The Complement Anaphylotoxin C5a Receptor Contributes to Obese Adipose Tissue Inflammation and Insulin Resistance

Julia Phieler, Kyoung-Jin Chung, Antonios Chatzigeorgiou, Anne Klotzsche-von Ameln, Ruben Garcia-Martin, David Sprott, Maria Moisidou, Theodora Tzanavari, Barbara Ludwig, Elena Baraban, Monika Ehrhart-Bornstein, Stefan R. Bornstein, Hassan Mziaut, Michele Solimena, Katia P. Karalis, Matina Economopoulou, John D. Lambris and Triantafyllos Chavakis

*J Immunol* published online 16 September 2013
http://www.jimmunol.org/content/early/2013/09/14/jimmunol.1300038

Supplementary Material

http://www.jimmunol.org/content/suppl/2013/09/16/jimmunol.1300038.DC1

Subscription

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
The Complement Anaphylatoxin C5a Receptor Contributes to Obese Adipose Tissue Inflammation and Insulin Resistance

Julia Phielker,*,†,‡,§ Kyoung-Jin Chung,*,†,1 Antonios Chatzigeorgiou,*,†,§,1 Anne Klotzsche-von Ameln,*,†,§ Ruben Garcia-Martin,*,†,§,1 David Sprott,*,†,§ Maria Moisidou,*, Theodora Tzanvari,*, Barbara Ludwig,‖ Elena Baraban,‖ Monika Ehrhart-Bornstein,‖ Stefan R. Bornstein,‖ Hassan Mziaut,‡ Michele Solimena,‡ Katia P. Karalis,*,†,§ Matina Economopoulou,‖ John D. Lambris,**,‡ and Triantafyllos Chavakis*,†,‡,§

Obese adipose tissue (AT) inflammation contributes critically to development of insulin resistance. The complement anaphylatoxin C5a receptor (C5aR) has been implicated in inflammatory processes and as regulator of macrophage activation and polarization. However, the role of C5aR in obesity and AT inflammation has not been addressed. We engaged the model of diet-induced obesity and found that expression of C5aR was significantly upregulated in the obese AT, compared with lean AT. In addition, C5a was present in obese AT in the proximity of macrophage-rich crownlike structures. C5aR-sufficient and -deficient mice were fed a high-fat diet (HFD) or a normal diet (ND). C5aR deficiency was associated with increased AT weight upon ND feeding in males, but not in females, and with increased adipocyte size upon ND and HFD conditions in males. However, obese C5aR−/− mice displayed improved systemic and AT insulin sensitivity. Improved AT insulin sensitivity in C5aR−/− mice was associated with reduced accumulation of total and proinflammatory M1 macrophages in the obese AT, increased expression of IL-10, and decreased AT fibrosis. In contrast, no difference in β cell mass was observed owing to C5aR deficiency under an HFD. These results suggest that C5aR contributes to macrophage accumulation and M1 polarization in the obese AT and thereby to AT dysfunction and development of AT insulin resistance. The Journal of Immunology, 2013, 191: 000–000.

Obesity is associated with the development of insulin resistance, type 2 diabetes, and cardiovascular diseases (1–5). Obesity is considered a state of chronic progressive low-grade inflammation. In particular, inflammation of the obese white adipose tissue (WAT) can directly contribute to the development of insulin resistance (1–3, 5, 6). A hallmark of obese WAT inflammation is increased accumulation of inflammatory cells, including cytotoxic T cells and macrophages (1–3, 5, 6). In the obese WAT, macrophages are skewed to the classically activated proinflammatory M1 macrophage phenotype (1–3, 5–9). In contrast, macrophages constitutively present in the lean WAT are, rather, alternatively activated anti-inflammatory M2-polarized macrophages. The latter alternatively activated M2 subtype (10–12) secretes anti-inflammatory cytokines, such as IL-10, whereas classically activated M1-polarized macrophages that accumulate in the WAT with increasing obesity secrete proinflammatory cytokines that can directly interfere with insulin signaling, rendering the WAT insulin resistant (13, 14).

A central component involved in the activation of innate immunity, inflammation, and tissue remodeling is the complement system. Cleavage of complement C3 into C3a and C3b is the point at which all three major complement activation pathways—the classical, alternative, and lectin pathways—merge (15, 16). The further cleavage of C5 results in generation of the active anaphylatoxin C5a, which acts through its cellular G-protein–coupled receptor complement C5a receptor (C5aR) (15), and the membrane attack complex initiating fragment C5b. Of interest, adipocytes can secrete several complement factors, such as C3 or factor B and factor D, that serve as activating factors of the alternative complement pathway, implying a role of complement in adipose tissue (AT) biology (15, 17, 18). In particular, factor D, also named adipin, leads to the production of acylation-stimulating protein (ASP); this pathway has been reported to act as a regulator of lipid metabolism (19). The absence of ASP, for example, in mice deficient in C3, resulted in reduced fat storage in adipocytes.

*Division of Vascular Inflammation, Diabetes and Kidney, Department of Medicine III, Dresden University, 01307 Dresden, Germany; †Institute of Physiology, Dresden University, 01307 Dresden, Germany; ‡Paul Langerhans Institute Dresden, Dresden University, 01307 Dresden, Germany; §Institute for Clinical Chemistry and Laboratory Medicine, Dresden University, 01307 Dresden, Germany; ¦Developmental Biology Section, Biomedical Research Foundation of the Academy of Athens, 115 27 Athens, Greece; ‖Division of Molecular Endocrinology, Department of Medicine III, Dresden University, 01307 Dresden, Germany; ‡Department of Ophthalmology, Dresden University, 01307 Dresden, Germany; and **Department of Pathology and Laboratory Medicine, University of Pennsylvania, Perelman School of Medicine, Philadelphia, PA 19104

1K.-J.C. and A.C. contributed equally to this work.
2J.D.L. and T.C. contributed equally as senior authors to this work.

Received for publication January 9, 2013. Accepted for publication August 13, 2013.

This work was supported by grants from the National Institutes of Health (Grant AI068730 to J.D.L.), the Else-Kröner Fresenius Stiftung (to T.C.), the Deutsche Forschungsgemeinschaft (to T.C.), and the German Federal Ministry of Education and Research to the German Center for Diabetes Research (to T.C.).

Address correspondence and reprint requests to Dr. Triantafyllos Chavakis, Department of Medicine III, Dresden University, Fetscherstrasse 74, 01307 Dresden, Germany. E-mail address: Triantafyllos.Chavakis@uniklinikum-dresden.de

The online version of this article contains supplemental material.

Abbreviations used in this article: ASP, acylation-stimulating protein; AT, adipose tissue; C5aR, C5a receptor; CLS, crownlike structure; DIO, diet-induced obesity; GGT, glucose tolerance test; gWAT, gonadal white adipose tissue; HFD, high-fat diet; IHIC, immunohistochemistry; ITT, insulin tolerance test; ND, normal diet; ORO, Oil Red O; PPA, paraformaldehyde; SVE, stromal vascular fraction; sWAT, subcutaneous white adipose tissue; WAT, white adipose tissue; WT, wild-type.

Copyright © 2013 by The American Association of Immunologists, Inc. 0022-1767/13/S16.00
and delayed triglyceride and fatty acid clearance from circulation after a fat meal (20). Furthermore, C5aR-deficient mice on a high-fat diet displayed reduced weight gain associated with decreased macrophage infiltration in WAT and showed reduced insulin resistance (21). In contrast to the aforementioned, the role of the C5a–C5aR axis in obesity and WAT inflammation has not been addressed so far. In this study, we engaged the model of diet-induced obesity (DIO) to elucidate the contribution of C5aR to obesity-associated WAT inflammation and insulin resistance. Our findings that C5aR-deficient mice displayed improved WAT insulin sensitivity and reduced WAT inflammation associated with decreased macrophage accumulation, reduced M1 macrophage polarization, and reduced fibrosis suggest that the C5a–C5aR axis contributes to WAT inflammation and insulin resistance.

Materials and Methods

Animal experiments

C5aR−/− mice were previously described (22, 23). C57BL/6 mice were from Janvier (Saint-Berthevin, France). Male mice 6 to 8 wk old were fed either a high-fat diet (HFD) or a normal diet (ND) (60% kcal from fat or 10% kcal from fat, respectively; Research Diets, New Brunswick, NJ) for different time periods up to 28 wk. Body weight was recorded weekly. Animal experiments were approved by the Landesdirektion Dresden, Germany.

For i.p. glucose tolerance tests (GTTs), mice were starved overnight (24–28) or, alternatively, for 6 h with free access to drinking water. Glucose level measurements were performed using an Accu-Chek glucose meter (Roche, Mannheim, Germany). Subsequently, mice were injected i.p. with 10% D-(+)-glucose (1 g/kg; Sigma-Aldrich, Munich, Germany), and glucose levels were monitored at indicated time points. For insulin tolerance tests (ITTs), mice were fasted for 5–6 h prior to i.p. injection of 0.75 U/kg insulin (Lilly, Bad Homburg, Germany) in male mice fed an ND and in female mice fed an HFD, or 1 U/kg insulin i.p. in male mice fed an ND and male and female mice were monitored at indicated time points. For serum insulin analysis, mice were starved for 6 h prior to blood collection, and insulin was measured using an insulin immunoassay (Chrysal Chem, Cologne, Germany). After euthanasia of mice, blood was collected, and the serum was analyzed using adiponectin and leptin immunoassays (R&D Systems, Wiesbaden-Nordenstadt, Germany).

Stromal vascular fraction isolation and FACS analysis

Previously described protocols were followed (3, 29), with some modifications. Frozen WAT samples were fixed in 4% paraformaldehyde (PFA) at 4˚C overnight before embedding in paraffin. For each individual mouse, 4–8 mm sections were cut and mounted, and staining was performed with Mayer’s hematoxylin (SA V, Flintsbach am Inn, Germany) counterstained with 1% eosin (Pharmacy of the University Clinic Carl-Gustav-Carus, Dresden, Germany). For assessing fibrosis, Masson’s trichrome staining was performed according to the manufacturer’s instructions (Sigma-Aldrich). Other sections were mounted on silane-treated slides (Marianfeld, Lauda-Königshofen, Germany) and stained with anti-F4/80 Ab (Novus Biologicals, Herford, Germany). For this purpose, Ab retrieval was performed with citrate buffer (Sigma-Aldrich) followed by peroxidase (Dako Deutschland, Hamburg, Germany) and proteinase K blocking (Sigma-Aldrich). The avidin–biotin complex was detected with a AEC Peroxidase Substrate Kit (Vector Laboratories, Peterborough, U.K.). Finally, slides were stained with hematoxylin and mounted.

For determination of adipocyte size, pictures of H&E-stained sections obtained at ×100 magnification. The diameter of ~200 adipocytes per slide was measured with the AxiosVision Rel. 4.8 software (Carl Zeiss MicroImaging, Jena, Germany). The diameters of both height and width were measured for each cell.

For histochemical staining of the liver with Oil Red O (ORO), 10-μm cryosections were prepared. Briefly, slides were fixed in ice-cold 10% formalin solution for 10 min, rinsed in dH2O, and stained for 15 min in ORO in 60% isopropanol solution (ORO:H2O:3, 2). They were rinsed in 60% isopropanol, and nuclei were counterstained with Mayer’s hematoxylin. Slides were rinsed in H2O and mounted with 95% glycerol.

For C5a staining, frozen gWAT derived from mice fed an ND or HFD for 18 wk was fixed in 4% PFA at 4˚C overnight before embedding in paraﬃn and sectioning. Ag demasking was performed with citrate buffer (pH = 6.8), 2% SDS, and 100 mM 2-ME (all from Sigma-Aldrich) in a water bath at 50˚C for 20 min, blocked with TBST containing 5% w/v skin milk, and incubated with an Ab against total Akt (Cell Signaling) prior to development and signal detection as described above. Insulin-induced phospho-Akt was evaluated by normalization over total Akt signaling, using ImageJ software.

Immunohistochemistry

We followed previously described protocols (13, 32, 33), with some modifications. Frozen WAT samples were fixed in 4% paraformaldehyde (PFA) at 4˚C overnight before embedding in paraffin. For each individual mouse, 4–8 mm sections were cut and mounted, and staining was performed with Mayer’s hematoxylin (SA V, Flintsbach am Inn, Germany) counterstained with 1% eosin (Pharmacy of the University Clinic Carl-Gustav-Carus, Dresden, Germany). For assessing fibrosis, Masson’s trichrome staining was performed according to the manufacturer’s instructions (Sigma-Aldrich). Other sections were mounted on silane-treated slides (Marianfeld, Lauda-Königshofen, Germany) and stained with anti-F4/80 Ab (Novus Biologicals, Herford, Germany). For this purpose, Ab retrieval was performed with citrate buffer (Sigma-Aldrich) followed by peroxidase (Dako Deutschland, Hamburg, Germany) and proteinase K blocking (Sigma-Aldrich). The avidin–biotin complex was detected with a AEC Peroxidase Substrate Kit (Vector Laboratories, Peterborough, U.K.). Finally, slides were stained with hematoxylin and mounted.

For determination of adipocyte size, pictures of H&E-stained sections obtained at ×100 magnification. The diameter of ~200 adipocytes per slide was measured with the AxiosVision Rel. 4.8 software (Carl Zeiss MicroImaging, Jena, Germany). The diameters of both height and width were measured for each cell.

For histochemical staining of the liver with Oil Red O (ORO), 10-μm cryosections were prepared. Briefly, slides were fixed in ice-cold 10% formalin solution for 10 min, rinsed in dH2O, and stained for 15 min in ORO in 60% isopropanol solution (ORO:H2O:3, 2). They were rinsed in 60% isopropanol, and nuclei were counterstained with Mayer’s hematoxylin. Slides were rinsed in H2O and mounted with 95% glycerol.

For C5a staining, frozen gWAT derived from mice fed an ND or HFD for 18 wk was fixed in 4% PFA at 4˚C overnight before embedding in paraﬃn and sectioning. Ag demasking was performed with citrate buffer (pH = 6.8), 2% SDS, and 100 mM 2-ME (all from Sigma-Aldrich) in a water bath at 50˚C for 20 min, blocked with TBST containing 5% w/v skin milk, and incubated with an Ab against total Akt (Cell Signaling) prior to development and signal detection as described above. Insulin-induced phospho-Akt was evaluated by normalization over total Akt signaling, using ImageJ software.

In vivo phospho-Akt signaling study

We followed previously published protocols (30, 31). Briefly, mice on an HFD were fasted for 5 h prior to i.p. injection of 2 U/kg insulin (Lilly). After 8 min, mice were euthanized, and gWAT, sWAT, and liver tissues were harvested and shock frozen in liquid nitrogen. For protein isolation from WAT, 100 mg tissue was homogenized with an IKA T10 basic Ultra-Turrax Homogenizer in 1 ml 1× RIPA lysis buffer [1% Triton X-100, 0.5% sodium deoxycholate; 0.1% SDS, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, Mini Protease Inhibitor and Phosphatase Inhibitor Cocktail Tablet (Roche)] and centrifuged at 14,000 × g for 15 min at 4˚C. The fatty layer was removed and the liquid phase transferred in a new tube. After incubation in an ultrasonic bath, the liquid phase was centrifuged two more times before measurement of its protein concentration using the BCA Protein Assay Kit (Thermo Scientific, Schwerte, Germany). For protein isolation from the liver, 50 mg tissue was homogenized in 1 ml 1× RIPA lysis buffer and centrifuged at 13,000 × g for 15 min at 4˚C. The liquid phase was transferred in a new tube, and protein concentration was determined.

Equal amounts of protein/tissue (15 μg for sWAT and gWAT, 25 μg for liver) were loaded per lane on a 10% SDS-PAGE gel. Protein was transferred to a nitrocellulose membrane (GE Healthcare, Munich, Germany). The membrane was blocked with TBST containing 5% v/v skin milk (BD, Heidelberg, Germany), incubated overnight with the first Ab against phospho-Akt (phospho-Akt–Ser473) (Cell Signaling/New England Biolabs, Frankfurt am Main, Germany), and then incubated with a goat anti-rabbit secondary Ab conjugated with HRP. The blot was developed with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific), and the signal was detected with a luminescent image analyzer, LAS-3000 (Fujifilm, Düsseldorf, Germany).

Membranes were stripped in a buffer containing 62.5 mM Tris-HCl (pH = 6.8), 2% SDS, and 100 mM 2-ME (all from Sigma-Aldrich) in a water bath at 50˚C for 20 min, blocked with TBST containing 5% w/v skin milk, and incubated with an Ab against total Akt (Cell Signaling) prior to development and signal detection as described above. Insulin-induced phospho-Akt was evaluated by normalization over total Akt signaling, using ImageJ software.
for different time periods up to 26 wk. C5aR mRNA expression increased upon DIO already at 12 wk on an HFD, continuing to increase and reaching a peak at 18 wk on an HFD and remaining significantly elevated at 26 wk on an HFD, compared with respective ND-fed mice (Fig. 1A). Thus, C5aR mRNA expression in the gWAT increased upon DIO in a time-dependent manner. Moreover, a significant upregulation of C5aR mRNA was observed in the sWAT of DIO mice (data not shown).

To analyze the presence of the C5aR ligand in the obese gWAT, we performed immunohistological studies for C5a at 18 wk on an HFD versus an ND, as this represented the time point with maximal C5aR upregulation in the gWAT. Of note, very little C5a was found in the gWAT of lean mice, whereas the presence of C5a in the gWAT of obese mice was substantial (Fig. 1B). Interestingly, the majority of C5a staining was observed as diffuse staining within crownlike structures (CLS), which are usually macrophage rich (Fig. 1B). These findings indicate that C5a and C5aR are elevated in the obese WAT and that activation of C5a and C5aR may correlate with WAT inflammation.

The role of C5aR in HFD-induced obesity

Next, C5aR-sufficient- and -deficient mice were fed a control ND or an HFD. Male C5aR−/− mice on ND displayed increased weight gain, compared with male wild-type (WT) mice; however, no weight difference was seen between WT and C5aR−/− mice upon feeding with an HFD (Fig. 1C, 1D). Consistently, the total weights of lean sWAT and lean gWAT isolated from C5aR-deficient male mice were

Ab (Abcam, Cambridge, U.K.). Ag retrieval was performed with pepsin (Sigma-Aldrich). Endogenous biotin block, streptavidin-HRP, and color development were performed with a peroxidase VECTASTAIN ABC Kit and a AEC Peroxidase Substrate Kit (Vector Laboratories). A negative control was included using Mayer’s hematoxylin and mounted with a water-based mounting medium. For determination of islet morphology, the areas of pancreas and islets were measured with the AxioVision Rel. 4.8 software (Carl Zeiss MicroImaging), and β cell mass was calculated by multiplying the percentage of measured pancreatic area occupied by β cells by the total pancreatic weight (34).

Quantitative real-time PCR

RNA from AT and liver of C5aR-sufficient and -deficient mice was extracted with TRIzol (Invitrogen, Darmstadt, Germany). cDNA was synthesized with an iScript cDNA Synthesis Kit (Bio-Rad, Munich, Germany), and mRNA expression levels of each gene were quantified by quantitative real-time PCR using SsoFast EvaGreen Supermix (Bio-Rad) and a Bio-Rad cycler system (Bio-Rad) using gene-specific primers and normalization to 18S mRNA. Relative gene expression was calculated with the ΔΔCt method (35). The fold-change ratio was calculated and expressed as mean ± SEM.

Statistical analysis

Data were analyzed with the independent Mann–Whitney U test.

Results

The presence of C5a and C5aR expression in obese AT

To determine the regulation of C5aR expression in the WAT, we analyzed C5aR mRNA in the gWAT obtained from mice that were fed an HFD. In particular, male WT mice were fed an ND or HFD

FIGURE 1. The role of C5aR in DIO. (A) RNA from gWAT of male WT mice fed an ND or an HFD (gray bars) for up to 26 wk was extracted. The respective C5aR mRNA expression was normalized against 18S. Data are displayed as mean ± SEM (n = 3–5 mice per group) and are shown as percentage of control. C5aR expression of lean gWAT (i.e., under ND conditions) at the respective time point represents the 100% control. *p ≤ 0.05, **p ≤ 0.01. (B) Representative IHC of gWAT from male WT mice fed an ND or HFD for 18 wk stained for C5a (red) and hematoxylin (blue) are depicted. Left panels, negative control; right panels, samples stained with anti-C5a Ab. Arrowheads indicate C5a+ staining. The majority of the diffuse C5a staining is localized to macrophage-rich CLSs. Scale bars represent 100 μm. (C and D) C5aR-deficient (KO) or -sufficient (WT) male mice were fed an ND or an HFD (n = 7–19 per group). (C) The absolute body weight in grams is shown. Data are displayed as mean ± SEM. *p ≤ 0.05. (D) The difference of body weight gain in grams is shown. Data are displayed as mean ± SEM. The weight difference between the two groups under ND conditions was significant starting in the fifth week of feeding and remained significant through the end of the experiment. *p ≤ 0.05. (E and F) C5aR-deficient (KO) or -sufficient (WT) female mice were fed an ND or an HFD (n = 5–17 per group). (E) The absolute body weight in grams is shown. Data are displayed as mean ± SEM. (F) The difference of body weight gain in grams is shown. Data are displayed as mean ± SEM.
higher, as compared with male C5aR-sufficient lean sWAT and lean gWAT; respectively, whereas no significant difference in male sWAT and gWAT weights was observed upon HFD feeding between C5aR deficiency and C5aR sufficiency (Fig. 2A, 2E). Moreover, the adipocyte size of gWAT from C5aR-deficient male mice was increased, compared with that of C5aR-sufficient male mice under both ND and HFD feeding (Fig. 2B–D, 2F–H), although there was no difference in adipocyte size from obese sWAT owing to C5aR deficiency (Supplemental Fig. 1A, 1B). The increased adipocyte size and AT weight of male mice caused by C5aR deficiency was apparently not due to altered lipogenesis in the AT, as the expression of lipogenesis genes, such as ACC (acetyl-CoA carboxylase), FAS (fatty acid synthase), SREBP-1c (sterol regulatory element-binding protein), or PPARγ (peroxisome proliferator–activated receptor γ), was unchanged (Supplemental Fig. 1C). Together, these findings suggest that in male mice C5aR deficiency is associated with increased adipocyte size of the gWAT.

However, we could not detect any C5aR deficiency–related difference in female mice with regard to weight gain upon feeding of an ND or HFD (Fig. 1E, 1F); to total weight of sWAT and gWAT of lean and obese mice and to total weight of liver of obese mice (Supplemental Fig. 1D); and to adipocyte size of sWAT and gWAT of obese and gWAT of lean mice (Supplemental Fig. 1E, 1G). Thus, the increase in adipocyte size of the gWAT associated with C5aR deficiency was present only in males.

Next, we assessed the effect of C5aR deficiency on glucose tolerance and insulin sensitivity. C5aR-deficient male mice, upon feeding of an HFD, displayed a mild improvement in early insulin sensitivity after 19 wk of HFD feeding (Fig. 3A). The improved insulin sensitivity of C5aR-deficient mice upon HFD feeding was accompanied by improved glucose tolerance at 18 wk of an HFD (Fig. 3B). In addition to a GTT after overnight starvation (shown in Fig. 3B), we also performed a GTT after a 6-h starvation period, which yielded similar results—that is, a mild but significant improvement in glucose tolerance in C5aR−/− mice compared with WT mice (data not shown). Data acquired in an experimental HFD feeding with female mice revealed a similar phenotype associated with C5aR deficiency—mildly improved insulin sensitivity (Fig. 3C) and glucose tolerance (Fig. 3D)—thereby strengthening the conclusion that C5aR partially contributes to insulin resistance. In the course of an ITT performed after a 6-h starvation period, a reduction in basal glucose values in C5aR−/− mice, compared with WT mice, was observed (data not shown). Besides slightly higher fasting glucose values in the GTT, C5aR-deficient male mice, despite being overweight, displayed no difference in glucose tol-

**FIGURE 2.** C5aR-deficient male mice have increased adipocyte size of the gWAT. (A) Tissue weights of sWAT, gWAT, or livers from male C5aR-deficient (KO, gray bars) or -sufficient (WT, black bars) mice fed an ND for 28 wk (n = 6–7) are depicted. Data are displayed as mean ± SEM. *p ≤ 0.05. (B) H&E staining of gWAT from male C5aR−/− (KO) or WT mice fed an ND is shown. Scale bars represent 100 μm. (C and D) The adipocyte diameters from H&E-stained gWAT of C5aR-deficient (KO, gray bars) or -sufficient (WT, black bars) male mice fed an ND (n = 5) were measured. (C) Mean diameter of adipocytes in micrometers is shown. Data are displayed as mean ± SEM. *p ≤ 0.05. (D) Distribution of adipocytes based on their diameter in micrometers is shown (the cell number is shown as percentage of total cells counted). (E) Tissue weights of sWAT, gWAT, or livers from male C5aR-deficient (KO, gray bars) or -sufficient (WT, black bars) mice fed an HFD for 28 wk (n = 4–5) are depicted. Data are displayed as mean ± SEM. (F) H&E staining of gWAT from male C5aR−/− (KO) or WT mice fed an HFD for 20 wk is shown. Scale bars represent 100 μm. (G and H) The adipocyte diameters from H&E-stained gWAT of C5aR-deficient (KO, gray bars) or -sufficient (WT, black bars) male mice fed an HFD for 20 wk (n = 4) were measured. (G) Mean diameter of adipocytes in micrometers is shown. Data are displayed as mean ± SEM. *p ≤ 0.05. (H) Distribution of adipocytes based on their diameter in micrometers is shown (the cell number is shown as percentage of total cells counted).

**FIGURE 3.** C5aR deficiency in obese mice results in mild improvement of insulin sensitivity. ITTs and GTTs were performed as described in Materials and Methods. (A) ITT of C5aR-deficient (KO) or -sufficient (WT) male mice after 19 wk on an HFD is shown. The blood glucose values are displayed as percentage of basal glucose. (B) GTT of C5aR-deficient (KO) or -sufficient (WT) male mice after 18 wk on an HFD is shown. The blood glucose values in milligrams per deciliter are shown. The blood glucose values are displayed as percentage of basal glucose. (C) ITT of C5aR-deficient (KO) or -sufficient (WT) female mice after 18 wk on an HFD is shown. The blood glucose values in milligrams per deciliter are shown. Data are displayed as mean ± SEM; n = 8–17 per group. *p ≤ 0.05, **p ≤ 0.001.
erance and insulin sensitivity under conditions of ND feeding, compared with C5aR-sufficient male mice (Supplemental Fig. 2A, 2B). Furthermore, no difference in fasting insulin levels (Supplemental Fig. 2C) or in leptin or adiponectin levels (data not shown) was observed upon HFD feeding, owing to C5aR deficiency.

To further verify and understand the partial contribution of C5aR to insulin resistance, we performed an in vivo insulin signaling assay. To this end, obese C5aR−/− or WT mice were fasted for 5 h prior to injection of insulin. Mice were euthanized 8 min after injection of insulin, and their gWAT, sWAT, and liver were collected and analyzed. We found that gWAT derived from C5aR−/− animals displayed a higher phosphorylated Akt/total Akt ratio, as assessed by Western blot analysis, thus indicating improved insulin sensitivity in C5aR deficiency (Fig. 4A, 4B). In contrast, insulin sensitivity of the obese sWAT or the liver was not altered owing to C5aR deficiency (Supplemental Fig. 2D, 2E). This finding is in accordance with the idea that, in obesity, visceral (gonadal) AT contributes more to insulin resistance than does subcutaneous AT. The weights of the C5aR-deficient and -sufficient mice engaged in insulin signaling experiments were not different (data not shown). These observations suggest that C5aR partially contributes to gWAT insulin resistance and not to resistance in other insulin target organs.

Consistently, we could not detect any difference in liver steatosis (Supplemental Fig. 3A) due to C5aR deficiency. Moreover, lipogenesis genes, such as ChREBP (carbohydrate-responsive element-binding protein), ACC, FAS, SREBP-1c, or PPARγ (Supplemental Fig. 3B), were indistinguishable between liver samples from C5aR-sufficient and -deficient mice.

Taken together, our findings suggest that, in DIO, C5aR partially contributes to insulin resistance via inhibition of insulin sensitivity of the gWAT.

Reduced AT inflammation and M1 macrophage polarization in C5aR-deficient mice upon HFD feeding

We then continued to explain the mild improvement in systemic and gWAT insulin sensitivity in obese C5aR-deficient mice. We have previously reported that the C5a–C5aR axis may promote macrophage polarization toward the proinflammatory M1 phenotype (23). The M1 cell type has recently been implicated in the development of insulin resistance (13, 14, 36). We therefore hypothesized that this function of C5aR could account for the observed phenotype of improved insulin sensitivity upon C5aR deficiency. First, we analyzed macrophage numbers in the WAT of HFD-fed C5aR-deficient and -proficient mice by IHC analysis for the macrophage marker F4/80 (Fig. 5). Indeed, reduced accumulation of F4/80+ macrophages in gWAT of C5aR−/− mice, compared with C5aR-proficient mice, accompanied by decreased formation of macrophage-related CLSs, was observed.

To further analyze changes in AT inflammation caused by C5aR deficiency, we determined the inflammatory profile of WAT by flow cytometry and quantitative PCR. The immune cell subpopulations in the SVF from sWAT and gWAT of C5aR-sufficient and -deficient mice fed an HFD were analyzed by flow cytometry. Although C5aR-sufficient and -deficient mice did not differ in the numbers of sWAT and gWAT total leukocytes (as assessed by staining for CD45) (Fig. 6A), intriguingly, the numbers of total macrophages (assessed as F4/80+CD11b+ cells), as well as the numbers of M1-like proinflammatory macrophages (assessed in two independent ways as F4/80+CD11b+CD11c+ or F4/80+CD11c+CD206− cells) in the sWAT and gWAT, were significantly reduced in C5aR-deficient compared with C5aR-sufficient mice (Fig. 6B–D). The number of anti-inflammatory M2-type macrophages (F4/80+CD11c−CD206+ cells) was statistically not different (Fig. 6E) between the two groups. Recent reports suggested that a subpopulation of CD11c+ M1-type macrophages exists that expresses the M2-type marker CD206 (11). These “intermediate” polarized macrophages (F4/80+CD11c+CD206− cells) are considered proinflamm-
CD4+CD8+ and CD4+ T cells in the sW AT and gW AT were unaltered in the respective tissues of C5aR-sufficient mice (Fig. 6F). In contrast, the proinflammatory cytokines IL-6 and TNF-α were increased in macrophages and total leukocytes between C5aR-deficient and C5aR-sufficient mice upon ND feeding, as assessed by flow cytometry (data not shown). Together, these data indicate a reduction of M1-like macrophages in the obese AT, owing to C5aR deficiency. Moreover, we tested whether changes in pancreatic islet morphology and function could contribute to the improvement in glucose tolerance of obese C5aR-deficient mice. To this end, we have analyzed islet morphology by insulin staining of the pancreas (Fig. 4C). The reduced WAT fibrosis could be linked to the decreased amount of “intermediate” polarized, proinflammatory F4/80+CD11c+CD206+ macrophages (Fig. 6F) in the gWAT of C5aR-deficient mice, as this cell type is often detected in CLSs of fibrotic visceral tissue and is thought to trigger remodeling of dysfunctional WAT (11, 37, 39).

C5aR deficiency does not affect pancreas morphology or β cell mass

An intimate link between obesity and WAT inflammation has been identified recently (1, 3, 13, 14, 40, 41). The inflamed microenvironment of the obese WAT triggers the accumulation of leukocytes, especially macrophages, in the WAT (1, 3, 13, 14, 40–42). Macrophages accumulate mainly in the visceral WAT, which is in keeping with the higher contribution of the visceral than of the subcutaneous fat to insulin resistance (40, 41). In this study, we identified a previously unknown component of the intriguing crosstalk between macrophage accumulation and activation in the WAT and insulin resistance development. Specifically, we found that the C5a–C5aR axis contributes to macrophage accumulation in the gWAT, which results in improved gWAT insulin resistance, whereas no alterations in the liver or the pancreatic islets were observed in C5aR-deficient mice.

**Figure 6.** C5aR deficiency results in reduced macrophage accumulation and M1 polarization in the obese WAT. The SVF of sWAT and gWAT from C5aR-deficient (KO, gray bars) or -sufficient (WT, black bars) male mice fed an HFD for 20 wk (n = 8–9 per group) was analyzed by flow cytometry. The absolute cell numbers of (A) total leukocytes (CD45+); (B) total macrophages (F4/80+CD11b+); (C) M1 macrophages, characterized as F4/80+CD11b+CD11c+; (D) M1 macrophages, characterized as F4/80+CD11c+CD206+; (E) M2 macrophages, characterized as F4/80+CD11c−CD206+; (F) proinflammatory “intermediate” M1/M2-type macrophages, characterized as F4/80+CD11c+CD206+; (G) CD8+ T cells (CD3+CD4−CD8+); and (H) CD4+ T cells (CD3+CD4+CD8−) are shown. Data are displayed as mean ± SEM. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

**Discussion**

The seeming discrepancy between increased AT weight, adipocyte size (Fig. 2), and mildly improved insulin sensitivity associated with impaired AT inflammation caused by C5aR deficiency in obese male mice prompted us to analyze WAT fibrosis, which is linked to AT inflammation and indicates AT dysfunction. WAT fibrosis occurs by remodeling of the extracellular matrix in the dysfunctional obese AT and is mediated by macrophages (11, 38). This process restricts expansion of adipocytes, limiting their size and capacity for triglyceride storage (11, 38). In other words, we hypothesized that increased adipocyte size in the less inflamed and more insulin-sensitive C5aR-deficient AT could partially be linked to reduced WAT fibrosis. By Masson’s trichrome staining, which is specific for collagen deposition, we found that gWAT of obese C5aR−/− mice displayed a striking reduction of fibrotic areas, compared with the gWAT of C5aR-sufficient mice (Supplemental Fig. 4C). The reduced WAT fibrosis could be linked to the decreased amount of “intermediate” polarized, proinflammatory F4/80+CD11c+CD206+ macrophages (Fig. 6F) in the gWAT of C5aR-deficient mice, as this cell type is often detected in CLSs of fibrotic visceral tissue and is thought to trigger remodeling of dysfunctional WAT (11, 37, 39).

C5aR deficiency does not affect pancreas morphology or β cell mass

Moreover, we tested whether changes in pancreatic islet morphology and function could contribute to the improvement in glucose tolerance of obese C5aR-deficient mice. To this end, we have analyzed islet morphology by insulin staining of the pancreas of WT and C5aR−/− male mice on DIO and by measuring β cell mass. No differences in islet morphology or β cell mass resulting from C5aR deficiency were detectable (Supplemental Fig. 3D, 3E).

Taken together, our data suggest that the mild improvement in glucose tolerance and insulin resistance in C5aR-deficient mice is due to reduced inflammation and fibrosis in the gWAT, which results in improved gWAT insulin resistance, whereas no alterations in the liver or the pancreatic islets were observed in C5aR-deficient mice.

**Figure 6.** C5aR deficiency results in reduced macrophage accumulation and M1 polarization in the obese WAT. The SVF of sWAT and gWAT from C5aR-deficient (KO, gray bars) or -sufficient (WT, black bars) male mice fed an HFD for 20 wk (n = 8–9 per group) was analyzed by flow cytometry. The absolute cell numbers of (A) total leukocytes (CD45+); (B) total macrophages (F4/80+CD11b+); (C) M1 macrophages, characterized as F4/80+CD11b+CD11c+; (D) M1 macrophages, characterized as F4/80+CD11c+CD206+; (E) M2 macrophages, characterized as F4/80+CD11c−CD206+; (F) proinflammatory “intermediate” M1/M2-type macrophages, characterized as F4/80+CD11c+CD206+; (G) CD8+ T cells (CD3+CD4−CD8+); and (H) CD4+ T cells (CD3+CD4+CD8−) are shown. Data are displayed as mean ± SEM. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

**Discussion**

An intimate link between obesity and WAT inflammation has been identified recently (1, 3, 13, 14, 40, 41). The inflamed microenvironment of the obese WAT triggers the accumulation of leukocytes, especially macrophages, in the WAT (1, 3, 13, 14, 40–42). Macrophages accumulate mainly in the visceral WAT, which is in keeping with the higher contribution of the visceral than of the subcutaneous fat to insulin resistance (40, 41). In this study, we identified a previously unknown component of the intriguing crosstalk between macrophage accumulation and activation in the WAT and insulin resistance development. Specifically, we found that the C5a–C5aR axis contributes to macrophage accumulation in the gWAT, to macrophage polarization to M1 cells, as well as to WAT fibrosis and thereby to WAT insulin resistance.

Whereas female C5aR-deficient mice did not show any difference in weight gain or adipocyte size upon ND or HFD feeding, male C5aR-deficient mice were prone to increased adipocyte size of the gWAT upon feeding of an ND and an HFD, as well as to increased weight gain under ND conditions, compared with C5aR-sufficient mice. Intriguingly, we observed a mild, yet significant, improvement in systemic insulin sensitivity in both sexes in C5aR deficiency. Whereas we found no influence of C5aR deficiency on the insulin sensitivity of the liver or on pancreatic β cell mass in the course of DIO, we found improved insulin sensitivity of the obese gWAT in C5aR deficiency, associated with decreased accumula-
tion of macrophages in the WAT—and their reduced polarization to proinflammatory M1 cells—as well as with decreased AT fibrosis. Moreover, IL-10 in the obese gWAT was higher owing to C5aR deficiency; consistent with our finding, IL-10 was recently shown to protect mice from DIO and glucose intolerance (43). The levels of C5aR were increased in the obese AT. Moreover, we found that C5a was activated in the obese AT, as opposed to lean AT; C5a in the obese AT localized mainly in the macrophage-rich CLSs of the obese AT. Together, these data suggest that C5a accumulated in the obese AT localizes in the proximity of AT macrophages and signals via C5aR on the latter, mediating further accumulation of macrophages, their polarization to the M1 proinflammatory phenotype, and AT inflammation and fibrosis—and thereby contributing to gWAT insulin resistance.

Our data on the role of C5aR in AT inflammation provide evidence for a novel function of the complement system in obesity. Previous studies mostly referred to the complement component ASP, a degradation product of C3a, as a regulator of adipogenesis. ASP can regulate fatty acid uptake and storage in adipocytes (15, 44, 45). In addition, disruption of the second C5a receptor (46), C5L2, in mice resulted in development of diet-induced insulin resistance, associated with altered ectopic fat deposition and a proinflammatory phenotype (47). These apparently distinct functions of the two C5a receptors—C5aR, as analyzed in the current study, and C5L2, as shown previously (15, 17, 19, 20, 44, 46–48)—in obesity, AT inflammation, and development of insulin resistance are intriguing and merit further investigation.

In fact, the increased adipocyte size in C5aR−/− male mice, as observed in this study, could be due to increased C5a-mediated signaling via C5L2, as well as the different expression and function of C5aR and C5L2 in distinct cell populations in the AT microenvironment. C5L2 deficiency has been associated with reduced adipocyte size, higher glucose uptake, and worsened insulin resistance (47, 49). Thus, enhanced activation of C5L2 by C5a in the absence of C5aR could lead to increased adipocyte size and weight gain in C5aR-deficient mice under ND conditions. We think that a future study should be performed that compares mice having a double deficiency of C5aR and C5L2 with mice having a single deficiency of either receptor, as well as with WT mice, to further address in detail the crosstalk between both C5a receptors in the AT.

A common denominator may exist for the reciprocal regulation of macrophage numbers and adipocyte size in the gWAT attributable to C5aR deficiency in males. Although increased adipocyte size positively correlates with the frequency of adipocyte death (50) leading to increased leukocyte recruitment in the AT (1, 3, 13, 14, 40–42), one function of macrophages in the obese WAT is the remodeling of the extracellular matrix around hypertrophic adipocytes (11, 38), rendering the tissue fibrotic, which could in turn limit adipocyte growth and the capacity for triglyceride storage. Consistently, more fibrosis is found in obese, macrophage-rich visceral WAT than in macrophage-poorer subcutaneous fat (11). In line with this finding, we detected fewer fibrotic areas in the gWAT from C5aR-deficient mice. This finding is likely linked not only to the decreased accumulation of the proinflammatory M1 macrophages but also to the reduced numbers of the profibrotic “intermediate” M1/M2-type F4/80+/CD11c−CD206+ macrophages in the WAT, owing to C5aR deficiency. Intriguingly, reduced fibrosis as a result of C5aR inhibition has been previously described for various tissues, including the heart, kidney, and lung (51–53). Besides its known role in leukocyte recruitment and tissue inflammation, C5a could also directly interact with tissue fibroblasts and alter their ability for tissue remodeling (51). In the case of WAT, it is conceivable that the reduced gWAT fibrosis of C5aR-deficient mice could potentially explain the phenotype of increased adipocyte size. Of interest, a similar phenotype (improved peripheral insulin sensitivity despite increased adipocyte size) was observed in GM-CSF–deficient mice, which had decreased macrophage numbers in the WAT as well (54). The potential reciprocal correlation between macrophage numbers and adipocyte size in obese AT needs to be addressed in future investigations.

Intriguingly, C5aR deficiency had no influence on islet morphology or β cell mass. Increased glucose levels and pancreatic amyloid deposition in obesity and diabetes may result in β cell death, a process that is regulated by inflammatory cells (55–57). Amyloid deposits can promote complement activation, thereby enhancing islet inflammation (48, 58). In our studies we could not detect any effect of C5aR deficiency on pancreatic islets in the course of DIO. Thus, the liver and the pancreas remained unaffected in obese C5aR-deficient mice, and only obese gWAT insulin sensitivity was improved in C5aR-deficiency, accompanied by decreased total and M1 macrophages in the WAT and reduced AT fibrosis. These findings are quite in keeping with the relatively mild metabolic phenotype in obesity attributable to C5aR deficiency, as altering only one of the many components contributing to insulin resistance, namely, WAT macrophages, could hardly influence whole-body insulin resistance more than the phenotype observed in this study. Our present findings also underline that insulin resistance can only be partially attributed to WAT macrophage activation and polarization. Consistent with our findings, in a very recent article, a C5aR antagonist inhibited DIO-induced metabolic dysfunction in rats (59), whereas another very recent study (60) did not find alterations in GTT and ITT owing to C5aR deficiency in mice, which is likely explained by the different high-fat diets used in the study by Roy et al. (60) and our study. Taken together, our data suggest that the complement C5a–C5aR axis contributes to macrophage accumulation and polarization to M1 cells in the obese WAT—and thereby to AT inflammation and fibrosis and the development of AT insulin resistance. Our findings may also have potential therapeutic implications: C5aR might represent a novel target to control progression of insulin resistance in obese individuals, a hypothesis worth testing in future studies.

Acknowledgments

We thank Sylvia Grossklaus, Janine Gebler, Marta Prucnal, Jindrich Chmelar, Christine Mund, and Bettina Gercken for technical assistance.

Disclosures

The authors have no financial conflicts of interest.

References

Suppl Fig. 1: Metabolic effects of C5aR-deficiency in male and female mice.

A and B) The adipocyte diameters from HE stained sWAT of male C5aR-deficient (KO, grey bars) or –sufficient (WT, black bars) mice fed a HFD for 20 weeks were measured (n = 5 / group). A) Mean diameter of adipocytes in μm is shown. Data are displayed as mean ± SEM. n.s. = not significant. B) Distribution of adipocytes based on their diameter in μm is shown (the cell number is shown as percentage of total cells counted).

C) RNA from gonadal (g) WAT of C5aR-deficient or -sufficient male mice fed a HFD for 20 weeks was extracted and analyzed with quantitative real-time PCR. The expression of lipogenesis-related genes ACC, FAS, SREBP-1c, PPARg and PPARg2 was studied. The respective mRNA was normalized against 18s. Data are displayed as mean ± SEM (n = 5–6 / group) and are shown as percentage of the WT control. The expression of ACC, FAS, SREBP-1c, PPARg or PPARg2 in WT mice represents the 100% control. n.s. = not significant.

D) Tissue weights of subcutaneous (s) WAT, gonadal (g) WAT from female C5aR-deficient (KO, grey bars) or –sufficient (WT, black bars) mice fed a ND for 16 weeks (left panel) and tissue weights of subcutaneous (s) WAT, gonadal (g) WAT or livers from female C5aR-deficient (KO, grey bars) or –sufficient (WT, black bars) mice fed a HFD (right panel) for 20 weeks are depicted. Data are displayed as mean ± SEM; (n = 5-17 / group). n.s. = not significant.

E) and F) The adipocyte diameters from gWAT (left panels) and sWAT (right panels) of C5aR-deficient (KO, grey bars) or –sufficient (WT, black bars) female mice fed a HFD for 20 weeks (n = 5 / group) were measured. E) Mean diameter of adipocytes in μm is shown. Data are displayed as mean ± SEM. n.s. = not significant. F) Distribution of adipocytes based on their diameter in μm is shown (the cell number is shown as percentage of total cells counted). There is no difference in adipocyte size due to C5aR-deficiency in female mice.
G) Representative images of HE stained gWAT from female C5aR-/- (KO) or –sufficient (WT) mice fed a ND for 16 weeks are shown. Scale bars represent 100 μm. There is no difference in adipocyte size due to C5aR-deficiency in female mice.

Suppl Fig. 2:

A and B) Glucose tolerance tests (GTT) and insulin tolerance tests (ITT) were performed as described under Material and Methods (n = 6 – 7 / group). Blood glucose is shown as mg/dl. A) GTT of C5aR-deficient (KO) or –sufficient (WT) male mice after 18 weeks on ND is shown. B) ITT of C5aR-deficient (KO) or –sufficient (WT) male mice after 19 weeks on ND is shown. Data are displayed as mean ± SEM. *P ≤ 0.05.

C) Sera obtained after a 5 h starvation period from C5aR-deficient (KO; grey bars) or –sufficient (WT; black bars) male mice before or after 9 weeks on HFD were tested by ELISA for mouse insulin. Data are displayed as mean ± SEM (n = 7 animals per group). n.s. = not significant.

D and E) C5aR-sufficient (WT) or –deficient (KO) male mice fed a HFD for 28 weeks were fasted and then injected i.p. with insulin; after 8 min, mice were euthanized, the subcutaneous (s) WAT and the liver were extracted and tissue lysates were analyzed for the phosphorylated Akt / total Akt ratio by western blot. Densitometric analysis from sWAT (D) or liver (E) of phosphorylated Akt / total Akt is shown. Data are mean ± SEM (n = 4-5) and are shown as percentage of control. The phosphorylated Akt / total Akt ratio in the WT mice represents the 100% control. n.s. = not significant.

Suppl Fig. 3: No differences in hepatic steatosis or pancreatic morphology due to C5aR-deficiency in diet-induced obesity.

A) Representative sections of livers from C5aR-/- (KO) or WT mice (20 weeks on HFD) stained with Oil Red O staining at 100x are depicted; liver steatosis associated lipid droplets
are stained red. C5aR/- mice showed no difference in liver steatosis as compared to C5aR-sufficient mice.

B) RNA from liver tissues of C5aR-deficient or -sufficient male mice fed a HFD for 20 weeks was extracted and analyzed with quantitative real-time PCR. The expression of the lipogenesis related genes ACC, FAS, SREBP-1c, ChREBP and PPARg was studied. The respective mRNA was normalized against 18s. Data are displayed as mean ± SEM (n = 6 / group) and are shown as percentage of the WT control. The expression of ACC, FAS, SREBP-1c, ChREBP or PPARg of WT mice represents the 100% control. n.s. = not significant.

C) RNA from liver tissue of C5aR-deficient or -sufficient male mice fed a HFD for 20 weeks was extracted and analyzed with quantitative real-time PCR. The expression of the pro-inflammatory markers CCL3, CCL4, CCL5, IL-6, MCP-1, TNF and of the anti-inflammatory cytokine IL-10 was studied. The respective mRNA was normalized against 18s. Data are displayed as mean ± SEM (n = 5–6 / group) and are shown as percentage of the WT control. The expression of CCL3, CCL4, CCL5, IL-6, MCP-1, TNF or IL-10 from WT mice represents the 100% control. n.s. = not significant.

D) IHC of pancreas stained for insulin and haematoxylin from male C5aR-deficient (KO) or -sufficient (WT) mice that were for 12 weeks on HFD are depicted. Scale bars represent 50 μm.

E) The pancreatic β-cell mass of C5aR-deficient (KO, grey bars) or –sufficient (WT, black bars) mice fed a HFD (n = 5 / group) was measured. β-cell mass in mg is shown. Data are displayed as mean ± SEM. n.s. = not significant.

Suppl Fig. 4: Effect of C5aR deficiency in WAT inflammation and WAT fibrosis. A-B) RNA from gonadal (g) WAT of C5aR-deficient or -sufficient male mice fed a HFD for 20 weeks was extracted and analyzed with quantitative real-time PCR. The expression of A) IL-10, or B) TNF and IL-6 was studied. The respective mRNA was normalized against 18s. Data
are displayed as mean ± SEM (n = 5–6 / group) and are shown as percentage of the WT control. The expression of IL-10, TNF or IL-6 of WT mice represents the 100% control. **P ≤ 0.01; n.s. = not significant. C) Representative IHC of gonadal WAT from male C5aR-/- (KO) or WT mice (20 weeks on HFD) stained for collagen deposition with Masson’s trichrome staining at 100x are depicted; C5aR-/- mice show reduced density of fibrotic areas, as compared to C5aR-sufficient mice.
Figures from Phieler et al., Supplemental Figure 2

Panel A: Glucose levels (mg/dl) over time (min) for KO and WT mice. There is a significant increase (*) at 15 minutes for KO mice.

Panel B: Glucose levels (mg/dl) over time (min) for KO and WT mice under different conditions.

Panel C: Insulin levels (ng/ml) for KO and WT mice at weeks 0 and 9 on a high-fat diet (HFD). There is no significant difference (n.s.) between the groups.

Panel D: Akt phosphorylation (%) for sWAT KO and WT mice. Akt phosphorylation is not significantly different (n.s.).

Panel E: Akt phosphorylation (%) for liver KO and WT mice. Akt phosphorylation is not significantly different (n.s.).
Phieler et al., Supplemental Figure 4

A

![Bar graph showing mRNA expression of IL-10.](image)

B

![Bar graph showing mRNA expression of IL-6 and TNF.](image)

C

![Images comparing KO and WT tissue.](image)

**KO**

**WT**

n.s. (not significant)