCA	GCTTGGTGACAAAAACTACAGC
Rplp0	GGACCCGAGAAGACCTCCTT	GCACATCACTCAGAATTTCAATGG
Tnfa	GATCGGTCCCCAAAGGGATG	CACTTGGTGGTTTGCTACGAC

Histology

Formalin fixed and paraffin embedded sections of gWAT were used for histological analysis. An anti-mouse IgG antibody (1:250) (Vector Labs, Brunschwig Chemie, Amsterdam, The Netherlands) was used to stain IgG in gWAT. Vectastain ABC (Vector laboratories, CA, US) was used for visualization of the antibody complex according to manufacturer's instructions. Haematoxylin staining of gWAT sections was done using a standard protocol. The area positive for IgG was quantified using Image J (NIH, Maryland, US).

Intra-venous glucose tolerance test

After 6 weeks of HFD, an intra-venous glucose tolerance test was performed after an 6 hour fast. Blood was drawn via the tail vein, after which mice received an intra-venous glucose injection (20% D-glucose, 2 g/kg total body weight, of which 50% of the glucose was [6,6-2H2]glucose (Sigma-Aldrich, Zwijndrecht, The Netherlands)). Blood samples were drawn after 5, 15, 30, 45, 60, 90 and 120 minutes after glucose injection for measurement of plasma glucose using a commercially available kit (Instruchemie, Delfzijl, The Netherlands). Simultaneously with blood sampling, whole blood was spotted on sample carrier paper (Sartorius Stedim, Goettingen, Germany). To analyze peripheral glucose uptake, blood spot glucose enrichment was measured as previously described (17).

Mass spectrometry analysis

Lean and obese WT mice were killed and perfused, after which gWAT was isolated and processed for mass spectrometry analysis. In short, gWAT was homogenized in PBS using a Potter-Elvehiem homogenizer. After centrifugation (3500 x g, 10 min) the fat fraction floating on the lysate was discarded. The lysate was passed through a cell strainer and then treated with detergent (0.5% Zwittergent, 3-12 (N-dodecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate), Sigma, USA) for 20 min at 0 °C. The lysate was cleared by centrifugation (16.000 x g, 20 min). IgG was isolated from the supernatant using protein A affinity chromatography, eluted with 10% acetic acid and freeze dried. For mass spectrometric analysis the eluted protein was reduced by addition of 5 mM DTT and denatured in 1% SDS for 10 min at 70°C. Next, the samples were alkylated by addition of 15 mM iodoacetamide and incubation for 20 min at RT. Proteins were digested with 2 µg trypsin (Worthington Enzymes, USA) in 100 µl 25 mM NH4HCO3 overnight at RT. Peptides were analyzed by on-line nano-liquid chromatography tandem mass spectrometry on an LTQ-FT Ultra (Thermo, Bremen, Germany) (18, 19). In a postanalysis process, raw data were first converted to peak lists using Bioworks Browser software v 3.2 (Thermo Electron, USA), then submitted to the UniProt database using Mascot v. 2.2.04 (www.matrixscience.com) for protein identification and finally sorted and compared using Scaffold software version 3.0.1 (www.proteomesoftware.com). Mascot searches were with 2 ppm and 0.5 Da deviation for precursor and fragment mass, respectively, and trypsin as enzyme. Carbamidomethyl was set as a fixed modification, and oxidation, and N-acetylation (protein N-terminus) were set as variable modifications. Scaffold filtered for identified proteins with at least 2 peptides with 95% confidence.

Statistical analysis

Data are presented as means ± SD. Statistical differences were calculated using the unpaired students T-test. $p<0.05$ was considered statistically significant.

Results

Adipose tissue IgG correlates positively with body weight

Absolute B lymphocyte numbers were significantly increased in gWAT from obese compared to lean mice (+600%, p<0.01; Fig. 1A). IgG staining in gWAT, as percentage of total area, showed a positive correlation with body weight (Fig. 1B). This was quantified from histological stainings of IgG in gWAT as shown in Figure 1C (left picture). IgG staining was located around adipocytes and co-localized with F4/80 positive cells (Fig. 1C). Mass spectrometry analysis of the protein A purified fraction from the AT, indicated that all IgG subtypes (IgG1, IgG2b, IgG2c, and IgG3) were present in gWAT from both lean and obese WT mice, with subtype IgG3 the most prominent one (Table 2). Furthermore, we found FcγR1 to be bound to the extracted IgG in the obese AT, which is the high-affinity FcγR present on macrophages. These data indicate that both B lymphocytes and IgG increase in gWAT during obesity.

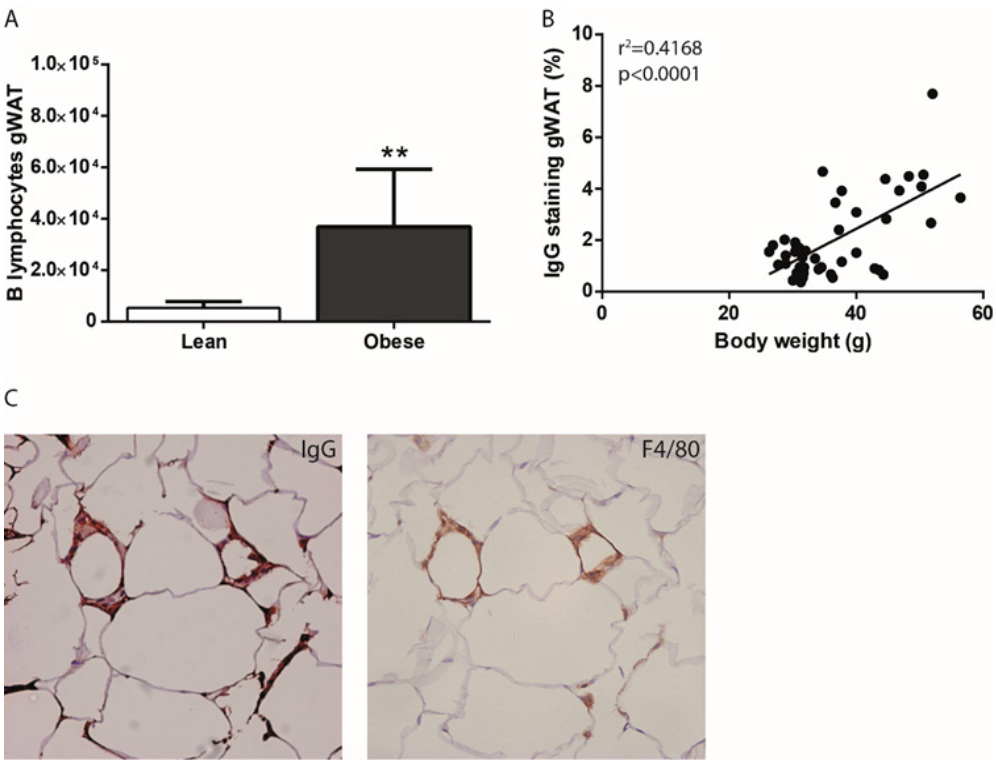

Figure 1. B cell numbers and IgG in gWAT increase during HFD-induced obesity.
WT mice were fed a HFD to induce obesity. Absolute B lymphocyte numbers were determined by flow cytometry analysis of gWAT from lean (<30 g, n=7) and obese (>40 g, n=14) WT mice (A). AT IgG was determined following histological staining of IgG in gWAT from WT mice with a varying body weight (26.3-56.4 g, n=44), IgG staining is represented as % of total area (B). Representative histological IgG staining in gWAT from WT mouse on HFD (C, left picture), as well as a macrophage (F4/80) staining of the same gWAT location, a 20x magnification is used (C, right picture). Error bars representing standard deviation of mean. ***p* <0.01 compared to lean.

Table 2. Hits in lean and obese AT from WT mice after Protein A column using mass spectrometry

Fragment	Lean AT	Obese AT
IgM	17	10
IgG3	12	9
IgG2B	9	9
IgG2C	7	5
IgG1	4	3
FcγR1	0	2

FcγR1234 deficiency has no effect on HFD-induced obesity or immune cell composition in gWAT

FcγR1234-/- mice were fed a HFD for 15 weeks to induce obesity. Bodyweight, lean and fat mass development were followed during the diet intervention. FcγR1234-/- mice gained comparable body weight compared to WT mice, with similar lean and fat mass (Fig. 2A). This was supported by comparable food intake and energy expenditure (data not shown). The isolated fat pads were similar in weight, with comparable adipocyte size and number (Supplementary Fig. 1A-C). gWAT adipocytes showed comparable basal and stimulated (8b-cAMP) lipolysis ex vivo, which was measured as glycerol release. Furthermore, inhibition of lipolysis by insulin was similar in the gWAT adipocytes of FcγR1234-/- mice compared to WT mice (Supplementary Fig. 1D).

WAT inflammation was determined after 15 weeks of HFD. IgG staining of gWAT tended to be increased compared to WT mice (+29%, $p<0.1$; Fig. 2B). Flow cytometry analysis showed reduced absolute leukocyte numbers in gWAT, which could primarily be attributed to lower macrophage numbers (Fig. 2C). vWAT and sWAT showed similar immune cell numbers and composition in WT and FcγR1234-/- mice (data not shown). Expression of several inflammatory genes (*Cd68, F4/80, Mcp1, Tnfa, Il6, Il10*) did not differ in gWAT from WT and FcγR1234-/- mice (Fig. 1D). Thus, FcγR1234 deficiency does not affect diet induced obesity, adipocyte size and functionality, or WAT inflammation.

FcγR1234 deficiency leads to increased HFD-induced systemic inflammation and decreased whole body glucose tolerance

Circulating immune cell numbers were determined in WT and FcγR1234-/- mice and revealed no difference in absolute leukocyte numbers (data not shown). However, within the T cell population, the percentage of cytotoxic T cells (CD8) was increased, whereas the percentage of T helper cells (CD4) was decreased in the FcγR1234-/- mice compared to the WT mice (data not shown). This led to a decreased CD4:CD8 ratio (Fig. 2E), which is indicative for increased systemic inflammation.

To determine if FcγRs play a role in diet-induced insulin resistance, glucose and insulin plasma levels were measured in 6 hour fasted FcγR1234-/- and WT mice, after 6 weeks of HFD. Glucose levels were comparable, whereas insulin levels were higher (+79%, $p<0.05$; Supplementary Fig. 1E, F) in FcγR1234-/- mice, indicating increased insulin resistance compared to WT mice. Furthermore, whole body glucose tolerance was determined by an intra-venous glucose tolerance test (ivGTT). FcγR1234-/- mice were less glucose tolerant than WT mice, indicated by the slower decay of glucose in plasma after glucose injection (Fig. 2F), and the increased area under the curve (AUC) of the plasma glucose levels

(+39%, p<0.05; data not shown) during the ivGTT. Stable isotope analysis from the ivGTT revealed that the difference in glucose tolerance could be attributed to decreased peripheral glucose uptake, rather than glucose production by the liver (data not shown).

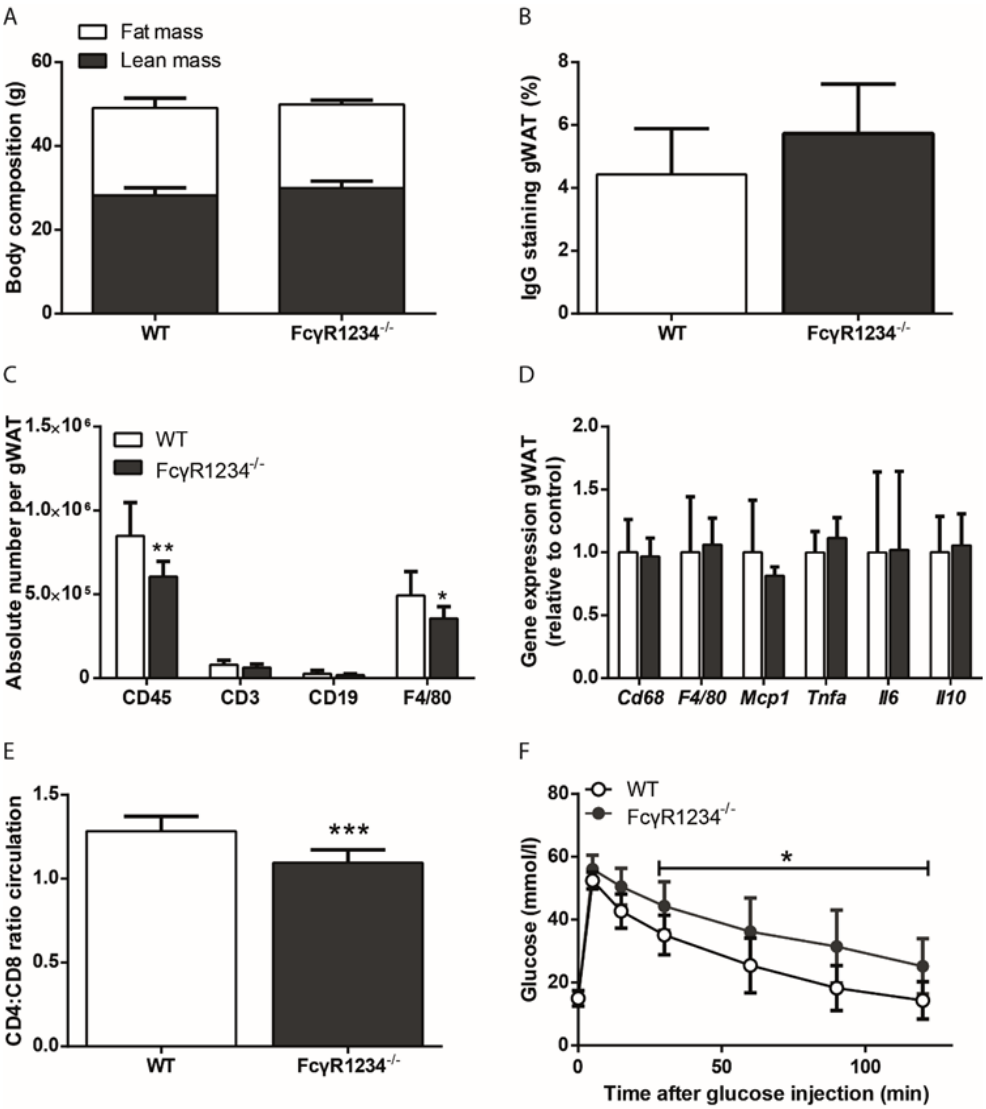

Figure 2. FcγR1234 deficiency has no effect on diet induced obesity, reduces immune cell numbers in gWAT, but deteriorates glucose tolerance.

WT and FcγR1234$^{-/-}$ mice were fed a HFD for 15 weeks to induce obesity (n=8 per group). Body composition was determined (A). IgG staining of gWAT was quantified as % of total area for WT FcγR1234$^{-/-}$ mice (B). Immune cell composition of gWAT was determined by flow cytometry analysis, absolute numbers of leukocytes (CD45), T cells (CD3), B cells (CD19), and macrophages (F4/80) were calculated per gonadal fat pad for WT and FcγR1234$^{-/-}$ mice (C). Expression of inflammatory genes was determined in gWAT from both groups (D). T helper (CD4) and cytotoxic T cell ratio of the circulation determined by flow cytometry analysis (E). After 6 weeks of HFD glucose tolerance was determined via an ivGTT (F). Error bars representing standard deviation of mean (n=8 per group). *p <0.05, **p <0.01, ***p<0.001 compared to WT.

FcγRIIb deficiency has no effect on HFD-induced obesity or immune cell composition in gWAT

To investigate the role of the inhibitory Fc-gamma receptor (FcγRIIb) on the development of diet induced obesity, FcγR2bflox (control group) and FcγR2b-/- mice were fed a HFD for 15 weeks to induce obesity. Both groups gained comparable body weight, with similar lean and fat mass (Fig. 3A). From gWAT, mWAT and sWAT the fat pad weight, and adipocyte size and number was determined for both groups. Although mWAT and sWAT did not differ in weight, adipocyte size and number, gWAT was significantly reduced in weight (-23%, $p<0.001$; Supplementary Fig. 2A-C) in FcγR2b-/- mice. However, this reduction in gWAT weight could not be attributed specifically to a decrease in hypertrophy or hyperplasia, as adipocyte size and number did not differ significantly (Supplementary Fig. 2B, C). Histological characterization of gWAT showed increased IgG in gWAT from FcγR2b-/- compared to control mice (+94%, $p<0.01$; Fig. 3B). Flow cytometry analysis showed comparable immune cell composition in gWAT (Fig. 3C). This was confirmed by comparable inflammatory gene expression in gWAT (Fig. 3D).

FcγRIIb deficiency has no effect on whole body glucose tolerance

Insulin inhibition of lipolysis was measured in gonadal adipocytes isolated after 15 weeks of HFD from FcγR2bflox and FcγR2b-/- mice. Gonadal adipocytes from both groups had comparable inhibition of lipolysis by insulin, which indicates similar insulin responsiveness (Supplementary Fig. 2D). Basal insulin and glucose levels were measured after 6 weeks of HFD and were comparable for FcγR2b-/- and control mice (Supplementary Fig. 2E, F). ivGTT was performed and showed no difference in whole body glucose tolerance between both groups (Fig. 3E).

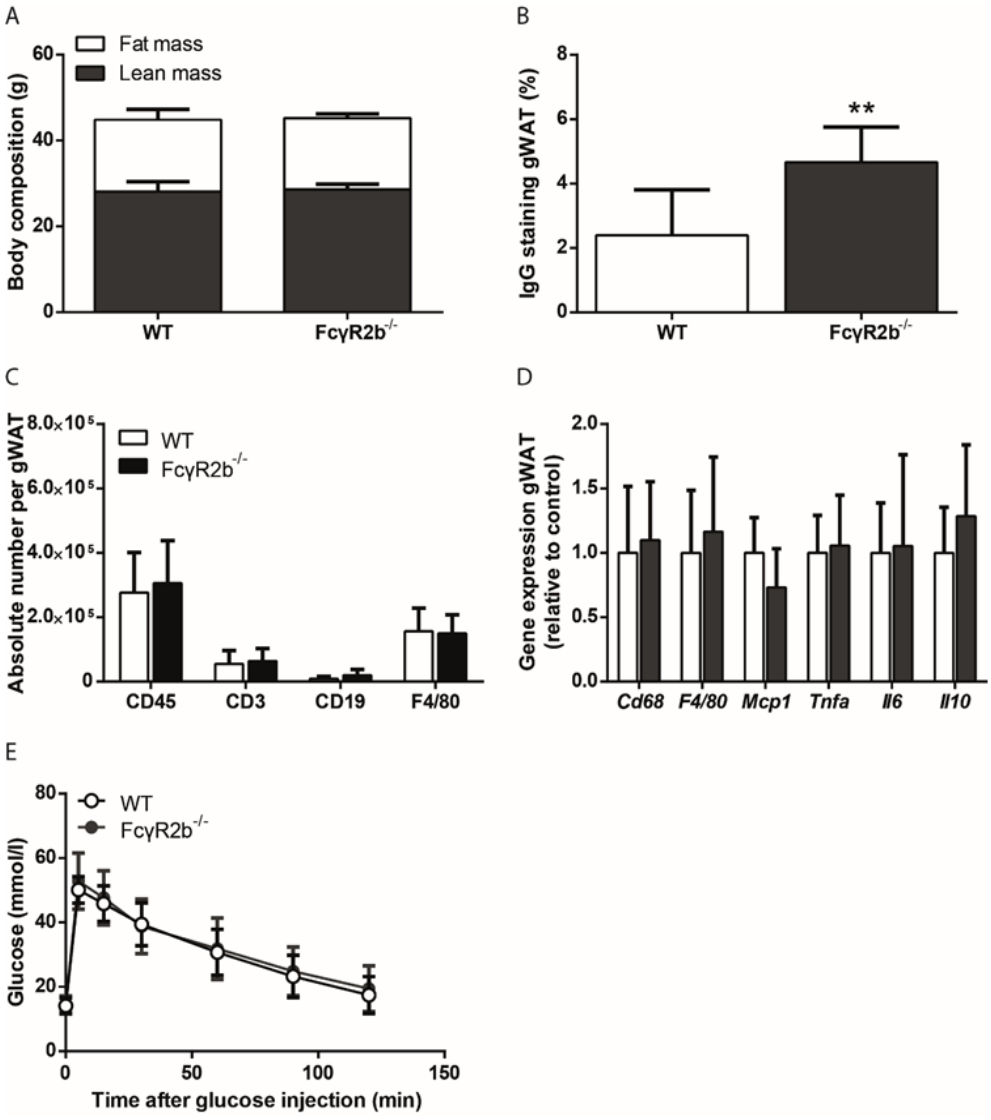

Figure 3. FcγR2b deficiency has no effect on diet induced obesity, increases IgG in gWAT, but has no effect on glucose tolerance.

FcγR2b^{flox} and FcγR2b$^{-/-}$ mice were fed a HFD for 15 weeks to induce obesity (n=12 per group). Body composition was determined (A). IgG staining of gWAT was quantified as % of total area for WT FcγR1234$^{-/-}$ mice (B). Immune cell composition of gWAT was determined by flow cytometry analysis, absolute numbers of leukocytes (CD45), T cells (CD3), B cells (CD19), and macrophages (F4/80) were calculated per gonadal fat pad for FcγR2b^{flox} and FcγR2b$^{-/-}$ mice (C). Expression of inflammatory genes was determined in gWAT from both groups. After 6 weeks of HFD glucose tolerance was determined via an ivGTT (E). Error bars representing standard deviation of mean (n=8 per group). *p <0.05, **p <0.01, ***p<0.001 compared to FcγR2b^{flox}.

Complement C3 deficiency has no effect on HFD-induced obesity or immune cell composition in gWAT

Complement C3-/- and WT mice were fed a HFD to induce obesity. Prior to the HFD-intervention mice were matched on lean mass, as C3-/- mice had a lower lean mass compared to WT mice (-5%, $p<0.01$; data not shown). After 15 weeks of HFD body weight and body composition were comparable (Fig. 4A). Fat pad weights of the different depots (gWAT, mWAT and sWAT) did not differ between the groups, neither did adipocyte size and number (Supplementary Fig. 3A-C). Furthermore, gWAT adipocytes from WT and C3-/- mice showed a comparable inhibition of lipolysis by insulin (Supplementary Fig. 2D). Adipose tissue inflammation was similar as flow cytometry analysis showed comparable immune cell composition (Fig. 4B) and expression of inflammatory genes (Fig. 4C) in gWAT from WT and C3-/- mice.

Complement C3 deficiency leads to increased HFD-induced systemic inflammation and has no effect on whole body glucose tolerance

Absolute leukocyte numbers in the circulation were comparable for WT and C3-/- mice (data not shown). T helper cell were comparable, whereas cytotoxic T cells were increased as percentage of the total T cell population for the C3-/- compared to WT mice (data not shown). Therefore, the CD4:CD8 ratio in the circulation was lower for the C3-/- mice (Fig. 4D), indicating increased systemic inflammation. Glucose and insulin levels were measured in plasma from WT and C3-/- mice after 6 weeks of HFD. Glucose levels were increased (+14%, $p<0.05$; Supplementary Fig. 2E) in the C3-/- mice compared to WT mice, indicating increased insulin resistance. Insulin levels were not significantly different (Supplementary Fig. 2F). By performing an ivGTT, whole body glucose tolerance of WT and C3-/- mice was determined. Glucose tolerance of C3-/- mice was not significantly different (Fig. 4E).

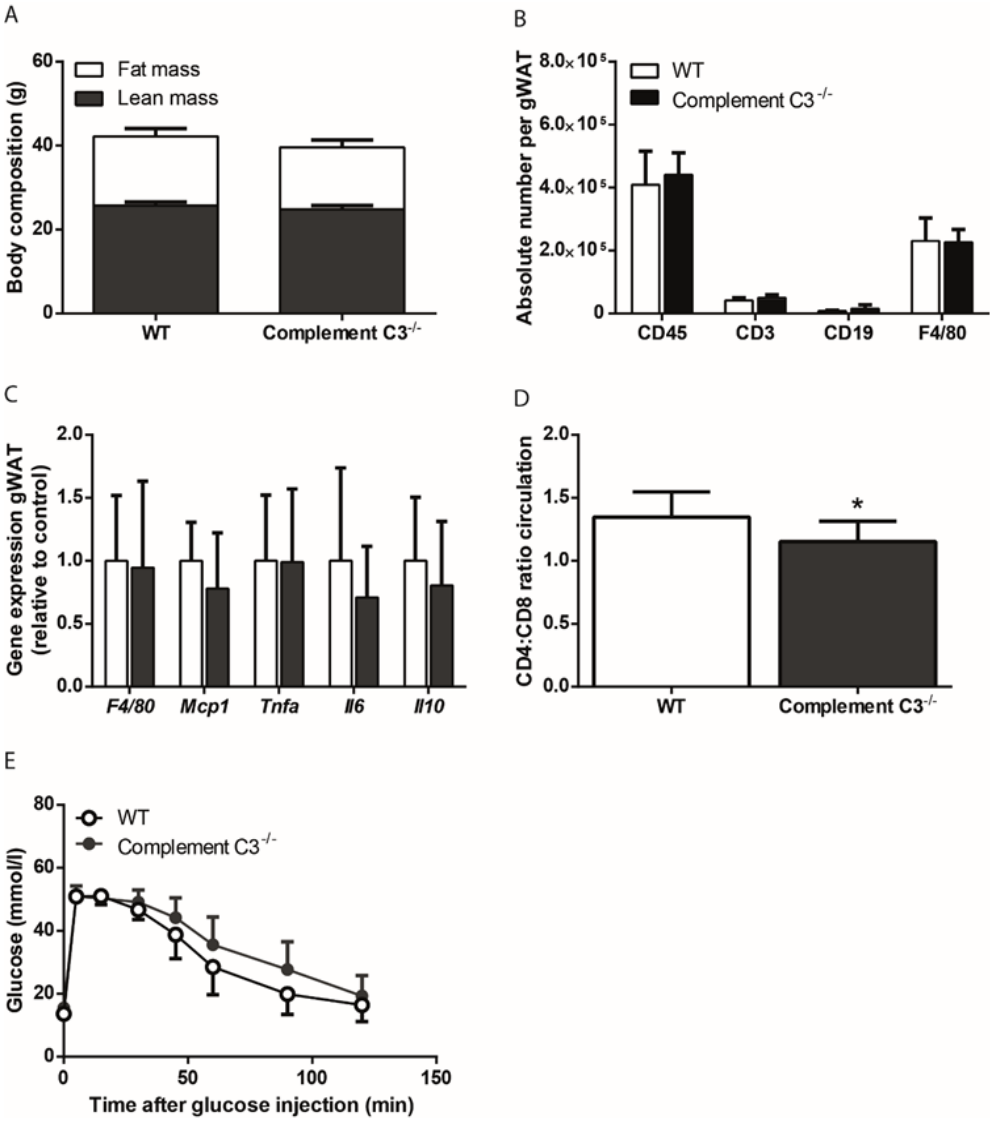

Figure 4. Complement C3 deficiency has no effect on diet induced obesity or adipose tissue inflammation, but slightly reduces glucose tolerance.

WT and Complement C3$^{-/-}$ mice were fed a HFD for 15 weeks to induce obesity (n=12 per group). Body composition was determined (A). Immune cell composition of gWAT was determined by flow cytometry analysis, absolute numbers of leukocytes (CD45), T cells (CD3), B cells (CD19), and macrophages (F4/80) were calculated per gonadal fat pad for WT and FcγR1234$^{-/-}$ mice (B). Expression of inflammatory genes was determined in gWAT from both groups (C). T helper (CD4) and cytotoxic T cell ratio of the circulation determined by flow cytometry analysis (D). After 6 weeks of HFD glucose tolerance was determined via an ivGTT (E). Error bars representing standard deviation of mean (n=8 per group). *p <0.05, **p <0.01, ***p<0.001 compared to WT.

Discussion

During obesity, B cell numbers and IgG antibodies increase in the plasma and the AT and play an important role in the development of IR (4, 10, 20, 21). The current study aimed to determine if Fc-receptors or activation of the complement system are the effector pathway by which IgG antibodies contribute to AT inflammation and IR. If so, we expect to see a beneficial effect on metabolism in either or both the FcγR and complement C3 deficient DIO mouse models. However, our data shows that neither FcγR nor complement C3 deficiency lead to reduced development of IR. Thus, this suggest that if obesity associated IgG antibodies contribute to the development of DIO-associated IR, this is not dependent of FcR or complement mediated pathways.

If obesity associated IgG antibodies activate the FcR or complement pathway and thereby affect insulin sensitivity, it may be that the inflammatory responses that are triggered via these pathways are marginal to the other inflammatory processes that also occur during HFD-induced obesity (e.g. attraction and activation of macrophages by adipocyte derived cytokines). Alternatively, it is possible that the FcR and the complement pathway are redundant. In another disease model, it has previously been shown that solitary FcR or complement deficiency did not exhibit the expected improved effect on IgG mediated pathology (22, 23), whereas a combined deficiency did (22). To establish if a compensatory effect might play a role in our DIO mouse models, we determined activated complement levels in gWAT of WT and FcγR1234-/- mice. Activated complement levels were slightly increased in WAT of FcγR1234-/- mice (data not shown), which hints towards a compensatory mechanism of the IgG mediated pathways. A combined FcγR and complement deficient model would need to be studied to determine whether this compensatory effect plays a role in DIO-associated pathology. Due to the complexity and multitude of loci to be knocked out simultaneously, we have not performed this experiment yet. Nevertheless, our data indicate that solitary FcR or complement deficiency does not improve metabolic health and therefore these pathways would not be effective therapeutic targets to reduce adipose tissue inflammation at least when targeted singularly.

Surprisingly, the FcγR1234-/-and C3-/-mice showed increased basal glucose or insulin levels, and decreased peripheral glucose tolerance, which actually indicates a deteriorated metabolic phenotype. As the Fc-receptors and complement pathway are important for immune complex clearance, there is likely impaired immune complex clearance in the FcγR1234-/-and C3-/- mice, which may lead to pro-inflammatory responses that contribute to the development of IR. Also, both the FcγR1234-/-and C3-/- mice showed higher cytotoxic T cell levels in blood which may indicate systemic inflammation. In both models the decreased glucose tolerance was caused by decreased peripheral glucose uptake rather than glucose production by the liver. As adipocyte functionality was not affected, but peripheral glucose tolerance was, this was most likely caused by decreased muscle glucose tolerance, as muscle is the principle organ storing and using glucose for generation of ATP.

We previously showed that FcRy-chain deficiency reduces the development of DIO and related pathologies (15). This model has diminished IgG and IgE mediated responses, but also exhibits other non-specific effects, as the y-chain is not exclusively associated with Fc-receptors. The FcγR1234-/-

is a Fc-receptor specific mouse model which can explicitly be used to study IgG induced effects. To our knowledge, deficiency of all four FcγR has not been studied yet with regard to DIO. The role of complement in obesity and related disorders has been studied before by others (24, 25). Cianflone et al., indicated the presence of a link between C3adesArg or ASP, which is a complement component produced by adipocytes, and the pathogenesis of obesity (25-27). They studied C3-/- mice for this purpose and observed reduced DIO with increased insulin sensitivity, which is conflicting with our results. An explanation for these contradictory results could be that we have matched our groups on lean mass, as C3-/- mice showed a reduced lean mass. C3-/- mice are known to have a decreased fetal weight (28), which could explain this phenotype. Lean mass is an important determinant of body weight gain during DIO and a reduced lean mass could explain the reduced DIO in C3-/- mice in the study of Cianflone. Additionally, the C3-/- mice they used had a different origin (29) than the mice we have used (14). Other groups previously indicated conflicting data on metabolic phenotype of C3-/- mice (30, 31), which they explained by the influence of the genetic background (32). Mice lacking C1q, the component of activation of the classical pathway of complement, showed improved glucose tolerance despite of a comparable body weight after DIO (33). Furthermore, expression of the C5aR was higher in the AT during obesity (34) whereas deficiency of C5aR led to reduced AT inflammation and IR (34). Taken together, several studies showed different outcomes regarding the role of complement in obesity. Interestingly, it has recently been shown that C5 can be activated in a C3-independent way (35, 36), indicating an additional compensatory pathway in the C3-/- mice.

Determining the antigens to which obesity induced IgG antibodies respond is of high therapeutic value. Winer et al., showed that the antigens in the circulation of obese humans that were associated with IR, were mainly intracellular proteins. However, they did not find specific obesity antigens in the circulation (10). Another group showed elevated IgG plasma levels against specific bacterial antigens in obese patients with diabetes as well as in DIO mice (37). We have attempted to unravel the presence of specific antigens in the obese AT, as this is possibly the source of the antigens, which lead to antibody production. IgG and antigens in AT of human subjects would be of our primary interest, however, as human AT cannot be perfused to eliminate blood from the tissue, the AT will be contaminated with IgG from the circulation which contains high levels of IgG. IgG-immune complexes were isolated from mouse AT by protein A affinity chromatography and measured using mass spectrometry. However, we did not find specific IgG bound antigens in the obese AT of mice (data not shown). IgG peptides were excessively present which made it hard to pick up specific antigen peptides by the method we used. Alternatively, this also suggests the presence of non-protein like antigens to which obesity induced IgG antibodies respond. These could be lipid antigens, which has previously been proposed by others (38, 39).

Since our mass spectrometry analysis of AT IgG detected peptides of the FcgR1, it is tempting to speculate that AT IgG is directly bound to FcRs on macrophages. Surprisingly, IgM was also abundantly present in the eluate of the protein A column. Since protein A is IgG specific, this indicates that IgM was directly bound to IgG or indirectly via an antigen they both bind. A direct interaction between IgG and

IgM antibodies is known to occur during rheumatoid arthritis. The auto-antibody rheumatoid factor acts on the Fc-portion of IgG and thereby forms immune complexes that contribute to the disease progress (40). IgG can also directly interfere with functional proteins, as is known to be happening during auto-immune diseases like myasthenia gravis or limbic encephalitis (reviewed by Huijbers et al. (41)). Interestingly, we found IgG accumulation co-localized with CLS in the AT of FcγR1234-/- mice. This indicates that the presence of IgG around CLS is partly Fc-receptor independent and may indicate direct binding of antibodies to adipocyte components or immune complex deposits.

In conclusion, our data demonstrate an increase of AT IgG with body weight in mice. Deficiency of one of the two downstream IgG mediated pathways, either FcγR-/- or complement C3-/-, has no effect on DIO, did not led to a reduction of AT inflammation and has no beneficial effect on metabolic parameters. Thus, if obesity-induced IgG antibodies contribute to the development of IR, this is not dependent of FcγR or complement mediated pathways.

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Supplemental figures

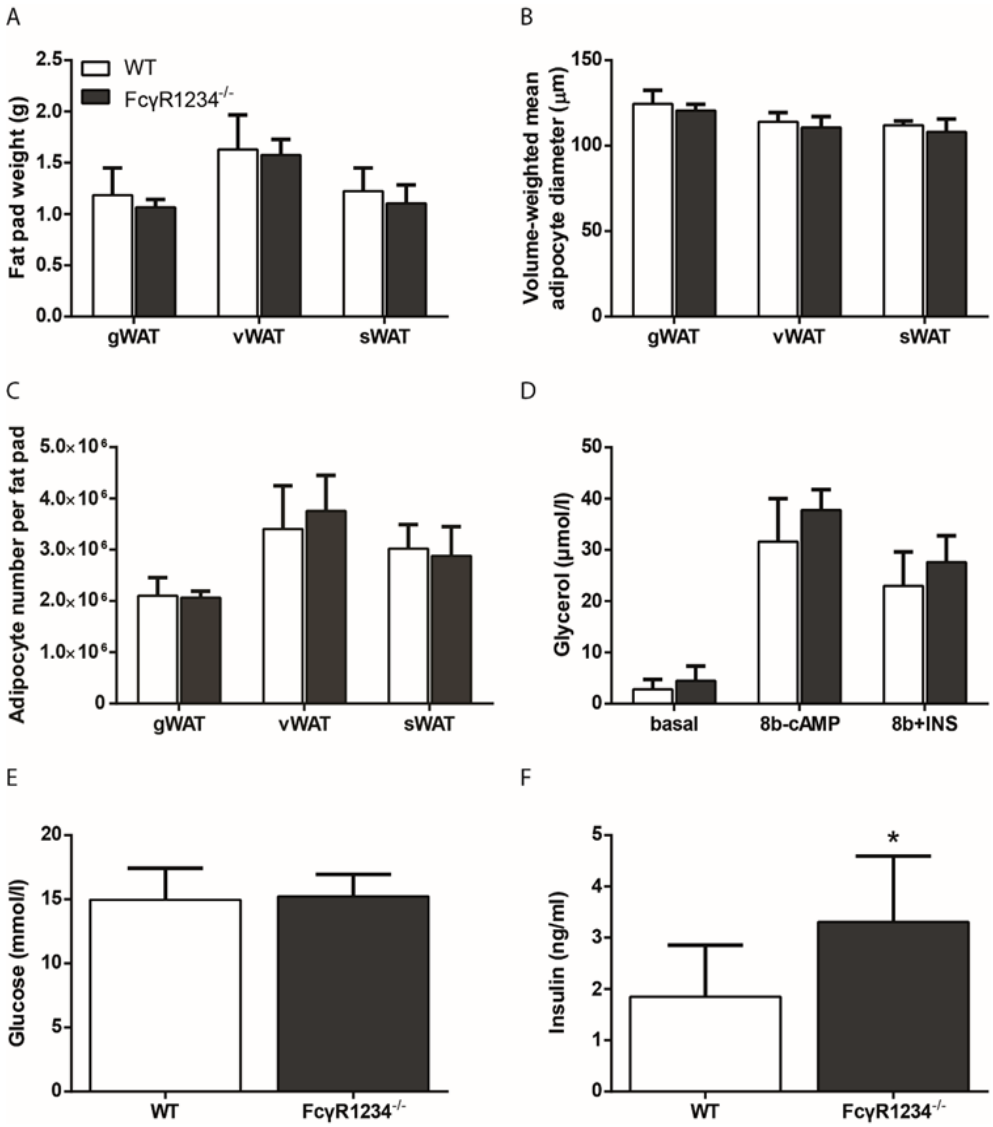

Figure S1. WT and FcγR1234$^{-/-}$ mice were fed a HFD for 15 weeks to induce obesity. Fat pad weight (A), adipocyte size (B) and adipocyte number per fat pad (C) were determined for gWAT, vWAT and sWAT from WT and FcγR1234$^{-/-}$ mice. Basal lipolysis, 8b-cAMP stimulated lipolysis and the insulin inhibition of 8b-cAMP stimulated lipolysis (8b+INS) of gonadal adipocytes were determined (D). Plasma glucose and insulin levels were measured in 6 hour fasted WT and FcγR1234$^{-/-}$ mice, after 6 weeks of HFD. Error bars representing standard deviation of mean (n=8 per group). *p <0.05, **p <0.01, ***p<0.001 compared to WT.

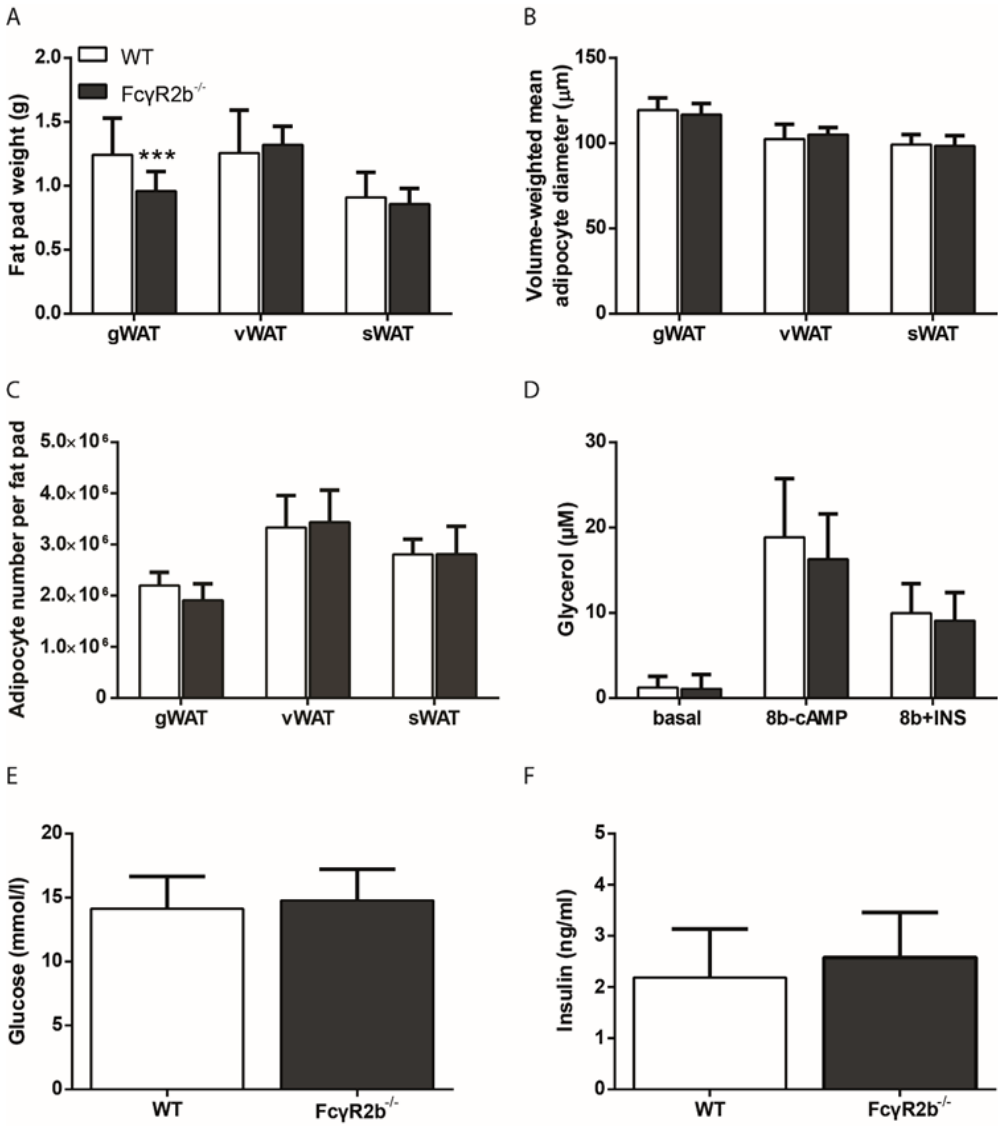

Figure S2. FcγR2b^{flox} and FcγR2b$^{-/-}$ mice were fed a HFD for 15 weeks to induce obesity. Fat pad weight (A), adipocyte size (B) and adipocyte number per fat pad (C) were determined for gWAT, vWAT and sWAT from WT and FcγR1234$^{-/-}$ mice. Basal lipolysis, 8b-cAMP stimulated lipolysis and the insulin inhibition of 8b-cAMP stimulated lipolysis (8b+INS) of gonadal adipocytes were determined (D). Plasma glucose and insulin levels were measured in 6 hour fasted FcγR2b^{flox} and FcγR2b$^{-/-}$ mice, after 6 weeks of HFD. Error bars representing standard deviation of mean (n=12 per group). *p <0.05, **p <0.01, ***p<0.001 compared to FcγR2b^{flox}.

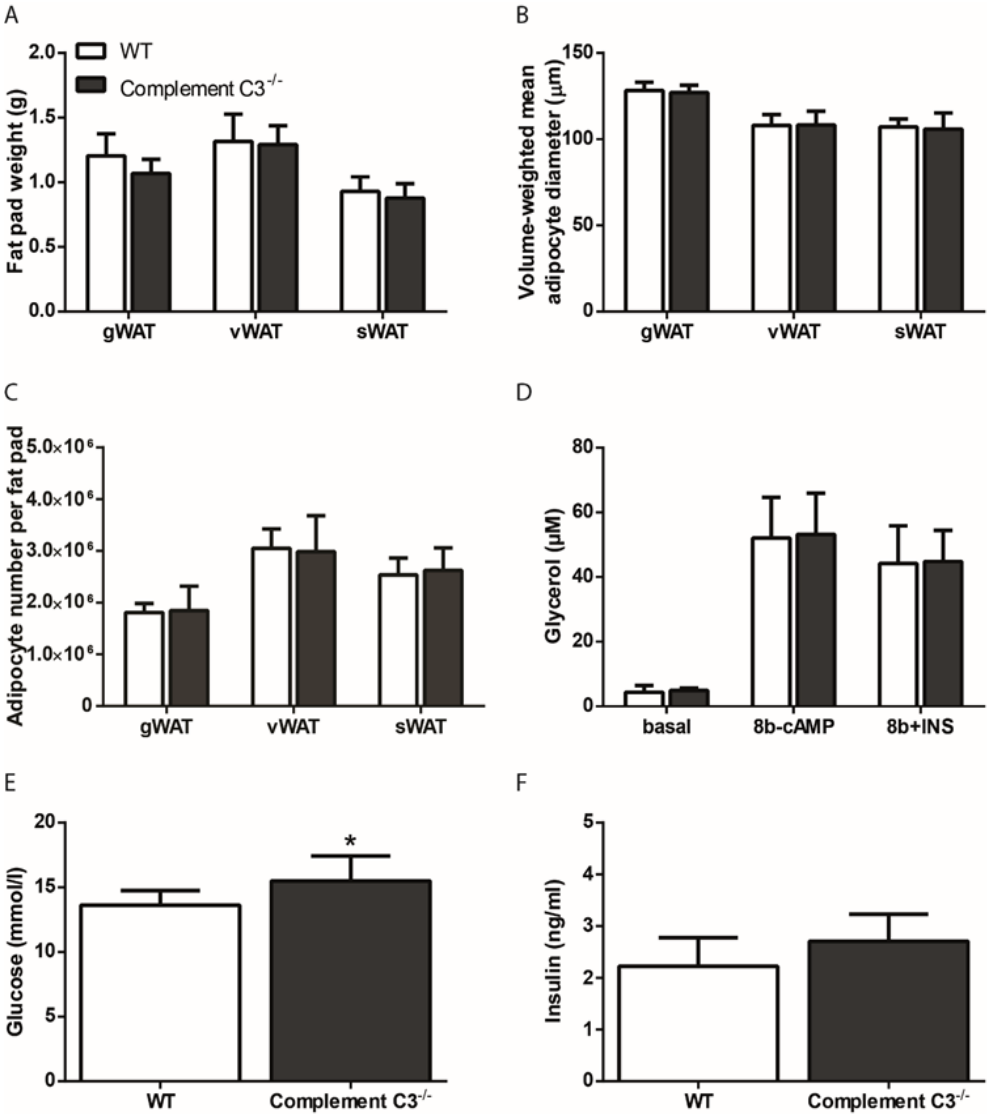

Figure S3. WT and Complement C3$^{-/-}$ mice were fed a HFD for 15 weeks to induce obesity. Fat pad weight (A), adipocyte size (B) and adipocyte number per fat pad (C) were determined for gWAT, vWAT and sWAT from WT and Complement C3$^{-/-}$ mice. Basal lipolysis, 8b-cAMP stimulated lipolysis and the insulin inhibition of 8b-cAMP stimulated lipolysis (8b+INS) of gonadal adipocytes were determined (D). Plasma glucose and insulin levels were measured in 6 hour fasted WT and FcγR1234$^{-/-}$ mice, after 6 weeks of HFD. Error bars representing standard deviation of mean (n=12 per group). *p <0.05, **p <0.01, ***p<0.001 compared to WT.

7

Summarizing discussion

Discussion

As the obesity epidemic is still increasing, strategies to prevent and treat obesity and related pathologies are urgently needed. Obesity-induced inflammation is thought to contribute to the development of metabolic disorders. Therefore, inflammatory pathways that play a role in obesity-induced inflammation are potential promising targets in the treatment of metabolic disorders. Extensive knowledge on obesity-induced inflammation and the role of inflammatory pathways in the development of metabolic disorders can benefit the development of these therapeutic strategies. Mouse models are widely used to study obesity and related disorders. However, to what extent mouse-derived results translate to humans has not been studied extensively yet. Obesity-induced inflammation and its role in the development of insulin resistance, as well as the similarities of these processes between humans and mice have been addressed in this thesis. The new findings described in this thesis will be summarized and discussed in this final chapter. Additionally, clinical implications of obesity-induced inflammation as target to treat metabolic disorders and future perspectives will be addressed.

Summary of the new findings

In this thesis, we determined to what extent metabolic health of obese humans is associated with obesity-related inflammation. WAT depot expandability and composition of the immune cells were determined during the development of obesity in mice, and the immune cell composition of the circulation and WAT were compared between obese humans and mice. Additionally, the role of Fc-receptors and the complement pathway in obesity-induced insulin resistance were studied. The main findings of this thesis are listed below:

- Metabolic health is associated with inflammatory status; obese women with T2DM have increased numbers of circulating leukocytes and higher levels of IL6 in the circulation as compared with metabolically healthy obese women (**Chapter 2**)
- DIO male C57Bl/6J mice exhibit metabolic dysfunction from a body weight of 40 grams onwards, which seems to be directed by the limited storage capacity of gWAT (**Chapter 3**)
- The different WAT depots of male C57Bl/6J mice differ with respect to expandability and immune cell composition (**Chapter 3**)
- The composition of immune cells in the circulation and WAT differs remarkably between obese humans and mice; mice have relatively higher numbers of B cells in both the circulation and WAT (**Chapter 4**)
- The effect of obesity on circulating immune cells shows similarities between humans and mice, including increased activation of lymphocytes and higher numbers of monocytes (**Chapter 4**)
- FcRγ-chain deficiency reduces the development of diet-induced obesity and associated WAT inflammation. This can, at least partly, be explained by increased lean mass and altered gut microbiota of the FcRγ-chain deficient mice (**Chapter 5**)

- IgG in WAT increases with increasing body weight in mice (**Chapter 6**)
- If obesity-induced IgG antibodies contribute to the development of insulin resistance, this is not dependent on FcγR or complement mediated pathways (**Chapter 6**)

Adipocytes in the development of metabolic disorders

Although obesity is known as a primary risk factor for the development of metabolic disorders, 20-25% of obese subjects are seemingly metabolically healthy, and it is not yet known why specifically some people remain healthy whereas others develop T2DM. Metabolically healthy obese subjects are considered as individuals with excessive weight, who are relatively insulin sensitive with a healthy lipid profile including low TG and fasting glucose levels, and who lack any signs of metabolic dysfunction (1). Adipocytes are thought to play an important role in the development of obesity related metabolic disorders. As long as obesity coincides with healthy normal functioning adipocytes, triglycerides (TG) can be properly stored in the adipose tissue and will not accumulate in peripheral tissues. Additionally, healthy non-stressed adipocytes produce less pro-inflammatory mediators compared to enlarged stressed adipocytes. Thus, normal functioning adipocytes that are still able to expand and thus can cope with the excessive lipid load during obesity, may be crucial for metabolic health. However, what are the determinants for adipocytes to expand without getting stressed, and how does this translate to metabolically healthy obesity? Metabolically healthy obese persons might have a genetic advantage that leads to better processing and storage of the increased lipid load. Whether metabolically healthy obese individuals always remain healthy seems questionable. Especially when obesity progresses, at some point the adipocytes will reach their limit for healthy expansion, and this will eventually and unavoidably lead to metabolic dysfunction.

Adipose tissue distribution is known as a major factor in the development of metabolic disorders and varies widely between individuals. Especially vWAT has been associated with the development of metabolic disorders (2), and is thought to exert more negative effects on health as compared with sWAT. Adipose tissue distribution might also contribute to the difference in metabolic health between equally obese individuals, however, the factors determining body fat mass and fat distribution are still largely unknown (3-5). A role for developmental genes in regulating body fat distribution has been suggested by several GWAS studies (6-9). Genetic variation in adipose tissue depot specific expression patterns of developmental transcription factors involved in embryological development, may contribute to differences in proliferation and differentiation capacity of the adipocytes in different depots and regulate fat distribution (10, 11). Inflammatory signals may also regulate adipose tissue depot specific growth and fat distribution. Recently, a role for the inflammatory component NLPR3-inflammasome has been described in the limited expansion capacity of sWAT and the development of insulin resistance in humans. The inflammasome down regulates adipogenesis (12). In **chapter 2** we found no differences in waist circumference, total body fat percentage, or adipocyte size of the different depots between morbidly obese women with and without T2DM. Thus, in our study population, fat distribution did not seem to be associated with metabolic health, or to explain the

difference in inflammation. However, as our study population is already morbidly obese for at least five years, it is possible that fat distribution is an important regulator of metabolic health primarily in the initial phase of the development of obesity. In **chapter 3**, we have determined inter-depot differences regarding adipocyte size and WAT depot expansion during the development of obesity in male C57Bl/6J mice. There we found an association between limited gWAT adipocyte expansion capacity and insulin resistance. This indicates that capacity of the gWAT depot to expand determines the development of metabolic disorders in mice. Whether humans have such a WAT depot expansion capacity threshold for the development of metabolic disorders requires further investigation. As humans have high inter-individual variability due to genetic and environmental variation, a general threshold for the development of metabolic disorders may be challenging to find.

Interestingly, the limited capacity of subcutaneous adipocytes to expand has recently been suggested as a determinant of metabolic disease and adipocyte dysfunction in humans (13). Comparable to what we showed in the gWAT depot in mice (**chapter 3**), in humans the limited expansion capacity of subcutaneous adipocytes seems to result in increased hypertrophy of visceral adipocytes and adipose tissue dysfunction. A previous study determined increased visceral adipose tissue and liver fat as the best predictor for insulin resistance (14). The expansion capacity of adipocytes from the human sWAT depot might therefore be an important factor in the development of metabolic disorders. To address the role of adipose tissue expansion, inflammation, and function in the development of metabolic disorders, longitudinal studies in humans during the development of obesity are crucial.

Adipocytes as a potential therapeutic target to treat metabolic disorders

Increasing the adipocyte plasticity and the adipose tissue expansion capacity might be a way to decrease WAT dysfunction and inflammation during obesity. There are several potential means to improve adipocyte and WAT functioning. The first option would be to increase the expandability of the individual adipocyte, which would lead to increased lipid storage capacity of the adipose tissue, without associated stress. Adipocyte expandability is limited by multiple factors, including intracellular lipid droplet size, oxygen supply and extracellular matrix remodelling (15). Adipocyte specific proteins like Plin1 and Fsp27 have been shown to be crucial for metabolic flexibility and cell growth of the adipocyte (16, 17). For instance, mice deficient for Fsp27 have small adipocytes and associated impaired lipid storage capacity, which leads to hepatic steatosis and insulin resistance upon HFD feeding (18). Fsp27 interacts with adipocyte triglyceride lipase (ATGL) and thus plays a role in the regulation of TG-storage. By increasing adipocyte specific proteins like Fsp27, adipocytes might be able to grow larger.

Another way to increase the lipid storage capacity of the adipose tissue, is to increase the number of adipocytes in the adipose tissue. Adipocyte hyperplasia can only be facilitated by adipogenesis, and thus increasing the number of pre-adipocytes differentiating into adipocytes. Adipocytes are typically derived from adipose tissue resident mesenchymal progenitor cells. Recently, it has been suggested that adipocytes could also be derived by de novo production from bone marrow progenitor cells,

which indicates an alternative lineage involved in adipogenesis (19). These new findings contribute to the possibilities to increase the availability of pre-adipocytes for recruitment in the expanding adipose tissue depots. Both adjustments would improve fat storage in the adipose tissue rather than in peripheral tissues, and additionally would lower the obesity induced adipocyte stress, which both improve metabolic health.

Obesity-induced inflammation in the development of metabolic disorders

In humans, the effect of obesity-associated inflammation has been extensively studied in the adipose tissue itself. Systemic inflammation has primarily been assessed by measuring circulating cytokine levels during obesity. However, the composition of the circulating immune cell pool and activation status thereof has not been examined thoroughly thus far. We performed an extensive analysis of immune cell composition and activation status in the circulation as described in **chapter 2**. Furthermore, metabolically healthy versus unhealthy obesity is poorly understood. We specifically addressed the hypothesis that obesity-induced inflammation is a determinant of the difference in metabolic health between obese individuals. In this thesis, we indeed showed that metabolic health is associated with obesity-related inflammation, as both adipose tissue and systemic inflammation was higher in obese women with T2DM as compared to metabolically healthy obese women (**chapter 2**). However, the factors that lead to the differences in inflammatory status of obese woman with and without T2DM have not been revealed yet.

Previous studies in humans showed increased adipose tissue as well as systemic inflammation during obesity (20-22). In mice, the effect of obesity on adipose tissue inflammation has been extensively studied, however, obesity-induced systemic inflammation has been under-investigated so far, as also reviewed by Ip et al (23). In **chapter 2 and 4** we showed that obesity is associated with increased lymphocyte activation and increased numbers of monocytes in the circulation of both humans and mice. Additionally, we showed higher numbers of T cells, B cells and macrophages in the adipose tissue of mice with increasing obesity (**chapter 3**). It thus seems that systemic inflammation reflects the inflammatory status of the adipose tissue. The local inflammatory response in the adipose tissue during obesity may result in a spillover of inflammatory mediators into the circulation. Cytokines and chemokines from the adipose tissue can be released into the circulation, thereby inducing low-grade systemic inflammation and also promoting inflammation in other tissues.

In addition to inflammatory cytokine from hypertrophic adipocytes, other possible inducers of inflammation are released. Stressed and hypertrophic adipocytes have a disturbed lipid metabolism that can directly contribute to a pro-inflammatory state. Saturated fatty acids derived from or poorly stored by adipocytes are able to increase inflammation by binding to Toll like receptors (24). Furthermore, saturated fatty acids have been shown to increase reactive oxygen species (ROS), which also can stimulate inflammatory pathways, like the inflammasome complex, which in turn results in increased levels of the pro-inflammatory cytokine IL-1β (25).

Recently, the role of the gut microbiota in the development of metabolic disorders received much

attention. It has been shown that obese human subjects have an altered bacterial composition in their gut, compared to lean human subjects (26, 27). Conversely, altering the microbiota of obese humans and mice by transfer from a healthier, lean phenotype, results in a better metabolic phenotype with increased insulin sensitivity (26, 28). This supports a role for the microbiota in the development of obesity induced inflammation and the development of metabolic disorders. Another gut related hypothesis that might contribute to inflammation is the leaky-gut syndrome. It is known that the altered gut microbiota in obesity are associated with changes in the digestion and absorption of food (29). This gut dysbiosis is also thought to lead to increased intestinal permeability, allowing endotoxins from the microbiota to enter the blood stream inducing systemic inflammation.

Antibodies in the development of metabolic disorders

In this thesis, we specifically focused on the role of IgG antibodies in the development of metabolic disorders. We showed in **chapter 2** an increased activation status of circulating B cells in obese women compared to age matched lean women. Furthermore, we found increased levels of IgG antibodies in the adipose tissue with increasing obesity (**chapter 6**). However, we could not prove that IgG antibodies are causative in the development of IR, as described in **chapter 5 and 6**. In addition, it remains unclear to which antigens these obesity-induced antibodies are directed. Stressed and dying adipocytes are suggested as a source of the antigens during obesity. It is generally known that dying adipocytes are surrounded by macrophages, characterized as CLS that phagocytose the cell debris. We showed that IgG antibodies co-localise with CLS, and are thus surrounding dying adipocytes. This co-localization consists of FcR mediated IgG-macrophage interaction, but is also partly FcR independent as we showed in **chapter 6**. This suggest that these antibodies are sampling antigens from these dying adipocytes to induce immune responses. We and others tried to reveal the antigens to which the obesity induced IgG antibodies respond (**chapter 6**, 30), however we were not able to identify specific obesity antigens yet. Winer did show increased IgG antibodies targeting general intracellular components in the circulation of insulin resistant versus insulin sensitive subjects. If the obesity related antigens originate from the AT, it might be a valuable approach to search for the antigens specifically in the human AT rather than in the circulation.

The underlying mechanisms driving obesity induced low-grade chronic inflammation are still poorly understood. Both the adaptive and innate immune responses have been recognized as key players in these inflammatory responses. Since autoantigens have been discovered to be present during obesity, the question arises if T2DM is possibly an autoinflammatory disease and thereby innate immune response driven, or an autoimmune disease and adaptive immunity driven. The presence of obesity related autoantibodies indicates that T2DM and insulin resistance may be autoimmune diseases. T2DM might thus be more similar to type 1 diabetes than originally thought, which is generally considered as a typical autoimmune disease. The innate immune system has also clearly proven its role in obesity induced inflammation. Especially macrophages have been implicated, which are known to take up and process excessive TG as well as to phagocytose dying and dead adipocytes. Both processes are able to

induce immune responses that can be recognized as autoinflammatory. With the current knowledge, I would not consider T2DM as a typical autoimmune nor a typical autoinflammatory disease, as it combines aspects of both.

Inflammation as a potential therapeutic target to treat metabolic disorders

As obesity-induced inflammation is known to contribute to the development of insulin resistance, inflammatory pathways seem promising targets to treat insulin resistance and T2DM. The anti-inflammatory drug aspirin seems to improve multiple metabolic measurements in T2DM patients (32), it may improve insulin sensitivity by preventing serine phosphorylation of the IRS proteins (33). However, the therapeutic value of high-doses of aspirin is limited by its side effects, including gastric ulcer formation and increased risk of bleeding and stroke. Salsalate, which similarly as aspirin belongs to the salicylate class of drugs, is not associated with these side effects. Salsalate is therefore recognized as a useful option in the treatment of insulin resistance and T2DM (34). Since 1876 salsalate has already been suggested as possible treatment for T2DM, and a recent study showed improved metabolic functioning in T2DM patients by salsalate (35). However, until now, salsalate has received limited study as potential treatment for T2DM, and larger clinical trials are needed.

Statins, generally used as lipid-lowering drugs, have been tested in clinical trials for a potential improvement of insulin sensitivity. Treatment with statins reduced cytokines and pro-inflammatory markers in the circulation, though no beneficial effect on glucose metabolism could be observed (36). In contrast, high-dose statin use is associated with increased risk for T2DM (37). Furthermore, blocking the Il1b signaling pathway by administration of neutralizing antibodies or administration of IL1 receptor antagonist does show beneficial effects on glucose metabolism in both obese humans and DIO-mice (38-42). Improved blood glucose levels after treatment of T2DM patients was attributed to enhanced pancreatic β-cell function, however, no effect on insulin sensitivity could be observed (43). TNF blockers did reduce blood glucose levels when used to treat patients with rheumatoid arthritis, as well as marginal effects in obese individuals (44-46). However, anti-TNF treatment has not been effective in improving glucose metabolism in T2DM patients thus far (47-50).

Another anti-inflammatory compound is dexamethasone, a member of glucocorticoid family of drugs which is considered the most effective compound to treat inflammatory diseases. However, dexamethasone therapy was associated with deteriorated insulin sensitivity in a clinical trial with healthy young males (51), likely due to systemic side effects. A strong reduction of inflammation may not only block the adverse, but also the beneficial effects of inflammation on energy metabolism and insulin sensitivity. It is suggested that inflammation regulates energy metabolism in a feedback manner by increasing energy expenditure (52). In a recent study, we studied the glucocorticoid receptor (GR) modulator C108297. This selective GR modulator combines both GR antagonism, which is known to reduce diet-induced obesity and GR agonism, which is known to reduce diet-induced inflammation (53). Selective GR modulation might thereby be a potential strategy to reduce obesity and related insulin resistance.

Anti-inflammatory therapies fail to improve insulin sensitivity in both animal models and clinical trials thus far (31). Failure of these drugs to treat insulin resistance is probably primarily caused by the complexity and comprehensiveness of obesity induced inflammation, with numerous inflammatory signals and immune responses involved. Targeting inflammatory pathways to treat T2DM seems more difficult than suggested, which may be explained by the fact that inflammatory pathways are involved in almost all physiological processes and diseases. Furthermore, it could also be risky to alter inflammatory responses, as the capacity to destroy invading pathogens may also be affected by anti-inflammatory agents. To circumvent interfering with general inflammatory processes in the body, targeting the inducer of obesity related inflammation instead of the inflammatory pathways itself, might be a relevant manner in the treatment of metabolic disorders. In this way, the obesity induced inflammation and thus the development of metabolic disorders can be diminished without affecting general inflammatory processes. This strengthens the value for identifying the antigens or other inducers of obesity related inflammation. Future studies should focus on unravelling the source and type of the inducers of obesity related inflammation.

Diet-induced obese mouse models to study metabolic disorders in humans

In this thesis, we have addressed mouse to human translatability, studying to what extent the situation in mice mimics the situation in humans in terms of WAT inflammation and development of insulin resistance during obesity. The expansion and immune cell composition of the different WAT depots during the development of obesity in mice has been extensively described in **chapter 3**. We found that the composition of the different WAT depots was very different, however, with increasing obesity, all depots had increasing numbers of macrophages, primarily of the pro-inflammatory M1 type. It is difficult to determine the immune cell populations in human adipose tissue over time with progressing obesity. We were therefore not able to perform a one to one comparison of the effect of obesity on the AT expansion and composition. However, we did compare systemic inflammation between lean and obese humans and mice (**chapter 4**). Even though the immune cell composition of the circulation is different between humans and mice, they both show increased lymphocyte activation and higher numbers of monocytes in the circulation in the obese condition. This indicates a similar effect of obesity on the immune cells, and thus a comparable obesity-induced inflammatory response in humans and mice. DIO mouse models can therefore be considered as a useful tool in the field of obesity research regarding obesity related inflammation.

We showed in **chapter 3** that gWAT is the depot to be primarily affected as well as the depot that shows an increase in all immune cell subtypes during the development of obesity. According to these data, the gWAT depot is most suitable to be analysed when studying the underlying mechanisms behind obesity induced WAT inflammation in mice. When studying the effect of immunological pathways on obesity-related disorders, immune deficient mouse models and immunological modulators might, next to changing immunological pathways, also affect body weight. This makes it very difficult to interpret the obesity-related results. When studying WAT inflammation and insulin resistance in mice,

differences in body weight should be recognized and if possible corrected for as this is an important confounding factor.

In this thesis, we have specifically focussed on the role of immunoglobulins (Ig) during obesity and primarily on IgG antibodies and related pathways, including the Fc-receptor pathway and complement system. For this purpose, we have used genetically modified mouse models as a tool to study the role of immune cells or immunological pathways in the development of obesity related inflammation. It has been shown that circulating IgG antibodies increase with obesity in mice (30). We additionally showed increased IgG in the adipose tissue of mice with increasing body weight (**chapter 6**). We found that mice have a much higher number of B cells in the circulation and the adipose tissue compared to humans (**chapter 4**). As IgG antibodies are produced by B cells, it is possible that the IgG effect induced by obesity is mouse specific, and may be of less importance in the human situation. However, although the number of B cells in the circulation is lower in humans, we did observe increased numbers of activated B cells in the human circulation with obesity (**chapter 2**). Furthermore, it has been shown that obesity increases systemic IgG levels in humans as well (54). Whether IgG plays a role in both mice and humans during obesity thus requires further investigation.

Concluding remarks

Thus far, the mechanisms driving obesity-indSuced inflammation and the contribution thereof to metabolic disorders are not fully understood. This thesis described obesity-induced systemic and WAT inflammation in mice, and showed comparable effects of obesity on circulating immune cells between humans and mice. Furthermore, we determined that inflammatory status is associated with metabolic health in obese human subjects. Despite the fact that human studies are more difficult to carry out, costly and time consuming, they are crucial for a proper understanding of human pathology and to find potential targets to treat metabolic diseases. Therefore, more effort should be put into performing large scale longitudinal clinical trials focussing simultaneously on systemic and WAT inflammation in lean and obese subjects, with and without metabolic disorders. Longitudinal studies might eventually provide us with insight in the starting point of adipose tissue dysfunction and metabolic disorders in humans and they might provide clues towards the driver(s) of these processes. Nevertheless, mouse models have contributed greatly to our current knowledge. The data described in this thesis indicates a similar response of immune cells from humans and mice in obesity, which highlights the value and relevance of animal models in obesity research. In my view, mechanistic animal studies and human clinical trials are highly complementary and should be performed side by side to improve relevance and applicability for human therapeutic target discovery.

In conclusion, although ideally diet and exercise would be the best way to prevent obesity and metabolic disorders, this is obviously not effective in reducing obesity in our current society. Immune cells as well as inflammatory factors that have been shown to contribute to obesity related pathologies seem attractive targets in the fight against obesity and metabolic disorders. However, as the immune system is a very delicate and complex system comprising interaction and compensation from other

inflammatory pathways, obesity-induced inflammation might be too ambitious as a target. Therefore, I suggest that instead of broadly decreasing inflammatory responses, targeting the inducers of the inflammation would be a more promising approach to decrease obesity related inflammation and metabolic disorders. Our data suggest that obesity related antigens represent novel targets to treat obesity related insulin resistance.

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Addendum

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Samenvatting

Obesitas en metabole ziekten

Het aantal mensen met obesitas neemt wereldwijd alsmaar toe. Obesitas is een medische aandoening waarbij zoveel lichaamsvet is opgehoopt dat dit een negatief effect op de gezondheid kan hebben. Overmatige vetstapeling wordt veroorzaakt door een verstoorde energiebalans. Wanneer de energie inname groter is dan het verbruik zal dit leiden tot gewichtstoename en als dit aanhoudt uiteindelijk tot obesitas. Personen met een body mass index (BMI) hoger dan 30 kg/m^2 worden beschouwd als obees. BMI wordt berekend door het lichaamsgewicht (kg) te delen door de lengte (m) in het kwadraat. Obesitas is de hoofdoorzaak van de ontwikkeling van het metabool syndroom, wat gedefinieerd wordt door de gecombineerde aanwezigheid van een serie gezondheidsrisico's, zoals abdominale obesitas (grote buikomtrek), verhoogde bloedsuikerspiegels, verhoogde bloeddruk en dyslipidemie (negatieve verandering van cholesterol en triglyceriden in het bloed). Het metabool syndroom is vooral een belangrijke risicofactor voor het ontstaan van type 2 diabetes en cardiovasculaire ziekten.

Het witte vetweefsel speelt een belangrijke rol in de ontwikkeling van het metabool syndroom. Onder normale omstandigheden wordt energieoverschot opgeslagen in het witte vetweefsel tot dat het nodig is voor gebruik door het lichaam. Echter, wanneer de energie inname het verbruik langdurig blijft overschrijden, zal het vetweefsel zich moeten uitbreiden om de overmaat aan energie op te slaan. Bij de vergroting van de opslagcapaciteit, groeien de vetcellen en dus ook de vetweefsels. Op een gegeven moment is het maximum bereikt en kan de overmaat aan energie niet meer efficiënt in het vetweefsel worden opgeslagen. Het vet wordt dan ook in andere organen opgeslagen zoals in de lever en spier. Dit staat bekend als ectopische vetopslag, oftewel vetopslag op plekken waar het normaal gesproken niet gebeurt. Ectopische vetopslag verstoort het normale metabolisme in het betreffende weefsel en verhoogt het risico op het ontwikkelen van insulineresistentie. Dit is een conditie waarbij organen als de lever, spier en het witte vetweefsel niet meer goed reageren op insuline. Insuline is een hormoon wat geproduceerd wordt door de beta-cellen in de alvleesklier na een maaltijd en reguleert de glucose spiegels in het lichaam. Wanneer weefsels insuline resistent zijn, is er meer insuline nodig voor de opname van glucose door deze organen en als gevolg zal de alvleesklier meer insuline produceren. Als deze conditie lange tijd aanhoudt zullen de beta-cellen van de alvleesklier uitgeput raken. Ze kunnen dan niet meer voldoende insuline produceren om de glucose levels in het bloed constant te houden, resulterend in hoge bloedsuikerspiegels. Disfunctionele beta-cellen zijn de directe oorzaak van type 2 diabetes mellitus, ook wel bekend als ouderdoms-suikerziekte.

Obesitas-geïnduceerde ontsteking en type 2 diabetes

Tijdens de ontwikkeling van obesitas kan de groei van het witte vetweefsel op twee manieren plaatsvinden. In eerste instantie expanderen (groeien) de al aanwezige vetcellen. Wanneer dit niet voldoende bijdraagt aan het vergroten van de vetopslagcapaciteit, zal mogelijk ook het aantal vetcellen toenemen doordat er nieuwe vetcellen worden aangemaakt. Het expanderen van witte vetcellen tijdens de ontwikkeling van obesitas is niet zonder gevolg. Te grote vetcellen ondervinden stress,

onder andere doordat de zuurstofvoorziening naar grote cellen minder efficiënt is. Dit resulteert in het afgeven van ontstekingssignalen door deze cellen. Deze signalen trekken nieuwe immuuncellen aan en activeren aanwezige immuuncellen in het vetweefsel, die nog meer ontstekingssignalen afgeven en de ontsteking verergeren. Doordat de ontstekingsfactoren vanuit het vetweefsel naar het bloed doorvloeien, zal langdurige vetweefselontsteking uiteindelijk systemische (in het bloed) ontsteking veroorzaken. Obesitas-geïnduceerde vetweefsel en systemische ontsteking spelen een belangrijke rol in de ontwikkeling van insulineresistentie en type 2 diabetes.

Onderzoek beschreven in dit proefschrift

In dit proefschrift is obesitas-geïnduceerde vetweefsel en systemische ontsteking bestudeerd. De rol hiervan in de ontwikkeling van insulineresistentie en type 2 diabetes is in zowel muis als mens onderzocht en de resultaten zijn beschreven in 5 hoofdstukken.

Hoewel obesitas sterk geassocieerd is met de ontwikkeling van insulineresistentie en type 2 diabetes, blijft een deel van de obese populatie relatief metabool gezond. In **hoofdstuk 2** is onderzocht of obesitas-geïnduceerde ontsteking geassocieerd kan worden met de mate van metabole gezondheid. Hiervoor zijn systemische en vetweefselontsteking bestudeerd in obese vrouwen met en zonder type 2 diabetes. Obese vrouwen met type 2 diabetes hadden verhoogde aantallen witte bloedcellen (immuuncellen) en de inflammatoire (ontstekings) cytokine, interleukine-6, in het bloed. Daarnaast hadden ze in het vetweefsel ook meer macrofagen (type witte bloed cel) in “crown-like structures” (meerdere macrofagen rondom een doodgaande of dode vetcel). We hebben in dit hoofdstuk dus verhoogde systemische én vetweefselontsteking gevonden in obese vrouwen met type 2 diabetes vergeleken met obese vrouwen met normale glucose tolerantie. Dit wijst erop dat ontsteking mogelijk een bepalende factor is in het wel dan niet ontwikkelen van type 2 diabetes tijdens obesitas.

In **hoofdstuk 3** zijn verschillende vetweefseldepots gekarakteriseerd tijdens de ontwikkeling van obesitas in muizen. De verschillen in groei en immuuncelinstroom tijdens gewichtstoename is hier beschreven voor de verschillende vetweefseldepots. In mannetjes muizen met een gewicht variërend van 25 tot 60 gram zijn drie verschillende vetweefseldepots geïsoleerd en geanalyseerd; subcutaan (onderhuids), gonadaal (rondom de testis) en visceraal (tussen de organen) vetweefsel. Subcutaan en visceraal vetweefsel blijft groeien tijdens gewichtstoename. Ook het gonadale vetweefsel groeit tijdens de eerste fase van de ontwikkeling van obesitas. Echter vanaf een lichaamsgewicht van 40 gram lijkt het gewicht van het gonadale vetweefsel depot een maximale grootte bereikt te hebben. Vanaf dat moment ontstaan er “crown-like structures” in het gonadale vetweefsel, als ook leververvetting en insulineresistentie. De vetweefseldepots verschillen niet alleen in groei, maar er zijn ook grote verschillen in immuuncelcompositie tussen de verschillende depots zoals beschreven in dit hoofdstuk. Een lichaamsgewicht van 40 gram blijkt een belangrijk omslagpunt te zijn in muizen en het bereiken van de opslag limiet van het gonadale vetweefsel gaat gepaard met metabole dysfunctie.

Obesitas-geassocieerde ontsteking wordt vaak bestudeerd in obese muizen die een hoog vet dieet hebben gehad. Het is echter onduidelijk in hoeverre vetweefsel en systemische ontsteking vergelijkbaar

zijn tussen muis en mens. **Hoofdstuk 4** beschrijft welke immuuncellen en hoe veel er voorkomen in het bloed en het vetweefsel van obese muizen en mensen. Hiervoor zijn verschillende vetweefseldepots vergeleken van muis en mens. Onze resultaten geven aan dat er significante verschillen zijn tussen muis en mens. Ook in het bloed vonden we andere hoeveelheden van bepaalde typen immuuncellen. Desondanks was het effect van obesitas op de activatie van circulerende immuuncellen vergelijkbaar tussen muis en mens.

Het lichaam beschikt over verschillende verdedigingssystemen om ziekteverwekkers (pathogenen) te bestrijden, namelijk het aangeboren en het adaptieve (verworven) immuunsysteem. Beide afweersystemen zijn belangrijk in het ontstaan van ontsteking als gevolg van obesitas. B-cellen zijn onderdeel van het adaptieve immuunsysteem en produceren antilichamen (immunoglobulines; Ig) tegen specifieke antigenen. Tijdens de ontwikkeling van obesitas worden pathogene Ig geproduceerd, welke bijdragen aan het ontstaan van insulineresistentie. Ig kunnen een immuunrespons teweegbrengen door immuuncelactivatie via Fc-receptoren (FcR), of via activatie van het complementsysteem. Specifieke Ig binden aan hun bijbehorende FcR (bv IgG bindt aan FcγR), welke zich bevinden zich op cellen van het aangeboren immuunsysteem (zoals macrofagen). Het complementsysteem bestaat uit een groep eiwitten die na activatie een kettingreactie veroorzaken die belangrijk is voor immuuncel activatie. In **hoofdstuk 5** is de rol van de FcRγ-chain in de ontwikkeling van dieet-geïnduceerde obesitas en gerelateerde metabole dysfunctie onderzocht. De FcRγ-chain is een essentieel onderdeel van onder andere de FcR, en muizen die de FcRγ-chain missen, hebben verschillende niet-functionele FcR. Hierdoor hebben ze verminderde IgG en IgE antilichaam gemedieerde responsen. We hebben gevonden dat FcRγ-chain deficiënte muizen beschermd zijn tegen dieet-geïnduceerde obesitas, als ook geassocieerde vetweefselontsteking en insulineresistentie. Deze resultaten suggereren dat FcRγ-chain gemedieerde mechanismen betrokken zijn bij de ontwikkeling van obesitas en insulineresistentie. Deze mechanismen vormen hiermee een mogelijk aangrijpingspunt voor nieuwe therapieën tegen obesitas en type 2 diabetes.

In **hoofdstuk 6** is verder onderzocht via welke specifieke FcR obesitas geassocieerde IgG antilichamen mogelijk bijdragen aan insulineresistentie. Daarnaast hebben we ook onderzocht of het complementsysteem mogelijk een rol speelt. Hiervoor zijn FcγR1234, FcγR2b en complement C3 deficiënte muizen bestudeerd tijdens de ontwikkeling van dieet-geïnduceerde obesitas. FcγR1234 deficiënte muizen missen alle FcR voor bijbehorende IgG en hebben daardoor geen IgG gemedieerde immuunresponsen. FcγR2b deficiënte muizen missen alleen de remmende FcγR wat normaalgesproken de IgG productie afremt. En C3 deficiënte muizen missen de centrale component C3 van complement, waardoor ze niet beschikken over een functioneel complementsysteem. Het missen van FcγR of C3 in deze muizen resulteerde niet in verminderde vetweefselontsteking of insulineresistentie. Dit suggereert dat wanneer obesitas geassocieerde IgG antilichamen daadwerkelijk een rol spelen in insulineresistentie, de werking hiervan niet afhankelijk is van FcγR of complement-gemedieerde reacties. Mogelijk is er sprake van een compensatiesysteem, waardoor beide mechanismen (FcR en complementsysteem) elkaars functies kunnen aanvullen en overnemen.

Samengevat laten de studies beschreven in dit proefschrift zien dat obesitas geassocieerde ontsteking een belangrijke rol speelt in de ontwikkeling van insulineresistentie. Ontsteking is mogelijk een bepalende factor voor metabole gezondheid tijdens obesitas. Het effect van obesitas op de verschillende vetweefsel depots in muis, als ook het verschil in vetweefsel en systemische ontsteking tussen muis en mens is nader bestudeerd en beschreven. Ook is de bijdrage van verschillende ontstekingsroutes aan de ontwikkeling van obesitas en metabole ziekten bestudeerd in verschillende immuundeficiënte muis modellen. Obesitas-geïnduceerde ontsteking, en dan vooral de factoren die de ontsteking veroorzaken, zijn een potentieel therapeutisch aangrijpingspunt in het gevecht tegen obesitas en gerelateerde ziekten.

List of Publications

BCG lowers plasma cholesterol levels and delays atherosclerotic lesion progression in mice.
Andrea D van Dam, Siroon Bekkering, Malou Crasborn, Lianne van Beek, Susan M van den Berg, Frank Vrieling, Simone A Joosten, Vanessa van Harmelen, Menno PJ de Winther, Dieter Lütjohann, Esther Lutgens, Mariëtte R Boon, Niels P Riksen, Patrick CN Rensen, Jimmy FP Berbée
Atherosclerosis, 2016

Identification of a selective glucocorticoid receptor modulator that prevents both diet-induced obesity and inflammation.
José K van den Heuvel, Mariëtte R Boon, Ingmar van Hengel, Emma Peschier-van der Put, Lianne van Beek, Vanessa van Harmelen, Ko Willems van Dijk, Alberto M Pereira, Hazel Hunt, Joseph K Belanoff, Patrick CN Rensen, Onno C Meijer
British Journal of Pharmacology, 2016

FcRγ-chain deficiency reduces the development of diet-induced obesity.
Lianne van Beek, Irene OCM Vroegrijk, Saeed Katiraei, Mattijs M Heemskerk, Andrea D van Dam, Sander Kooijman, Patrick CN Rensen, Frits Koning, J Sjef Verbeek, Ko Willems van Dijk, Vanessa van Harmelen
Obesity, 2015

Splenic autonomic denervation increases inflammatory status, but does not aggravate atherosclerotic lesion development.
Sander Kooijman, Illiana Meurs, Lianne van Beek, P Padmini SJ Khedoe, Annabel Giezekamp, Karin Pike-Overzet, Cathy Cailotto, Guy Boeckxstaens, Jimmy FP Berbée, Patrick CN Rensen
AJP Heart and Circulatory Physiology, 2015

The limited storage capacity of gonadal adipose tissue directs the development of metabolic disorders in male C57Bl/6J mice.
Lianne van Beek, Jan B van Klinken, Amanda CM Pronk, Andrea D van Dam, Eline Dirven, Patrick CN Rensen, Frits Koning, Ko Willems van Dijk, Vanessa van Harmelen
Diabetologia, 2015

Chronic helminth infection and helminth-derived egg antigens promote adipose tissue M2 macrophages and improve insulin sensitivity in obese mice.
Leonie Hussaarts, Noemí García-Tardón, Lianne van Beek, Mattijs M Heemskerk, Simone Haeberlein, Gerard C van der Zon, Arifa Ozir-Fazalalikhan, Jimmy FP Berbée, Ko Willems van Dijk, Vanessa van Harmelen, Maria Yazdanbakhsh, Bruno Guigas
The FASEB Journal, 2015

Short-term high-fat diet increases macrophage markers in skeletal muscle accompanied by impaired insulin signaling in healthy male subjects.
Mariette R Boon, Leontine EH Bakker, Mariëlle C Haks, Edwin Quinten, Gert Schaart, Lianne van Beek, Yanan Wang, Linda van Schinkel, Vanessa van Harmelen, A Edo Meinders, Tom HM Ottenhoff, Ko Willems van Dijk, Bruno Guigas, Ingrid M Jazet, Patrick CN Rensen
Clinical Science, 2014

Mannose-Binding Lectin is required for the effective clearance of apoptotic cells by adipose tissue macrophages during obesity.
Rinke Stienstra, Wieneke Dijk, Lianne van Beek, Henry Jansen, Mattijs Heemskerk, Riekelt H Houtkooper, Simone Denis, Vanessa van Harmelen, Ko Willems van Dijk, Cees J Tack, Sander Kersten
Diabetes, 2014

Increased systemic and adipose tissue inflammation differentiates obese women with T2DM from obese women with normal glucose tolerance.
Lianne van Beek, Mirjam A Lips, Annemieke Visser, Hanno Pijl, Andreea Ioan-Facsinay, René Toes, Frits J Berends, Ko Willems van Dijk, Frits Koning, Vanessa van Harmelen
Metabolism, 2014

Comparison of osteoblast and cardiomyocyte differentiation in the embryonic stem cell test for predicting embryotoxicity in vivo.
Esther de Jong, Lianne van Beek, Aldert H Piersma
Reproductive Toxicology, 2014

Osteoblast differentiation of murine embryonic stem cells as a model to study the embryotoxic effect of compounds.
Esther de Jong, Lianne van Beek, Aldert H Piersma
Toxicology In Vitro, 2012

Curriculum Vitae

Lianne van Beek werd geboren op 22 juli 1987 te Arnhem. In 2005 behaalde zij haar vwo diploma aan het Olympus college te Arnhem. In september van dat jaar begon zij aan de studie Biomedische Wetenschappen aan de Radboud Universiteit, te Nijmegen. Na het afronden van de Bachelor startte zij de Master Toxicologie.

Tijdens de masteropleiding heeft zij een onderzoeksstage gedaan bij de afdeling Biomedical Sciences aan de universiteit van Bradford (UK), onder begeleiding van dr. Martin Brinkworth. Lianne deed daar onderzoek naar apoptose van mannelijke geslachtscellen, na embryonale *N*-ethyl-*N*-nitroso-ureum (ENU) blootstelling in muizen. Haar afstudeerstage was bij het laboratorium voor Gezondheidsbeschermingsonderzoek (GBO) van het Rijksinstituut voor Volksgezondheid en Milieu (RIVM). Zij onderzocht het effect van triazolen op osteoblast differentiatie van embryonale stamcellen. Dit onderzoek werd uitgevoerd onder begeleiding van prof. dr. Aldert Piersma en dr. Esther de Jong. In 2011 haalde zij haar Master of Science-diploma.

In juni 2011 is Lianne begonnen met haar promotieonderzoek bij de afdeling Humane Genetica van het Leids Universitair Medisch Centrum, onder begeleiding van prof. dr. Ko Willems van Dijk, prof. dr. Frits Koning en dr. Vanessa van Harmelen. De resultaten van het promotieonderzoek zijn beschreven in dit proefschrift getiteld “Systemic and adipose tissue inflammation in obesity and insulin resistance”. Sinds 1 oktober 2015 werkt Lianne als scientist bij Charles River, te Leiden.

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