Systemic and white adipose tissue inflammation in obesity and insulin resistance

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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.

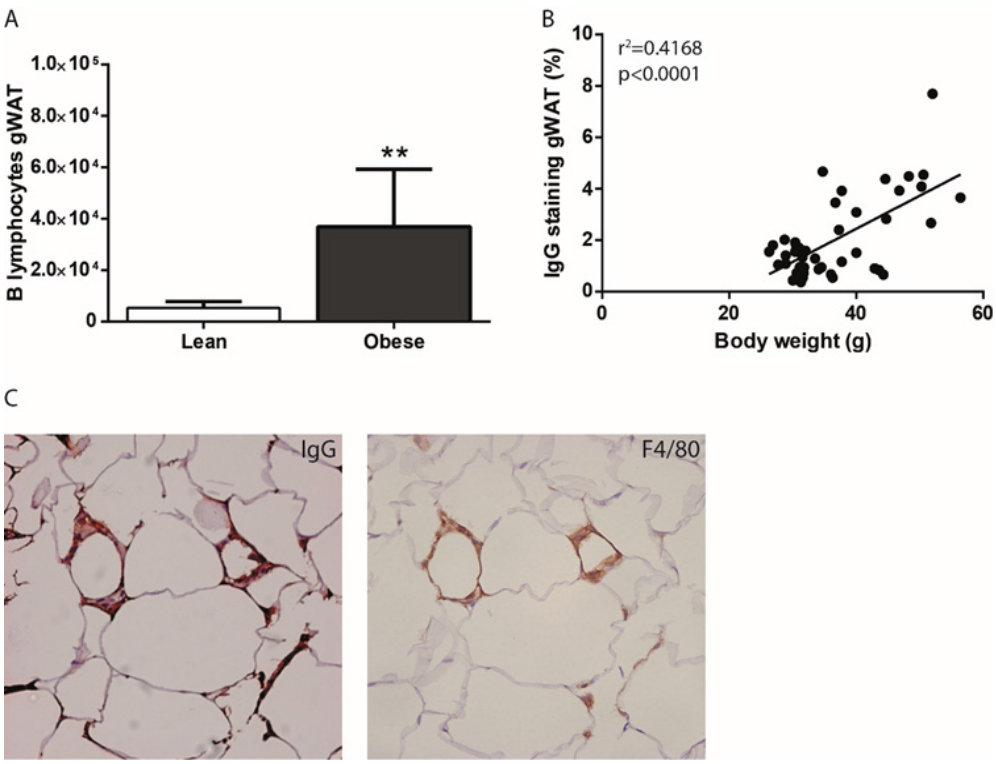

Figure 1. B cell numbers and IgG in gWAT increase during HFD-induced obesity.
WT mice were fed a HFD to induce obesity. Absolute B lymphocyte numbers were determined by flow cytometry analysis of gWAT from lean (<30 g, n=7) and obese (>40 g, n=14) WT mice (A). AT IgG was determined following histological 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).

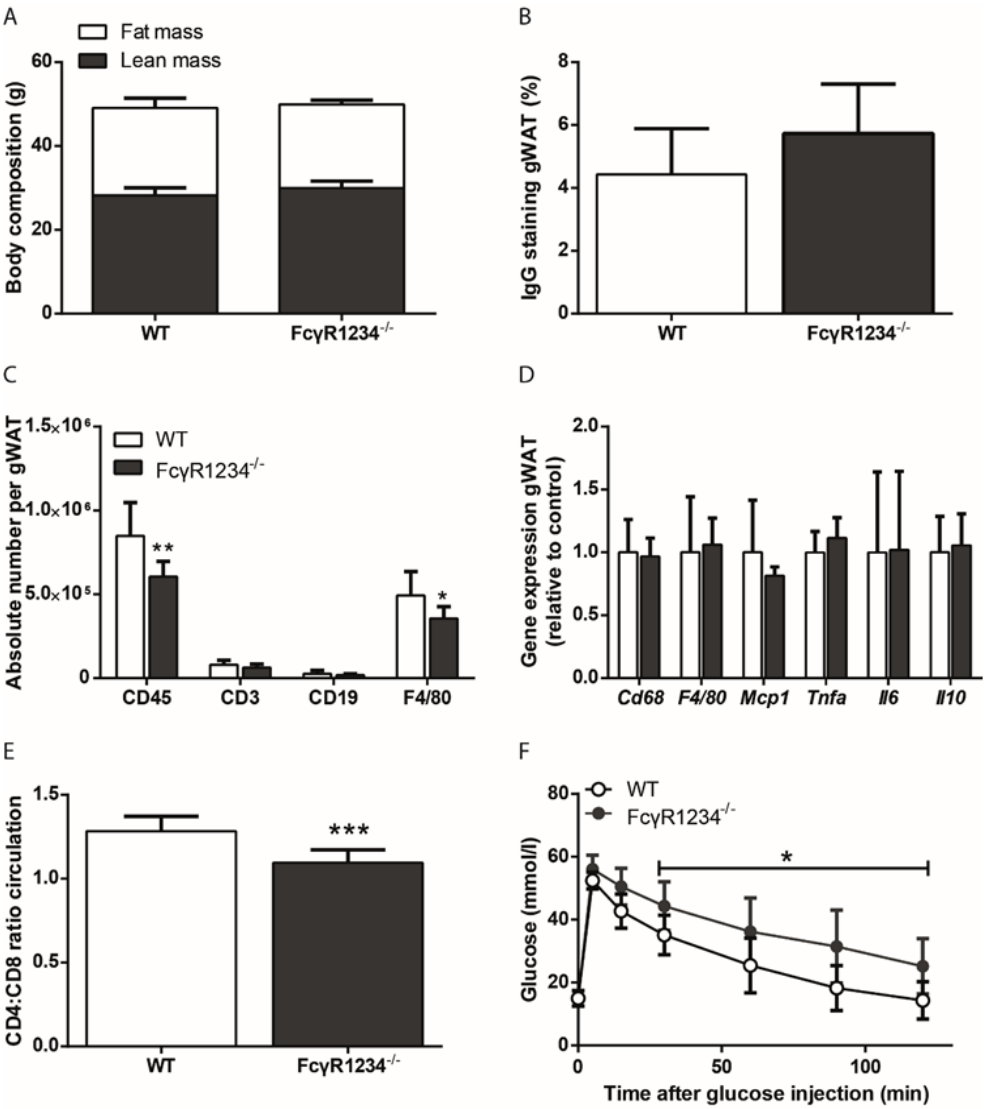

Figure 2. FcγR1234 deficiency has no effect on diet induced obesity, reduces immune cell numbers in gWAT, but deteriorates glucose tolerance.

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).

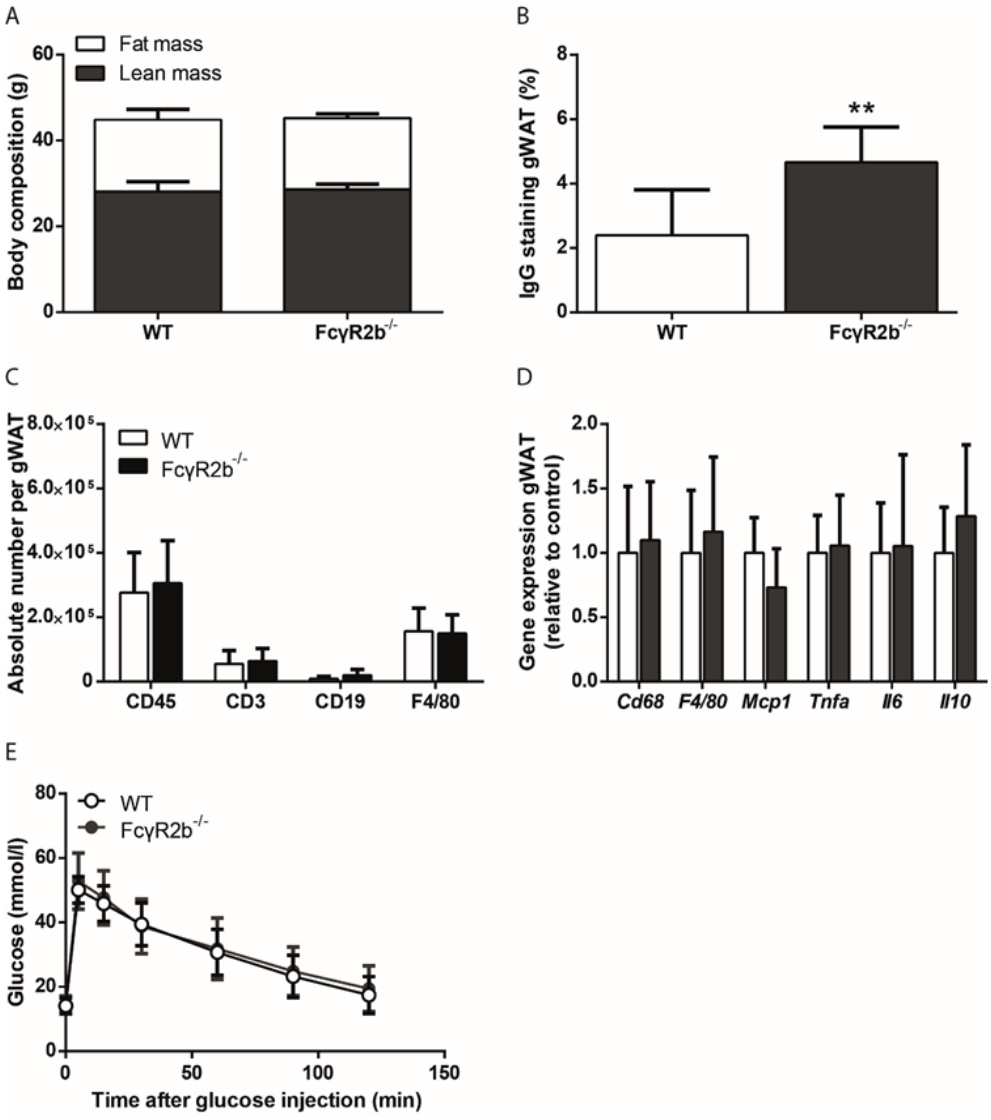

Figure 3. FcγR2b deficiency has no effect on diet induced obesity, increases IgG in gWAT, but has no effect on glucose tolerance.

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).

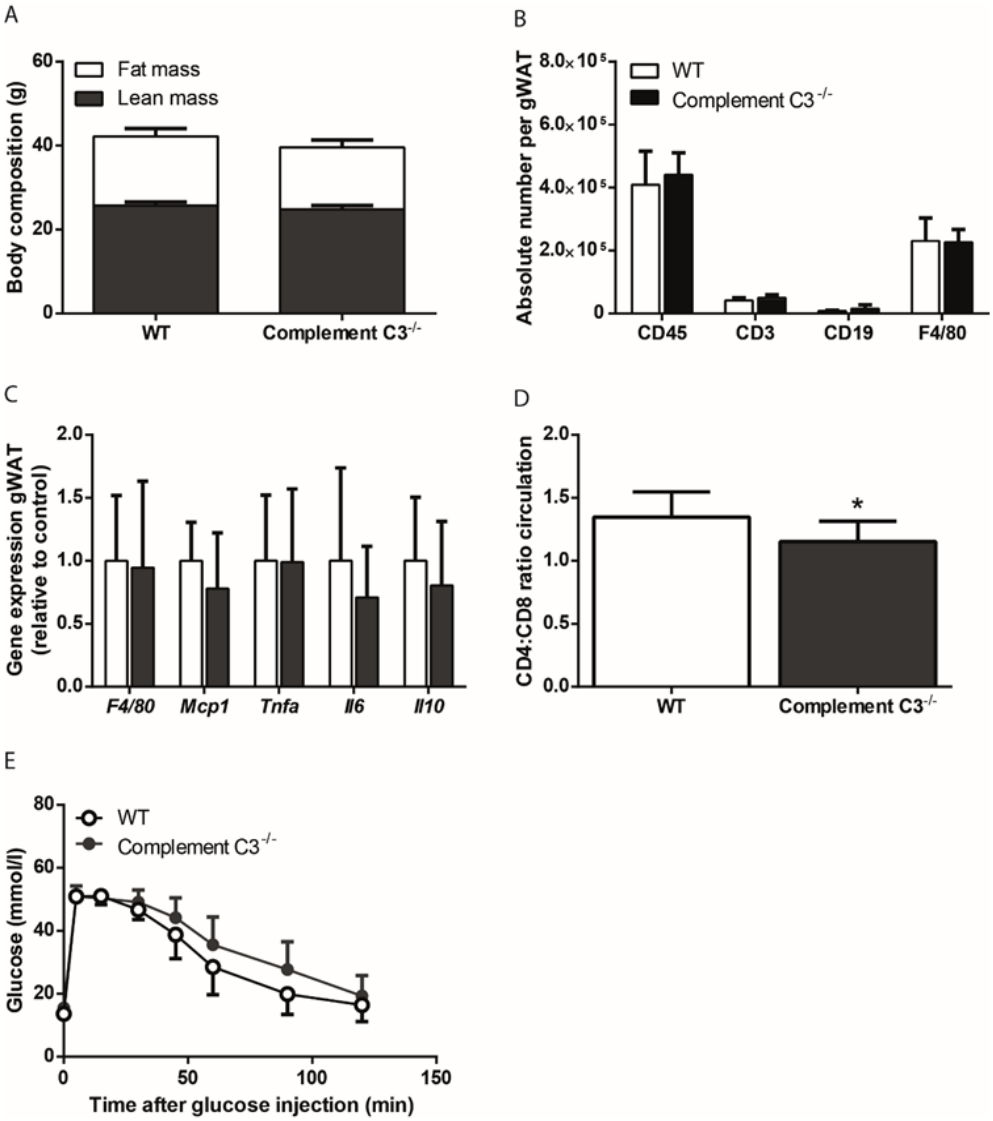

Figure 4. Complement C3 deficiency has no effect on diet induced obesity or adipose tissue inflammation, but slightly reduces glucose tolerance.

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.

Reference list

1. Olefsky JM, Glass CK. Macrophages, inflammation, and insulin resistance. Annual review of physiology. 2010;72:219-46.
2. Nishimura S, Manabe I, Nagasaki M, Eto K, Yamashita H, Ohsugi M, et al. CD8+ effector T cells contribute to macrophage recruitment and adipose tissue inflammation in obesity. Nature medicine. 2009;15(8):914-20.
3. Feuerer M, Herrero L, Cipolletta D, Naaz A, Wong J, Nayer A, et al. Lean, but not obese, fat is enriched for a unique population of regulatory T cells that affect metabolic parameters. Nature medicine. 2009;15(8):930-9.
4. Duffaut C, Galitzky J, Lafontan M, Bouloumie A. Unexpected trafficking of immune cells within the adipose tissue during the onset of obesity. Biochemical and biophysical research communications. 2009;384(4):482-5.
5. Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW, Jr. Obesity is associated with macrophage accumulation in adipose tissue. The Journal of clinical investigation. 2003;112(12):1796-808.
6. Lumeng CN, Bodzin JL, Saltiel AR. Obesity induces a phenotypic switch in adipose tissue macrophage polarization. The Journal of clinical investigation. 2007;117(1):175-84.
7. Ilan Y, Maron R, Tukpah AM, Maioli TU, Murugaiyan G, Yang K, et al. Induction of regulatory T cells decreases adipose inflammation and alleviates insulin resistance in ob/ob mice. Proceedings of the National Academy of Sciences of the United States of America. 2010;107(21):9765-70.
8. van Beek L, Lips MA, Visser A, Pijl H, Ioan-Facsinay A, Toes R, et al. Increased systemic and adipose tissue inflammation differentiates obese women with T2DM from obese women with normal glucose tolerance. Metabolism: clinical and experimental. 2014;63(4):492-501.
9. Mallat Z. The B-side story in insulin resistance. Nature medicine. 2011;17(5):539-40.
10. Winer DA, Winer S, Shen L, Wadia PP, Yantha J, Paltser G, et al. B cells promote insulin resistance through modulation of T cells and production of pathogenic IgG antibodies. Nature medicine. 2011;17(5):610-7.
11. Ravetch JV, Bolland S. IgG Fc receptors. Annual review of immunology. 2001;19:275-90.
12. Sarma JV, Ward PA. The complement system. Cell and tissue research. 2011;343(1):227-35.
13. Boross P, Arandhara VL, Martin-Ramirez J, Santiago-Raber ML, Carlucci F, Flierman R, et al. The inhibiting Fc receptor for IgG, FcgammaRIIB, is a modifier of autoimmune susceptibility. Journal of immunology. 2011;187(3):1304-13.
14. Wessels MR, Butko P, Ma M, Warren HB, Lage AL, Carroll MC. Studies of group B streptococcal infection in mice deficient in complement component C3 or C4 demonstrate an essential role for complement in both innate and acquired immunity. Proceedings of the National Academy of Sciences of the United States of America. 1995;92(25):11490-4.
15. van Beek L, Vroegrijk IO, Katiraei S, Heemskerk MM, van Dam AD, Kooijman S, et al. FcRgamma-chain deficiency reduces the development of diet-induced obesity. Obesity. 2015;23(12):2435-44. Epub 2015/11/03.
16. van Beek L, van Klinken JB, Pronk AC, van Dam AD, Dirven E, Rensen PC, et al. The limited storage capacity of gonadal adipose tissue directs the development of metabolic disorders in male C57Bl/6J mice. Diabetologia. 2015.
17. Hussaarts L, Garcia-Tardon N, van Beek L, Heemskerk MM, Haeberlein S, van der Zon GC, et al. Chronic helminth infection and helminth-derived egg antigens promote adipose tissue M2 macrophages and improve insulin sensitivity in obese mice. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2015.
18. Rombouts Y, Willemze A, van Beers JJ, Shi J, Kerkman PF, van Toorn L, et al. Extensive glycosylation of ACPA-IgG variable domains modulates binding to citrullinated antigens in rheumatoid arthritis. Annals of the rheumatic diseases. 2015.
19. Meiring HD, van der Heeft E, ten Hove GJ, de Jong APJM. Nanoscale LC-MS(n): technical design and applications to peptide and protein analysis. . J Sep Sci 2002(25):557–68.
20. Kawasaki K, Abe M, Tada F, Tokumoto Y, Chen S, Miyake T, et al. Blockade of B-cell-activating factor signaling enhances hepatic steatosis induced by a high-fat diet and improves insulin sensitivity. Laboratory investigation; a journal of technical methods and pathology. 2013;93(3):311-21.
21. Bassols J, Prats-Puig A, Gispert-Sauch M, Crehuet-Almirall M, Carreras-Badosa G, Diaz-Roldan F, et al. Increased serum IgG and IgA in overweight children relate to a less favourable metabolic phenotype. Pediatric obesity. 2014;9(3):232-8.

22. Trcka J, Moroi Y, Clynes RA, Goldberg SM, Bergtold A, Perales MA, et al. Redundant and alternative roles for activating Fc receptors and complement in an antibody-dependent model of autoimmune vitiligo. Immunity. 2002;16(6):861-8.

23. Shannon JG, Cockrell DC, Takahashi K, Stahl GL, Heinzen RA. Antibody-mediated immunity to the obligate intracellular bacterial pathogen Coxiella burnetii is Fc receptor- and complement-independent. BMC immunology. 2009;10:26.

24. Stienstra R, Dijk W, van Beek L, Jansen H, Heemskerk M, Houtkooper RH, et al. Mannose-binding lectin is required for the effective clearance of apoptotic cells by adipose tissue macrophages during obesity. Diabetes. 2014;63(12):4143-53.

25. Murray I, Havel PJ, Sniderman AD, Cianflone K. Reduced body weight, adipose tissue, and leptin levels despite increased energy intake in female mice lacking acylation-stimulating protein. Endocrinology. 2000;141(3):1041-9.

26. Murray I, Sniderman AD, Havel PJ, Cianflone K. Acylation stimulating protein (ASP) deficiency alters postprandial and adipose tissue metabolism in male mice. The Journal of biological chemistry. 1999;274(51):36219-25.

27. Xia Z, Sniderman AD, Cianflone K. Acylation-stimulating protein (ASP) deficiency induces obesity resistance and increased energy expenditure in ob/ob mice. The Journal of biological chemistry. 2002;277(48):45874-9.

28. Chow WN, Lee YL, Wong PC, Chung MK, Lee KF, Yeung WS. Complement 3 deficiency impairs early pregnancy in mice. Molecular reproduction and development. 2009;76(7):647-55.

29. Circolo A, Garnier G, Fukuda W, Wang X, Hidvegi T, Szalai AJ, et al. Genetic disruption of the murine complement C3 promoter region generates deficient mice with extrahepatic expression of C3 mRNA. Immunopharmacology. 1999;42(1-3):135-49.

30. Wetsel RA, Kildsgaard J, Zsigmond E, Liao W, Chan L. Genetic deficiency of acylation stimulating protein (ASP(C3ades-Arg)) does not cause hyperapobetalipoproteinemia in mice. The Journal of biological chemistry. 1999;274(27):19429-33.

31. Murray I, Sniderman AD, Cianflone K. Mice lacking acylation stimulating protein (ASP) have delayed postprandial triglyceride clearance. Journal of lipid research. 1999;40(9):1671-6.

32. Cianflone K, Xia Z, Chen LY. Critical review of acylation-stimulating protein physiology in humans and rodents. Biochimica et biophysica acta. 2003;1609(2):127-43.

33. Hillian AD, McMullen MR, Sebastian BM, Roychowdhury S, Kashyap SR, Schauer PR, et al. Mice lacking C1q are protected from high fat diet-induced hepatic insulin resistance and impaired glucose homeostasis. The Journal of biological chemistry. 2013;288(31):22565-75.

34. Phieler J, Chung KJ, Chatzigeorgiou A, Klotzsche-von Ameln A, Garcia-Martin R, Sprott D, et al. The complement anaphylatoxin C5a receptor contributes to obese adipose tissue inflammation and insulin resistance. Journal of immunology. 2013;191(8):4367-74.

35. Auger JL, Haasken S, Binstadt BA. Autoantibody-mediated arthritis in the absence of C3 and activating Fcgamma receptors: C5 is activated by the coagulation cascade. Arthritis research & therapy. 2012;14(6):R269.

36. Huber-Lang M, Sarma JV, Zetoune FS, Rittirsch D, Neff TA, McGuire SR, et al. Generation of C5a in the absence of C3: a new complement activation pathway. Nature medicine. 2006;12(6):682-7.

37. Mohammed N, Tang L, Jahangiri A, de Villiers W, Eckhardt E. Elevated IgG levels against specific bacterial antigens in obese patients with diabetes and in mice with diet-induced obesity and glucose intolerance. Metabolism: clinical and experimental. 2012;61(9):1211-4.

38. Wolf D, Jehle F, Ortiz Rodriguez A, Dufner B, Hoppe N, Colberg C, et al. CD40L deficiency attenuates diet-induced adipose tissue inflammation by impairing immune cell accumulation and production of pathogenic IgG-antibodies. PloS one. 2012;7(3):e33026.

39. Huh JY, Kim JI, Park YJ, Hwang IJ, Lee YS, Sohn JH, et al. A novel function of adipocytes in lipid antigen presentation to iNKT cells. Molecular and cellular biology. 2013;33(2):328-39.

40. Lee AN, Beck CE, Hall M. Rheumatoid factor and anti-CCP autoantibodies in rheumatoid arthritis: a review. Clinical laboratory science : journal of the American Society for Medical Technology. 2008;21(1):15-8.

41. Huijbers MG, Querol LA, Niks EH, Plomp JJ, van der Maarel SM, Graus F, et al. The expanding field of IgG4-mediated neurological autoimmune disorders. European journal of neurology : the official journal of the European Federation of Neurological Societies. 2015.

Supplemental figures

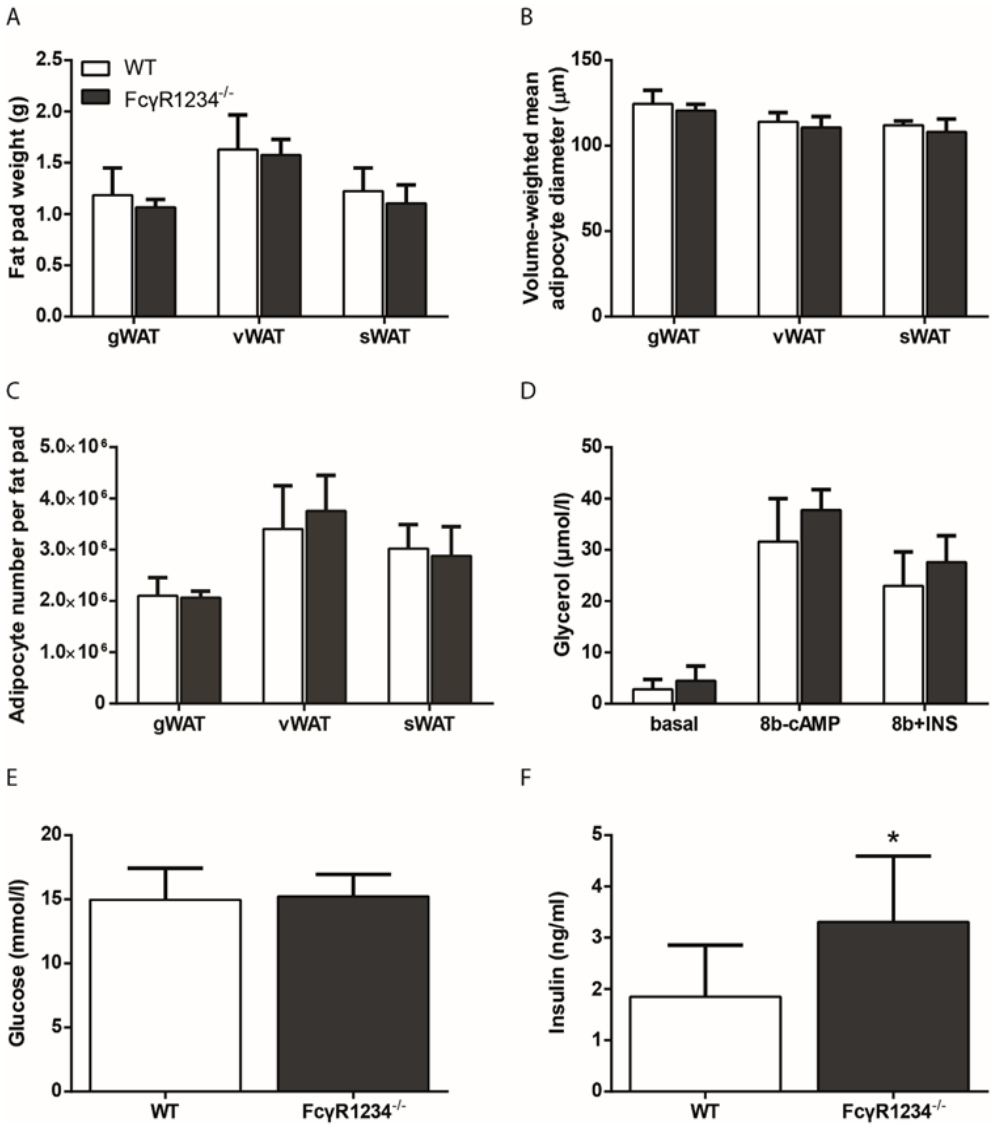

Figure S1. WT and FcγR1234$^{-/-}$ mice were fed a HFD for 15 weeks to induce obesity. Fat pad weight (A), adipocyte size (B) and adipocyte number per fat pad (C) were determined for gWAT, vWAT and sWAT from WT and FcγR1234$^{-/-}$ mice. Basal lipolysis, 8b-cAMP stimulated lipolysis and the insulin inhibition 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.

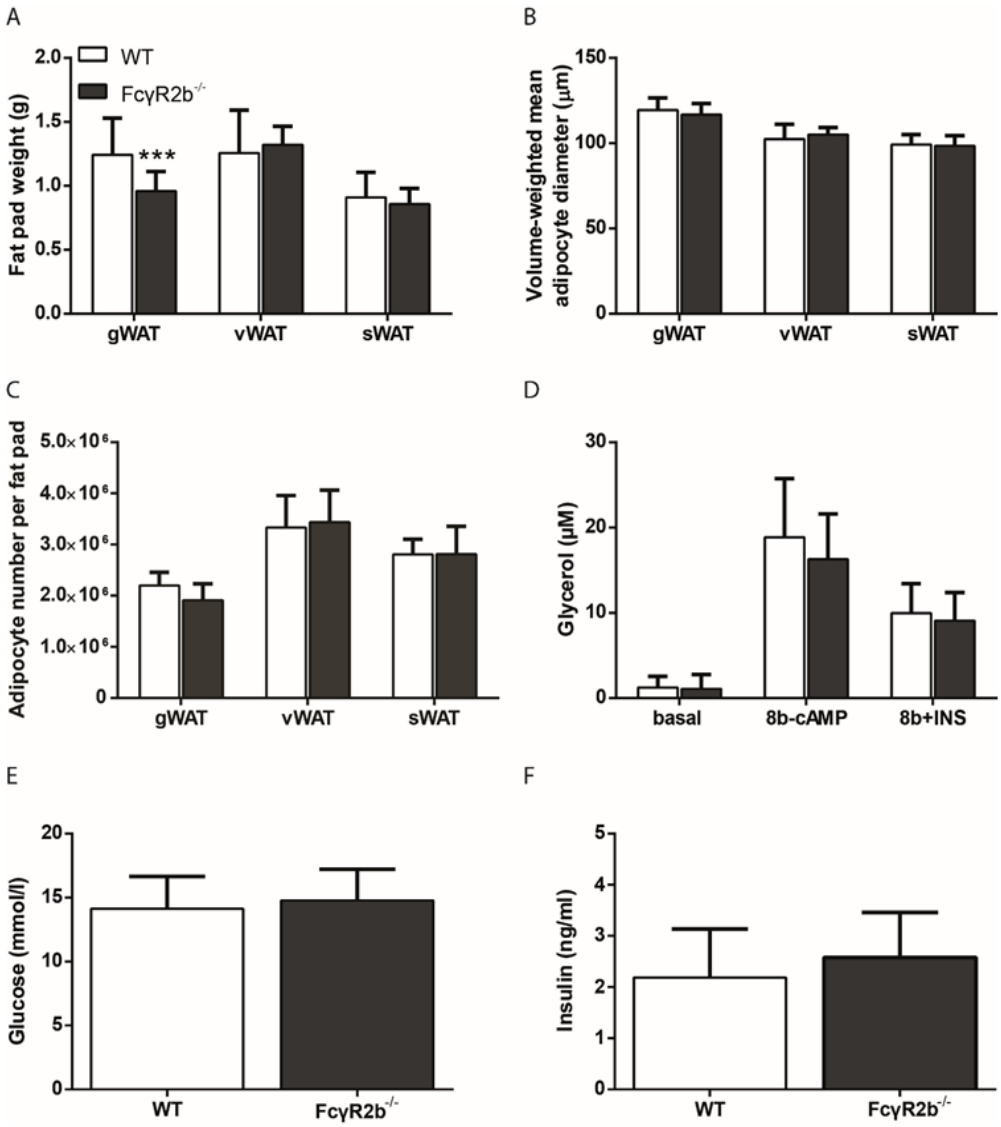

Figure S2. FcγR2b^{flox} and FcγR2b$^{-/-}$ mice were fed a HFD for 15 weeks to induce obesity. Fat pad weight (A), adipocyte size (B) and adipocyte number per fat pad (C) were determined for gWAT, vWAT and sWAT from WT and FcγR1234$^{-/-}$ mice. Basal lipolysis, 8b-cAMP stimulated lipolysis and the 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}.

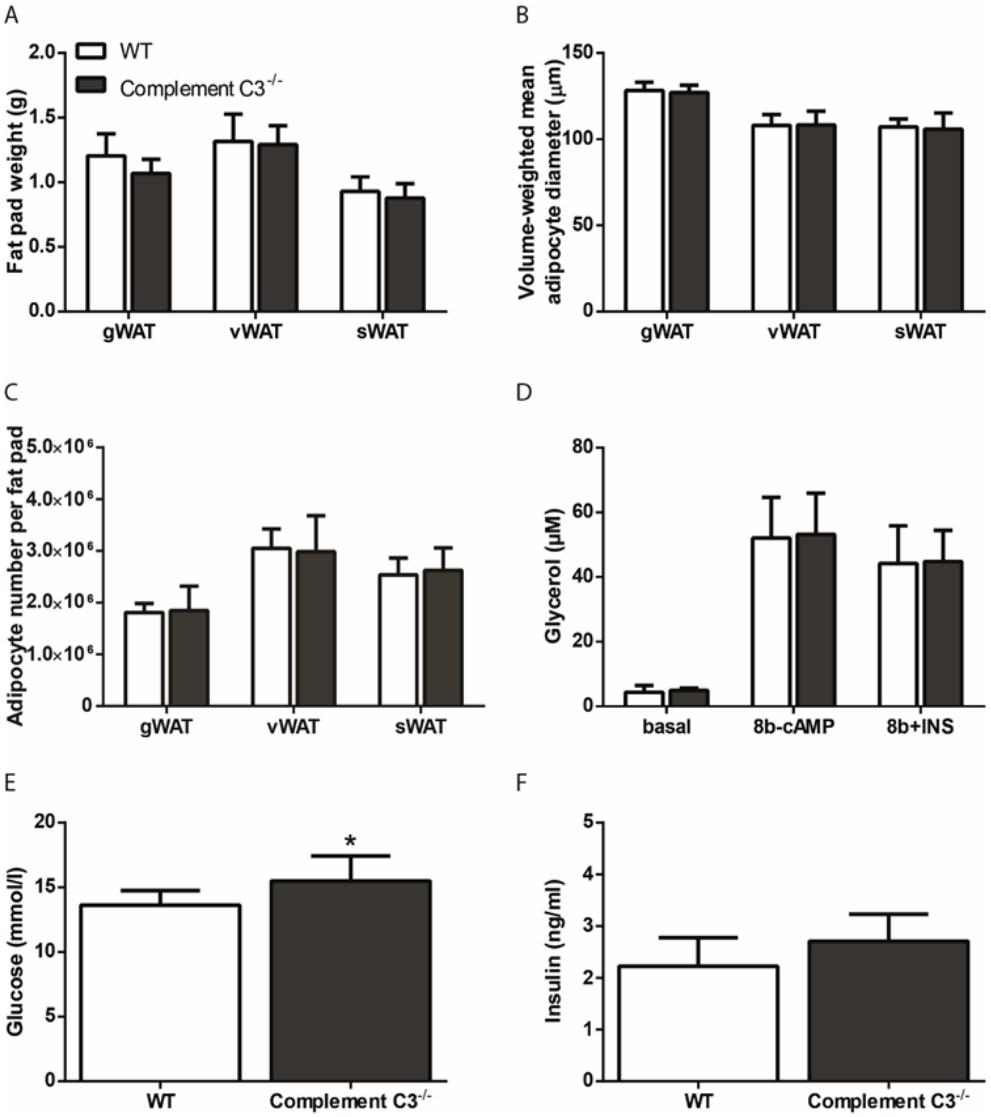

Figure S3. WT and Complement C3$^{-/-}$ mice were fed a HFD for 15 weeks to induce obesity. Fat pad weight (A), adipocyte size (B) and adipocyte number per fat pad (C) were determined for gWAT, vWAT and sWAT from WT and Complement C3$^{-/-}$ mice. Basal lipolysis, 8b-cAMP stimulated lipolysis and the 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.