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

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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: 4367–4374.

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 C5aR 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 adipsin, 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

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

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and delayed triglyceride and fatty acid clearance from circulation after a fat meal (20). Furthermore, C3aR-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 Cedex, France). Mice 6 to 8 wk old were fed 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 levels were measured at baseline via tail-vein blood sampling with 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 HFD, and glucose levels 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 (Chrystal 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. Briefly, mice were euthanized, and subcutaneous white adipose tissue (sWAT) and gonadal WAT (gWAT) fat pads were isolated, minced, and digested with collagenase type I (2 mg/ml per milligram of tissue; Life Technologies, Darmstadt, Germany) for 45 min. The suspension was resuspended in DMEM containing 0.5% fatty acid-poor BSA (Sigma-Aldrich) and 1% penicillin/streptomycin, filtered through a 100- μ m cell strainer (BD, Heidelberg, Germany), and centrifuged at $300 \times g$ for 5 min to separate the floating adipocyte fraction from the pelleted SV fraction. For FACS analysis, the stromal vascular fraction (SVF) was resuspended in HBSS (Invitrogen) with 0.1% fatty acid-poor BSA and 0.1% NaN₃, filtered through a 40- μ m cell strainer. For staining, the following Abs were used: Fc receptor-blocking Ab 2.4G2, CD11b-APC, CD11c-PE (BD); CD45-Alexa Fluor 488, CD11c-APC (BioLegend, Fell, Germany); CD31-APC, F4/80-Alexa Fluor 488 (eBioscience, Frankfurt, Germany); CD206/MRC1-PE (Acris Abs, Herford, Germany); and CD8a-APC, CD4-FITC, CD3e-PE (Miltenyi Biotec, Bergisch Gladbach, Germany). Staining of CD206 was performed after overnight fixation of cells with a fixation/permeabilization buffer (eBioscience, Frankfurt, Germany). FACS was carried out on a FACSCanto II (BD) and analyzed with FACSDiva Version 6.1.3 software. For triple staining for F4/80/CD11b/CD11c, events being double positive for F4/80-Alexa Fluor 488 and CD11b-APC were selected and regated for CD11c positivity. For triple staining for F4/80/CD11c/CD206, events being positive for F4/80-Alexa Fluor 488 were selected and regated for being either positive for CD11c-APC, positive for CD206-PE or positive for both CD11c and CD206.

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 $\times$ 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 $14,000 \times g$ 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 $\times$ RIPA lysis buffer and centrifuged at $13,000 \times g$ 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 μ g for sWAT and gWAT, 25 μ g 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% w/v skim 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, Dusseldorf, 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 skim 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- μ m sections were cut and mounted, and staining was performed with Mayer's hematoxylin (SAV, 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 (Marienfeld, Lauda-Königshofen, Germany) and stained with anti-F4/80 Ab (Novus Biologicals, Herford, Germany). For this purpose, Ag 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 were obtained at $\times 100$ magnification. The diameter of ~ 200 adipocytes per slide was measured with the AxioVision 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- μ m 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. Sections were stained with a rabbit anti-C5a Ab in blocking buffer at 4°C overnight. This anti-C5a Ab was generated using KLH-conjugated synthetic peptide encompassing a sequence within the N-terminal region of human C5a (thus, detection of C5 is not excluded) and was purified by protein-G affinity chromatography (1:200; Abbiotec, San Diego, CA). Specificity of the anti-C5a Ab was tested by showing that the anti-C5a reactivity could be blocked by preincubation of the Ab with C5a. In the negative control, the first Ab was omitted. After washing with PBS, the sections were probed with an HRP-conjugated goat anti-rabbit Ab (1:250; Sigma-Aldrich) in blocking buffer for 1 h. For signal detection, we used the AEC Peroxidase Substrate Kit (Vector Laboratories) according to the instructions of the manufacturer. Sections were counterstained with hematoxylin. Coverslipped slides were examined using a microscope (Zeiss, Oberkochen, Germany).

Freshly isolated pancreata were fixed in 4% PFA at 4°C for up to 4 d before embedding in paraffin. Sections were mounted on silane-treated slides (Marienfeld), and immunohistochemistry (IHC) staining was performed with an anti-insulin Ab and a secondary biotinylated goat anti-guinea pig

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). Finally, slides were counterstained with Mayer's hematoxylin and mounted with a water-based mounting medium. For determination of islet morphology, pictures were obtained as mosaics at $\times 100$ magnification. 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 cyclor system (Bio-Rad) using gene-specific primers and normalization to 18S mRNA. Relative gene expression was calculated with the $\Delta\Delta C_t$ method (35). The fold-change ratio was calculated and expressed as mean $\pm$ 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

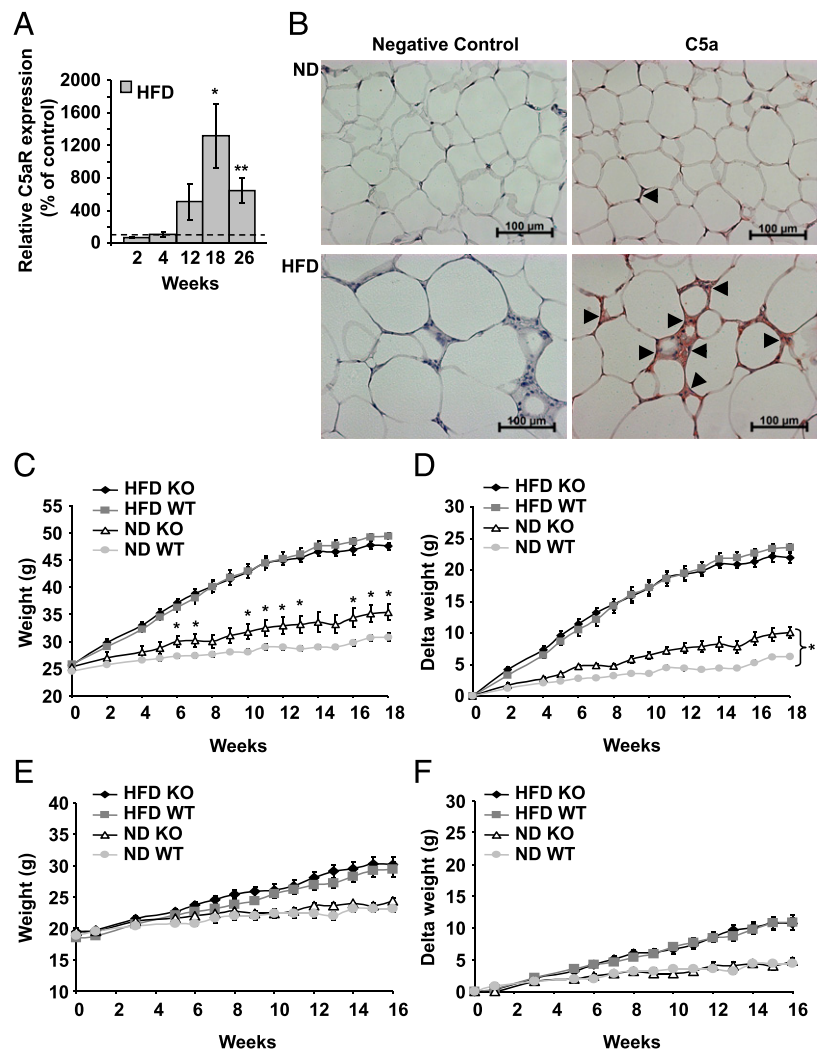
for different time periods up to 26 wk. C5aR mRNA expression increased upon DIO already at 12 wk on an HFD, continuing to increase and reaching a peak at 18 wk on an HFD and remaining significantly elevated at 26 wk on an HFD, compared with respective ND-fed mice (Fig. 1A). Thus, C5aR mRNA expression 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

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 $\pm$ 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. * $p \leq 0.05$, ** $p \leq 0.01$. **(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 μ m. **(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 $\pm$ SEM. * $p \leq 0.05$. **(D)** The difference of body weight gain in grams is shown. Data are displayed as mean $\pm$ 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. * $p \leq 0.05$. **(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 $\pm$ SEM. **(F)** The difference of body weight gain in grams is shown. Data are displayed as mean $\pm$ 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 PPAR γ (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-

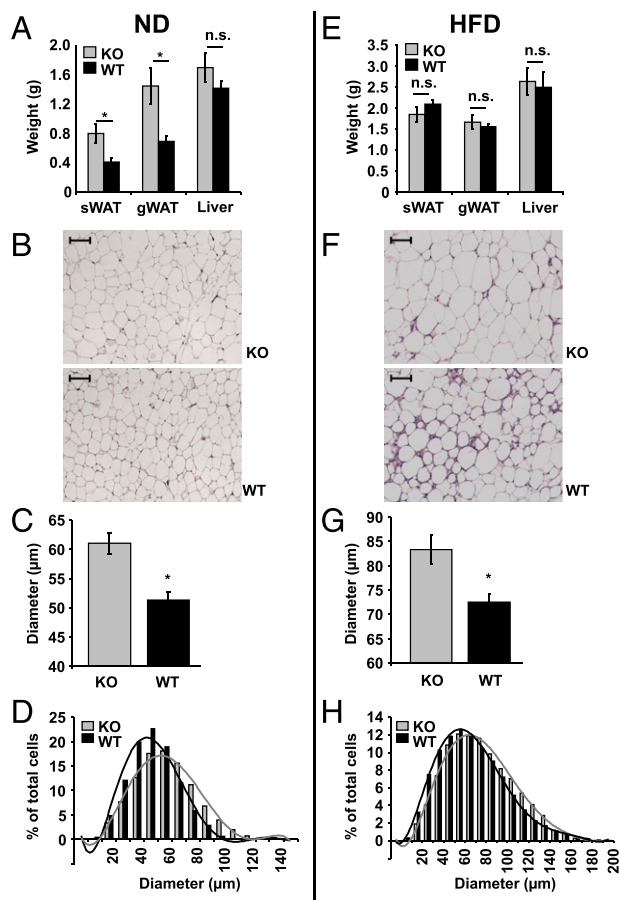


FIGURE 2. C5aR-deficient male mice have increased adipocyte size of the gWAT. (A) Tissue weights of sWAT, gWAT, or livers from male C5aR-deficient (KO, gray bars) or -sufficient (WT, black bars) mice fed an ND for 28 wk ($n = 6$ –7) are depicted. Data are displayed as mean $\pm$ SEM. $*p \leq 0.05$. (B) H&E staining of gWAT from male C5aR^{-/-} (KO) or WT mice fed an ND is shown. Scale bars represent 100 μ m. (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 $\pm$ SEM. $*p \leq 0.05$. (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 $\pm$ 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 μ m. (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 $\pm$ SEM. $*p \leq 0.05$. (H) Distribution of adipocytes based on their diameter in micrometers is shown (the cell number is shown as percentage of total cells counted).

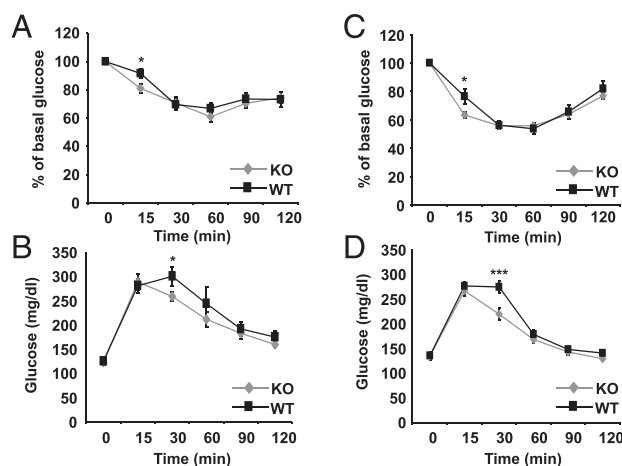


FIGURE 3. C5aR deficiency in obese mice results in mild improvement of insulin sensitivity. ITTs and GTTs were performed as described in *Materials and Methods*. (A) ITT of C5aR-deficient (KO) or -sufficient (WT) male mice after 19 wk on an HFD is shown. The blood glucose values are displayed as percentage 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. (C) ITT of C5aR-deficient (KO) or -sufficient (WT) female mice after 17 wk on an HFD is shown. The blood glucose values are displayed as percentage of basal glucose. (D) GTT of C5aR-deficient (KO) or -sufficient (WT) female mice after 16 wk on an HFD is shown. The blood glucose values in milligrams per deciliter are shown. Data are displayed as mean $\pm$ SEM; $n = 8$ –17 per group. $*p \leq 0.05$, $***p \leq 0.001$.

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 PPAR γ (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⁺) 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 proinflam-

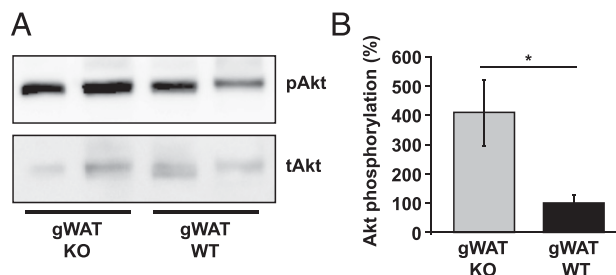


FIGURE 4. C5aR deficiency leads to improved insulin sensitivity in obese gWAT. C5aR-sufficient (WT) or -deficient (KO) male mice fed an HFD for 28 wk were fasted and then injected i.p. with insulin; after 8 min, mice were euthanized, the gWAT was extracted, and tissue lysates were analyzed for the phosphorylated Akt/total Akt ratio by Western blot. (A) Representative Western blot analysis from gWAT of insulin-induced Akt phosphorylation, as well as of total Akt, is shown. Membranes were stripped and reprobed with the total Akt Ab. (B) Densitometric analysis from gWAT of phosphorylated Akt/total Akt is shown. Data are mean $\pm$ SEM ($n = 4$) and are shown as percentage of control. The phosphorylated Akt/total Akt ratio in the WT mice represents the 100% control. * $p \leq 0.05$.

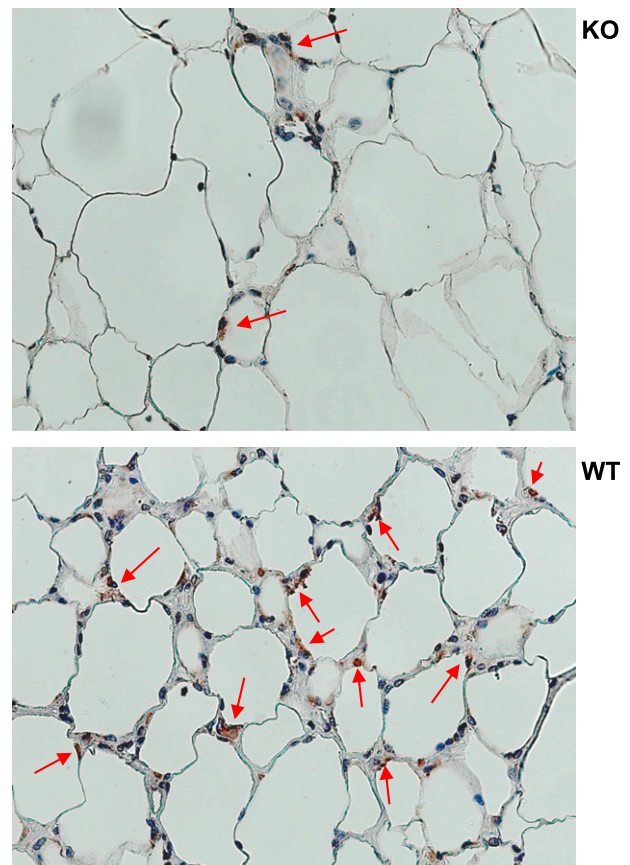


FIGURE 5. C5aR deficiency results in reduced macrophage accumulation in the obese gWAT. Representative IHC studies of gWAT from male C5aR^{-/-} (KO) or WT mice (20 wk on HFD) stained for F4/80 and hematoxylin at original magnification $\times 200$ are depicted. Arrows indicate F4/80⁺ macrophages. C5aR^{-/-} mice have fewer macrophages and macrophage-related CLSs, compared with C5aR-sufficient mice.

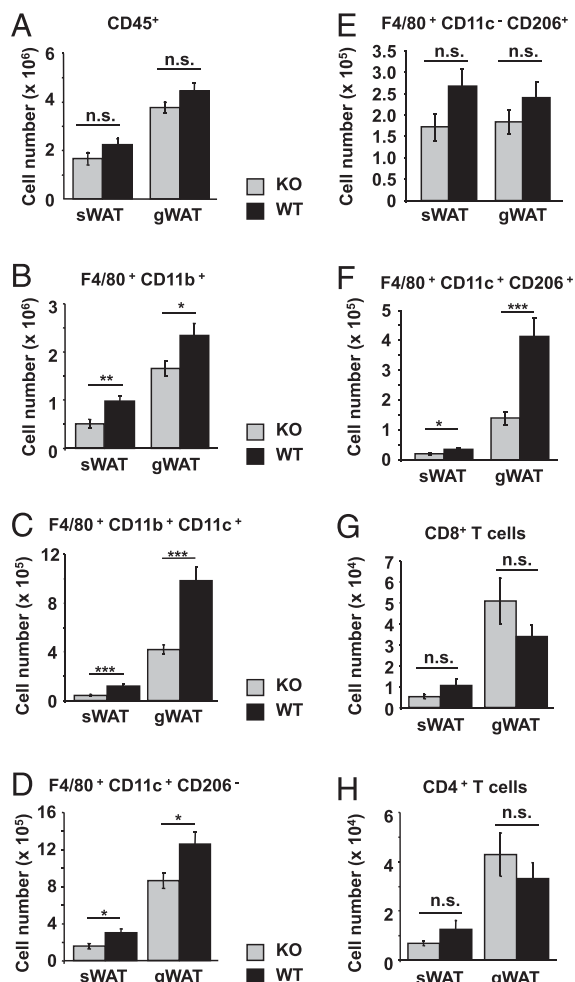


FIGURE 6. C5aR deficiency results in reduced macrophage accumulation and M1 polarization in the obese WAT. The SVF of sWAT and gWAT from C5aR-deficient (KO, gray bars) or -sufficient (WT, black bars) male mice fed an HFD for 20 wk ($n = 8-9$ per group) was analyzed by flow cytometry. The absolute cell numbers of (A) total leukocytes ($CD45^+ CD31^-$); (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 $\pm$ SEM. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

matory and are implicated in AT remodeling and dysfunction (11, 37). Of interest, the number of these macrophages was also reduced in the sWAT and gWAT of C5aR $^{-/-}$ mice, compared with the respective tissues of C5aR-sufficient mice (Fig. 6F). In contrast, $CD8^+$ and $CD4^+$ T cells in the sWAT and gWAT were unaltered owing to C5aR deficiency (Fig. 6G, 6H). No significant differences in macrophages and total leukocytes between C5aR-deficient and -sufficient mice were observed 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.

Furthermore, by quantitative PCR analysis, we found that the expression of anti-inflammatory IL-10 was higher in the obese gWAT, but not in the obese sWAT, of C5aR-deficient mice, compared with WT mice (Supplemental Fig. 4A; sWAT data not shown). In contrast, the proinflammatory cytokines IL-6 and TNF were not significantly lower owing to C5aR deficiency (Supple-

mental Fig. 4B). No differences in the cytokine milieu of the liver caused by C5aR deficiency were observed (Supplemental Fig. 3C).

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 deficiency 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 deficiency.

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 WAT, 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.

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Disclosures

The authors have no financial conflicts of interest.

References

1. Schenk, S., M. Saberi, and J. M. Olefsky. 2008. Insulin sensitivity: modulation by nutrients and inflammation. *J. Clin. Invest.* 118: 2992–3002.
2. Odegaard, J. I., and A. Chawla. 2008. Mechanisms of macrophage activation in obesity-induced insulin resistance. *Nat. Clin. Pract. Endocrinol. Metab.* 4: 619–626.
3. Weisberg, S. P., D. McCann, M. Desai, M. Rosenbaum, R. L. Leibel, and A. W. Ferrante, Jr. 2003. Obesity is associated with macrophage accumulation in adipose tissue. *J. Clin. Invest.* 112: 1796–1808.
4. Weber, C., and H. Noels. 2011. Atherosclerosis: current pathogenesis and therapeutic options. *Nat. Med.* 17: 1410–1422.
5. Chatzigeorgiou, A., K. P. Karalis, S. R. Bornstein, and T. Chavakis. 2012. Lymphocytes in obesity-related adipose tissue inflammation. *Diabetologia* 55: 2583–2592.
6. Chmelar, J., K. J. Chung, and T. Chavakis. 2013. The role of innate immune cells in obese adipose tissue inflammation and development of insulin resistance. *Thromb. Haemost.* 109: 399–406.
7. Ferrante, A. W., Jr. 2007. Obesity-induced inflammation: a metabolic dialogue in the language of inflammation. *J. Intern. Med.* 262: 408–414.
8. Chawla, A., K. D. Nguyen, and Y. P. Goh. 2011. Macrophage-mediated inflammation in metabolic disease. *Nat. Rev. Immunol.* 11: 738–749.
9. Lumeng, C. N., S. M. Deyoung, J. L. Bodzin, and A. R. Saltiel. 2007. Increased inflammatory properties of adipose tissue macrophages recruited during diet-induced obesity. *Diabetes* 56: 16–23.

10. Mantovani, A., A. Sica, S. Sozzani, P. Allavena, A. Vecchi, and M. Locati. 2004. The chemokine system in diverse forms of macrophage activation and polarization. *Trends Immunol.* 25: 677–686.
11. Morris, D. L., K. Singer, and C. N. Lumeng. 2011. Adipose tissue macrophages: phenotypic plasticity and diversity in lean and obese states. *Curr. Opin. Clin. Nutr. Metab. Care* 14: 341–346.
12. Lumeng, C. N., J. B. DelProposto, D. J. Westcott, and A. R. Saltiel. 2008. Phenotypic switching of adipose tissue macrophages with obesity is generated by spatiotemporal differences in macrophage subtypes. *Diabetes* 57: 3239–3246.
13. Xu, H., G. T. Barnes, Q. Yang, G. Tan, D. Yang, C. J. Chou, J. Sole, A. Nichols, J. S. Ross, L. A. Tartaglia, and H. Chen. 2003. Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. *J. Clin. Invest.* 112: 1821–1830.
14. Lumeng, C. N., S. M. Deyoung, and A. R. Saltiel. 2007. Macrophages block insulin action in adipocytes by altering expression of signaling and glucose transport proteins. *Am. J. Physiol. Endocrinol. Metab.* 292: E166–E174.
15. Ricklin, D., G. Hajishengallis, K. Yang, and J. D. Lambris. 2010. Complement: a key system for immune surveillance and homeostasis. *Nat. Immunol.* 11: 785–797.
16. Hamad, O. A., J. Bäck, P. H. Nilsson, B. Nilsson, and K. N. Ekdahl. 2012. Platelets, complement, and contact activation: partners in inflammation and thrombosis. *Adv. Exp. Med. Biol.* 946: 185–205.
17. Patrick, M., J. Luckett, L. Yue, and C. Stover. 2009. Dual role of complement in adipose tissue. *Mol. Immunol.* 46: 755–760.
18. Choy, L. N., B. S. Rosen, and B. M. Spiegelman. 1992. Adipsin and an endogenous pathway of complement from adipose cells. *J. Biol. Chem.* 267: 12736–12741.
19. Maslowska, M., H. Vu, S. Phelis, A. D. Sniderman, B. M. Rhode, D. Blank, and K. Cianflone. 1999. Plasma acylation stimulating protein, adiponin and lipids in non-obese and obese populations. *Eur. J. Clin. Invest.* 29: 679–686.
20. MacLaren, R., W. Cui, and K. Cianflone. 2008. Adipokines and the immune system: an adipocentric view. *Adv. Exp. Med. Biol.* 632: 1–21.
21. Mamane, Y., C. Chung Chan, G. Lavallee, N. Morin, L. J. Xu, J. Huang, R. Gordon, W. Thomas, J. Lamb, E. E. Schadt, et al. 2009. The C3a anaphylatoxin receptor is a key mediator of insulin resistance and functions by modulating adipose tissue macrophage infiltration and activation. *Diabetes* 58: 2006–2017.
22. Höpken, U. E., B. Lu, N. P. Gerard, and C. Gerard. 1996. The C5a chemoattractant receptor mediates mucosal defence to infection. *Nature* 383: 86–89.
23. Langer, H. F., K. J. Chung, V. V. Orlova, E. Y. Choi, S. Kaul, M. J. Kruhlak, M. Alatsianios, R. A. DeAngelis, P. A. Roche, P. Magotti, et al. 2010. Complement-mediated inhibition of neovascularization reveals a point of convergence between innate immunity and angiogenesis. *Blood* 116: 4395–4403.
24. Kim, D. H., M. A. Sartor, J. R. Bain, D. Sandoval, R. D. Stevens, M. Medvedovic, C. B. Newgard, S. C. Woods, and R. J. Seeley. 2012. Rapid and weight-independent improvement of glucose tolerance induced by a peptide designed to elicit apoptosis in adipose tissue endothelium. *Diabetes* 61: 2299–2310.
25. Stanford, K. I., R. J. Middelbeek, K. L. Townsend, D. An, E. B. Nygaard, K. M. Hitchcox, K. R. Markan, K. Nakano, M. F. Hirshman, Y. H. Tseng, and L. J. Goodyear. 2013. Brown adipose tissue regulates glucose homeostasis and insulin sensitivity. *J. Clin. Invest.* 123: 215–223.
26. Jonker, J. W., J. M. Suh, A. R. Atkins, M. Ahmadian, P. Li, J. Whyte, M. He, H. Jugulion, Y. Q. Yin, C. T. Phillips, et al. 2012. A PPAR γ -FGF1 axis is required for adaptive adipose remodelling and metabolic homeostasis. *Nature* 485: 391–394.
27. Kiechl, S., J. Wittmann, A. Giacari, M. Knoflach, P. Willeit, A. Bozec, A. R. Moschen, G. Muscogiuri, G. P. Sorice, T. Kireva, et al. 2013. Blockade of receptor activator of nuclear factor- κ B (RANKL) signaling improves hepatic insulin resistance and prevents development of diabetes mellitus. *Nat. Med.* 19: 358–363.
28. Kornfeld, J. W., C. Baitzel, A. C. Köhner, H. T. Nicholls, M. C. Vogt, K. Hermanns, L. Scheja, C. Haumaitre, A. M. Wolf, U. Knippschild, et al. 2013. Obesity-induced overexpression of miR-802 impairs glucose metabolism through silencing of Hnf1b. *Nature* 494: 111–115.
29. Choi, E. Y., V. V. Orlova, S. C. Fagerholm, S. M. Nurmi, L. Zhang, C. M. Ballantyne, C. G. Gahmberg, and T. Chavakis. 2008. Regulation of LFA-1-dependent inflammatory cell recruitment by Cbl-b and 14-3-3 proteins. *Blood* 111: 3607–3614.
30. Agouni, A., C. Owen, A. Czepek, N. Mody, and M. Delibegovic. 2010. In vivo differential effects of fasting, re-feeding, insulin and insulin stimulation time course on insulin signaling pathway components in peripheral tissues. *Biochem. Biophys. Res. Commun.* 401: 104–111.
31. Bajzer, M., M. Olivieri, M. K. Haas, P. T. Pfluger, I. J. Magrisso, M. T. Foster, M. H. Tschöp, K. A. Krawczewski-Carhuatanta, D. Cota, and S. Obici. 2011. Cannabinoid receptor 1 (CB1) antagonism enhances glucose utilisation and activates brown adipose tissue in diet-induced obese mice. *Diabetologia* 54: 3121–3131.
32. Eskin, M. A., R. Jotwani, T. Abe, J. Chmela, J. H. Lim, S. Liang, P. A. Ciero, J. L. Krauss, F. Li, M. Rauner, et al. 2012. The leukocyte integrin antagonist Del-1 inhibits IL-17-mediated inflammatory bone loss. *Nat. Immunol.* 13: 465–473.
33. Langer, H. F., E. Y. Choi, H. Zhou, R. Schleicher, K. J. Chung, Z. Tang, K. Göbel, K. Bdeir, A. Chatzigeorgiou, C. Wong, et al. 2012. Platelets contribute to the pathogenesis of experimental autoimmune encephalomyelitis. *Circ. Res.* 110: 1202–1210.
34. Jiang, H., H. Zhu, X. Chen, Y. Peng, J. Wang, F. Liu, S. Shi, B. Fu, Y. Lu, Q. Hong, et al. 2007. T1P-1 transgenic mice recover from diabetes induced by multiple low-dose streptozotocin. *Diabetes* 56: 49–56.
35. Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2 Δ (Δ CT) Method. *Methods* 25: 402–408.
36. Kanda, H., S. Tateya, Y. Tamori, K. Kotani, K. Hiasa, R. Kitazawa, S. Kitazawa, H. Miyachi, S. Maeda, K. Egashira, and M. Kasuga. 2006. MCP-1 contributes to macrophage infiltration into adipose tissue, insulin resistance, and hepatic steatosis in obesity. *J. Clin. Invest.* 116: 1494–1505.
37. Shaul, M. E., G. Bennett, K. J. Strissel, A. S. Greenberg, and M. S. Obin. 2010. Dynamic, M2-like remodeling phenotypes of CD11c+ adipose tissue macrophages during high-fat diet-induced obesity in mice. *Diabetes* 59: 1171–1181.
38. Khan, T., E. S. Muise, P. Iyengar, Z. V. Wang, M. Chandalia, N. Abate, B. B. Zhang, P. Bonaldo, S. Chua, and P. E. Scherer. 2009. Metabolic dysregulation and adipose tissue fibrosis: role of collagen VI. *Mol. Cell. Biol.* 29: 1575–1591.
39. Wentworth, J. M., G. Naselli, W. A. Brown, L. Doyle, B. Phipson, G. K. Smyth, M. Wabitsch, P. E. O'Brien, and L. C. Harrison. 2010. Pro-inflammatory CD11c+ CD206+ adipose tissue macrophages are associated with insulin resistance in human obesity. *Diabetes* 59: 1648–1656.
40. Cancellou, R., J. Tordjman, C. Poitou, G. Guilhem, J. L. Bouillot, D. Hugol, C. Coussieu, A. Basdevant, A. Bar Hen, P. Bedossa, et al. 2006. Increased infiltration of macrophages in omental adipose tissue is associated with marked hepatic lesions in morbid human obesity. *Diabetes* 55: 1554–1561.
41. Lumeng, C. N., J. L. Bodzin, and A. R. Saltiel. 2007. Obesity induces a phenotypic switch in adipose tissue macrophage polarization. *J. Clin. Invest.* 117: 175–184.
42. Yu, J., L. Shi, H. Wang, P. J. Bilan, Z. Yao, M. C. Samaan, Q. He, A. Klip, and W. Niu. 2011. Conditioned medium from hypoxia-treated adipocytes renders muscle cells insulin resistant. *Eur. J. Cell Biol.* 90: 1000–1015.
43. Gao, M., C. Zhang, Y. Ma, L. Bu, L. Yan, and D. Liu. 2013. Hydrodynamic delivery of miR10 gene protects mice from high-fat diet-induced obesity and glucose intolerance. *Mol. Ther.* 10.1038/mt.2013.125.
44. Cui, W., S. Pagliarlunga, D. Kalant, H. Lu, C. Roy, M. Laplante, Y. Deshaies, and K. Cianflone. 2007. Acylation-stimulating protein/C5L2-neutralizing antibodies alter triglyceride metabolism in vitro and in vivo. *Am. J. Physiol. Endocrinol. Metab.* 293: E1482–E1491.
45. Kalant, D., S. A. Cain, M. Maslowska, A. D. Sniderman, K. Cianflone, and P. N. Monk. 2003. The chemoattractant receptor-like protein C5L2 binds the C3a des-Arg77/acylation-stimulating protein. *J. Biol. Chem.* 278: 11123–11129.
46. Cui, W., M. Simaan, S. Laporte, R. Lodge, and K. Cianflone. 2009. C5a- and ASP-mediated C5L2 activation, endocytosis and recycling are lost in S3231-C5L2 mutation. *Mol. Immunol.* 46: 3086–3098.
47. Fisette, A., M. N. Munkonda, K. Oikonomopoulou, S. Pagliarlunga, J. D. Lambris, and K. Cianflone. 2013. C5L2 receptor disruption enhances the development of diet-induced insulin resistance in mice. *Immunobiology* 218: 127–133.
48. Phielers, J., R. Garcia-Martin, J. D. Lambris, and T. Chavakis. 2013. The role of the complement system in metabolic organs and metabolic diseases. *Semin. Immunol.* 25: 47–53.
49. Pagliarlunga, S., P. Schrauwen, C. Roy, E. Moonen-Kornips, H. Lu, M. K. Hesselink, Y. Deshaies, D. Richard, and K. Cianflone. 2007. Reduced adipose tissue triglyceride synthesis and increased muscle fatty acid oxidation in C5L2 knockout mice. *J. Endocrinol.* 194: 293–304.
50. Cinti, S., G. Mitchell, G. Barbatelli, I. Murano, E. Ceresi, E. Faloia, S. Wang, M. Fortier, A. S. Greenberg, and M. S. Obin. 2005. Adipocyte death defines macrophage localization and function in adipose tissue of obese mice and humans. *J. Lipid Res.* 46: 2347–2355.
51. Addis-Lieser, E., J. Köhl, and M. G. Chiaramonte. 2005. Opposing regulatory roles of complement factor 5 in the development of bleomycin-induced pulmonary fibrosis. *J. Immunol.* 175: 1894–1902.
52. Hillebrandt, S., H. E. Wasmuth, R. Weiskirchen, C. Hellerbrand, H. Keppeler, A. Werth, R. Schirin-Sokhan, G. Wilkens, A. Geier, J. Lorenzen, et al. 2005. Complement factor 5 is a quantitative trait gene that modifies liver fibrogenesis in mice and humans. *Nat. Genet.* 37: 835–843.
53. Iyer, A., T. M. Woodruff, M. C. Wu, C. Stylianou, R. C. Reid, D. P. Fairlie, S. M. Taylor, and L. Brown. 2011. Inhibition of inflammation and fibrosis by a complement C5a receptor antagonist in DOCA-salt hypertensive rats. *J. Cardiovasc. Pharmacol.* 58: 479–486.
54. Kim, D. H., D. Sandoval, J. A. Reed, E. K. Matter, E. G. Tolod, S. C. Woods, and R. J. Seeley. 2008. The role of GM-CSF in adipose tissue inflammation. *Am. J. Physiol. Endocrinol. Metab.* 295: E1038–E1046.
55. Donath, M. Y., M. Böni-Schnetzler, H. Ellingsgaard, and J. A. Ehses. 2009. Islet inflammation impairs the pancreatic beta-cell in type 2 diabetes. *Physiology (Bethesda)* 24: 325–331.
56. Donath, M. Y., D. M. Schumann, M. Faulenbach, H. Ellingsgaard, A. Perren, and J. A. Ehses. 2008. Islet inflammation in type 2 diabetes: from metabolic stress to therapy. *Diabetes Care* 31(Suppl 2): S161–S164.
57. Hull, R. L., G. T. Westermark, P. Westermark, and S. E. Kahn. 2004. Islet amyloid: a critical entity in the pathogenesis of type 2 diabetes. *J. Clin. Endocrinol. Metab.* 89: 3629–3643.
58. Klegeris, A., and P. L. McGeer. 2007. Complement activation by islet amyloid polypeptide (IAPP) and alpha-synuclein 112. *Biochem. Biophys. Res. Commun.* 357: 1096–1099.
59. Lim, J., A. Iyer, J. Y. Suen, V. Seow, R. C. Reid, L. Brown, and D. P. Fairlie. 2013. C5aR and C3aR antagonists each inhibit diet-induced obesity, metabolic dysfunction, and adipocyte and macrophage signaling. *FASEB J.* 27: 822–831.
60. Roy, C., A. Gupta, A. Fisette, M. Lapointe, P. Poursharifi, D. Richard, H. Lu, B. Lu, N. Gerard, C. Gerard, and K. Cianflone. 2013. C5a receptor deficiency alters energy utilization and fat storage. *PLoS One* 8: e62531.

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 $\pm$ 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, PPAR α and PPAR γ was studied. The respective mRNA was normalized against 18s. Data are displayed as mean $\pm$ SEM (n = 5–6 / group) and are shown as percentage of the WT control. The expression of ACC, FAS, SREBP-1c, PPAR α or PPAR γ 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 $\pm$ 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 $\pm$ 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 μ m. 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 $\pm$ SEM. * $P \leq 0.05$.

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 $\pm$ 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 $\pm$ 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 PPAR γ was studied. The respective mRNA was normalized against 18s. Data are displayed as mean $\pm$ SEM (n = 6 / group) and are shown as percentage of the WT control. The expression of ACC, FAS, SREBP-1c, ChREBP or PPAR γ of WT mice represents the 100% control. n.s. = not significant.

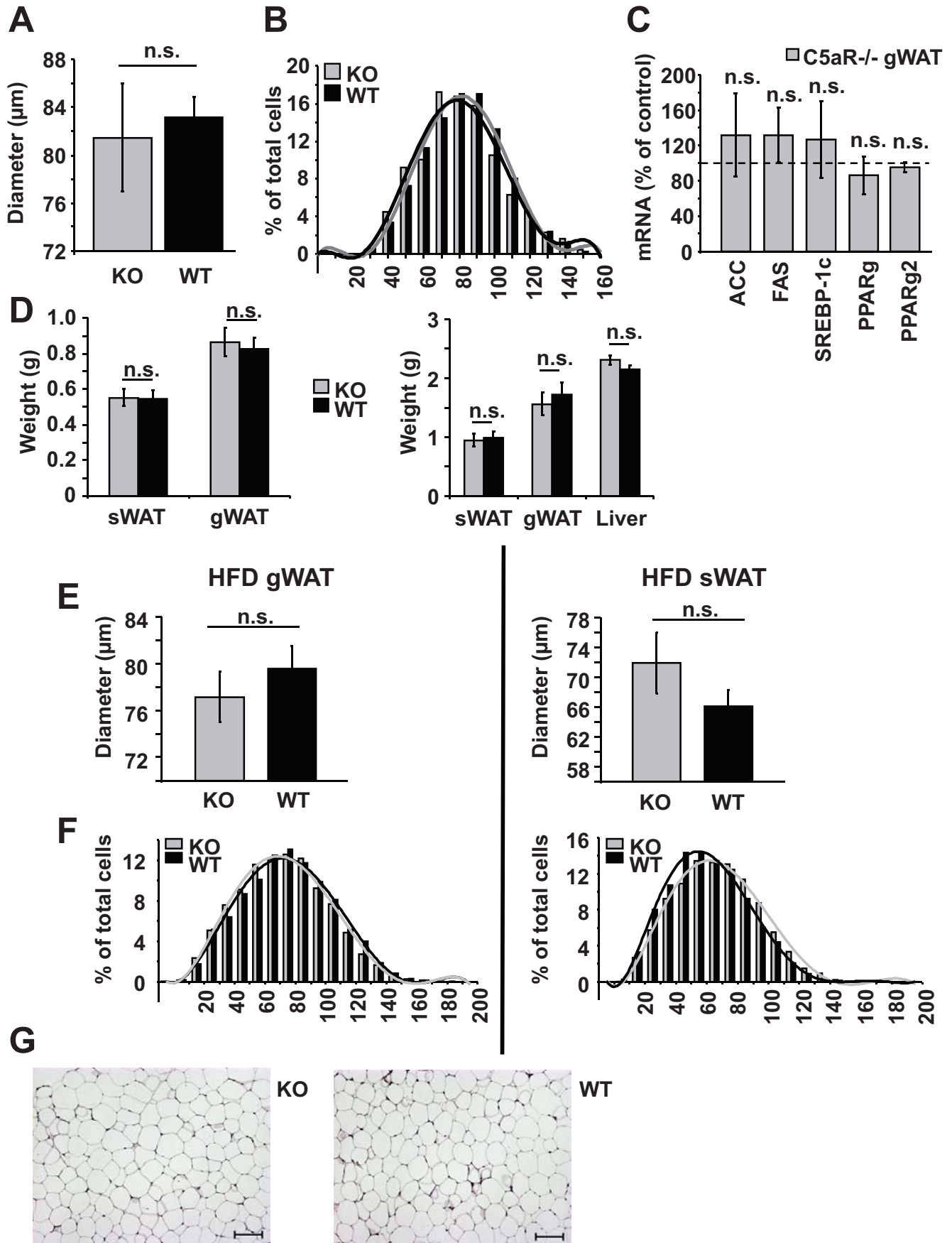
C) RNA from liver tissue of C5aR-deficient or -sufficient male mice fed a HFD for 20 weeks was extracted and analyzed with quantitative real-time PCR. The expression of the pro-inflammatory markers CCL3, CCL4, CCL5, IL-6, MCP-1, TNF and of the anti-inflammatory cytokine IL-10 was studied. The respective mRNA was normalized against 18s. Data are displayed as mean $\pm$ 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.

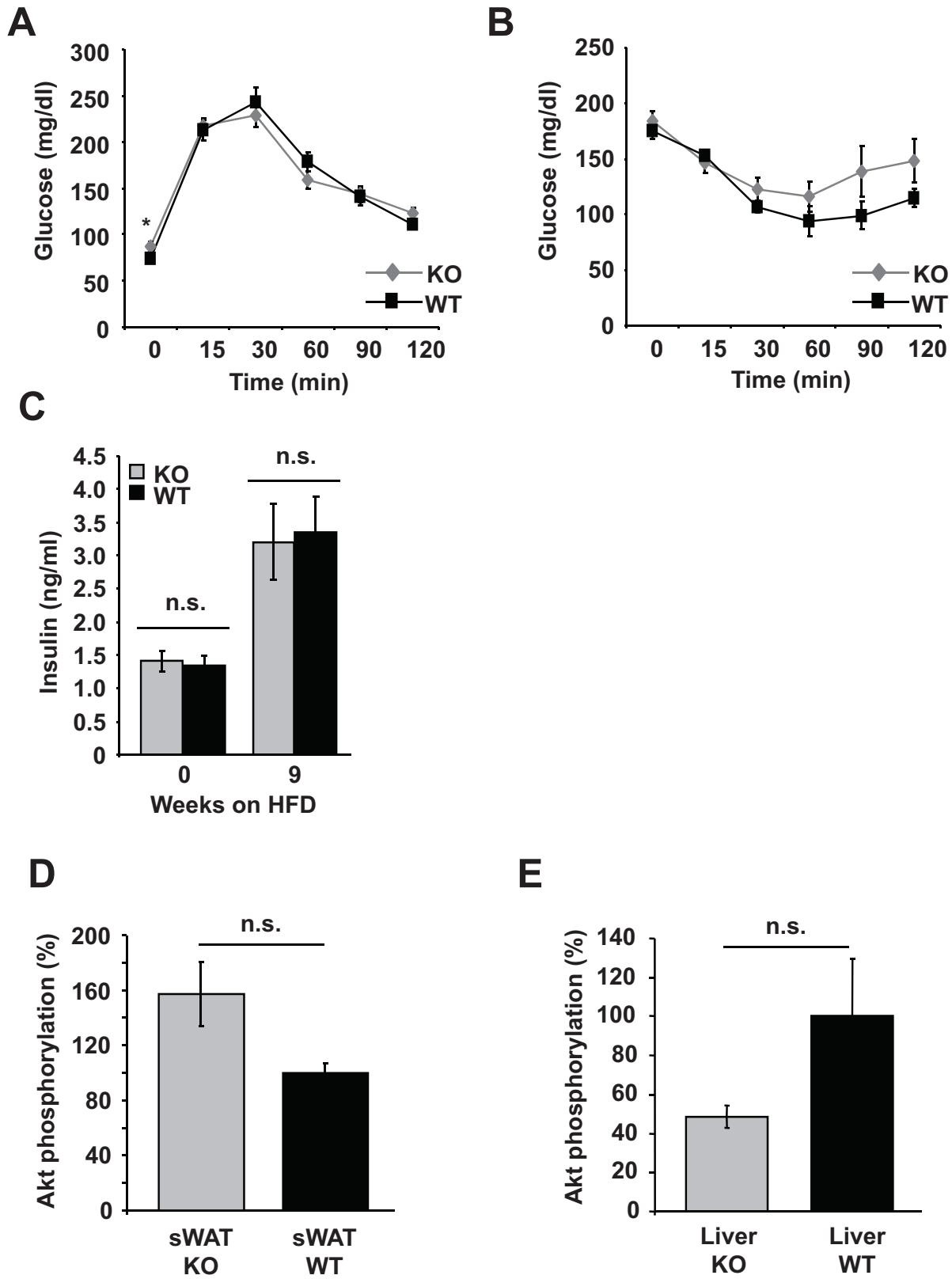
D) IHC of pancreas stained for insulin and haematoxylin from male C5aR-deficient (KO) or -sufficient (WT) mice that were for 12 weeks on HFD are depicted. Scale bars represent 50 μ m.

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 $\pm$ SEM. n.s. = not significant.

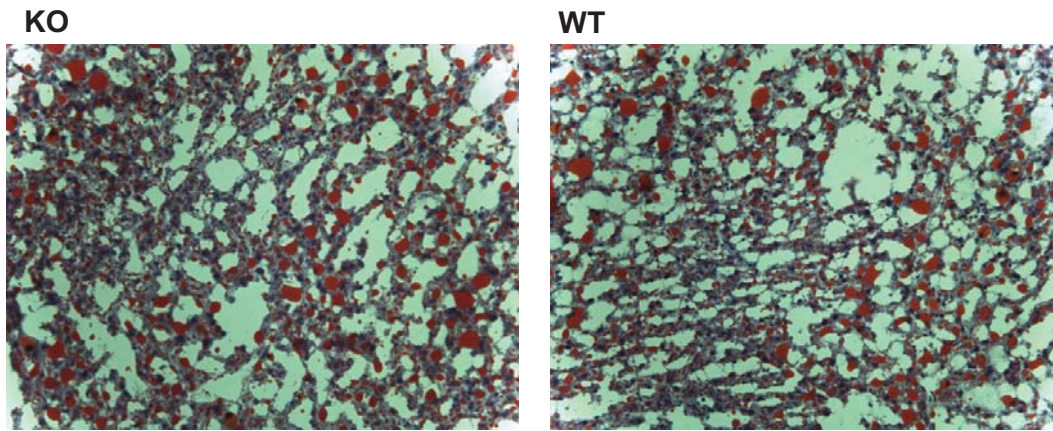
Suppl Fig. 4: Effect of C5aR deficiency in WAT inflammation and WAT fibrosis. A-B) RNA from gonadal (g) WAT of C5aR-deficient or -sufficient male mice fed a HFD for 20 weeks was extracted and analyzed with quantitative real-time PCR. The expression of A) IL-10, or B) TNF and IL-6 was studied. The respective mRNA was normalized against 18s. Data

are displayed as mean $\pm$ 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. $**P \leq 0.01$; 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.

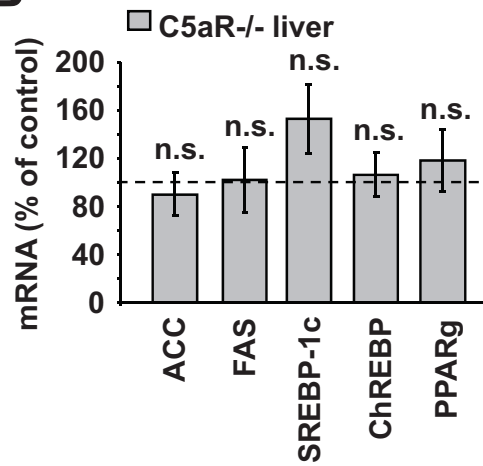




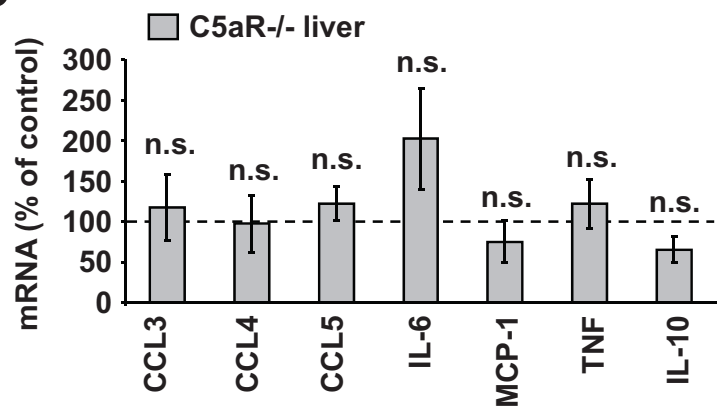
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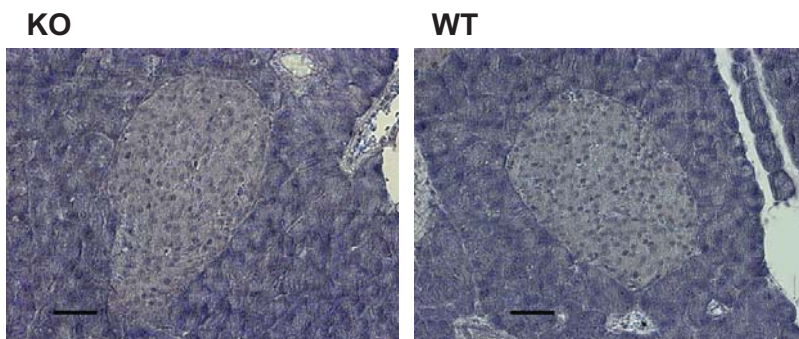
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D



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