Inflammation, but not recruitment, of adipose tissue macrophages requires signalling through Mac-1 (CD11b/CD18) in diet-induced obesity (DIO)

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Summary

Cell accumulation is a prerequisite for adipose tissue inflammation. The leukocyte integrin Mac-1 (CD11b/CD18, $\alpha_M\beta_2$) is a classic adhesion receptor critically regulating inflammatory cell recruitment. Here, we tested the hypothesis that a genetic deficiency and a therapeutic modulation of Mac-1 regulate adipose tissue inflammation in a mouse model of diet-induced obesity (DIO). C57Bl6/J mice genetically deficient (Mac-1$^{-/-}$) or competent for Mac-1 (WT) consumed a high fat diet for 20 weeks. Surprisingly, Mac-1$^{-/-}$ mice presented with increased diet-induced weight gain, decreased insulin sensitivity in skeletal muscle and in the liver in insulin-clamps, insulin secretion deficiency and elevated glucose levels in fasting animals, and dyslipidaemia. Unexpectedly, accumulation of adipose tissue macrophages (ATMs) was unaffected, while gene expression indicated less inflamed adipose tissue and macrophages in Mac-1$^{-/-}$ mice. In contrast, inflammatory gene expression at distant locations, such as in skeletal muscle, was not changed. Treatment of ATMs with an agonistic anti-Mac-1 antibody, M1/70, induced pro-inflammatory genes in cell culture. *In vivo*, treatment with M1/70 induced a hyper-inflammatory phenotype with increased expression of IL-6 and MCP-1, whereas accumulation of ATMs did not change. Finally, inhibition of Mac-1's adhesive interaction to CD40L by the peptide inhibitor cM7 did not affect myeloid cell accumulation in adipose tissue. We present the surprising finding that adhesive properties of the leukocyte integrin Mac-1 are not required for macrophage accumulation in adipose tissue. Instead, Mac-1 modulates inflammatory gene expression in macrophages. These findings question the net effect of integrin blockade in cardio-metabolic disease.

Keywords

Obesity, metabolic disorders, inflammation, macrophage, adhesion molecules

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Introduction

The metabolic syndrome (MS) comprises a cluster of cardiovascular risk factors including obesity, insulin resistance, hypertension, non-alcoholic hepatic steatosis (NASH), and dyslipidaemia. MS strongly associates with atherosclerosis, myocardial infarction, and stroke (1). Chronic, low-grade inflammation and infiltration of visceral adipose tissue (VAT) with immune and inflammatory cells accompanies MS (2, 3). Genetic modulation of the inflammatory response in adipose tissue has proven effective in insulin resistance and dysmetabolism in animal models (4, 5). A hallmark of adipose tissue inflammation is the accumulation and activation of mono-

cyte-derived adipose tissue macrophages (ATMs) (6). Alternatively activated macrophages (AAMs) reside in lean adipose tissue, share M2 expression markers such as arginase-1, and secrete the anti-inflammatory cytokine IL-10, which protects from inflammation and insulin resistance (7, 8). It has been postulated that during obesity new macrophages are being recruited from bone marrow-derived monocytes, which eventually turn into pro-inflammatory M1-like macrophages expressing tumour necrosis factor (TNF)α, interleukin (IL)-6, and, inducible nitric oxide synthase (iNOS), which further fuel the inflammatory response, insulin resistance, and adipocyte dysfunction (4, 5, 7). Chemotactic signals, such as the adipocyte- and macrophage-expressed chemokine monocyte chemoattractant protein (MCP)-1 have been discussed to promote the recruitment of monocytes into adipose tissue (9). On a cellular level, leukocyte integrins, such as Mac-1 ($\alpha_M\beta_2$, CD11b/CD18) regulate leukocyte recruitment, trans-endothelial migration, and various myeloid cell effector functions (10–12). Mac-1 is expressed on most monocytes, macrophages, neutrophils, natural killer (NK) cells, and to a smaller extent on activated lymphocytes. The diversity of Mac-1's functions is reflected by a broad variety of ligands, including intercellular adhesion molecule (ICAM)-1 (13), fibrinogen (14), glycoprotein (GP)Ibα (15), and CD40L (16). Notably, inhibition of Mac-1 by neutralising antibodies or by inhibiting its interaction to CD40L attenuated atherosclerotic lesion formation in mice by impairing macrophage accumulation (16, 17). Anti-Mac-1 therapy has therefore been suggested in inflammatory pathologies, but its exact function remains unproven in adipose tissue inflammation (18, 19). Clinically, Mac-1 expression is enhanced in patients with the metabolic syndrome (20). Surprisingly, two studies proposed that genetic deficiency of Mac-1 induces an unexpected obese phenotype in mice after consumption of a high fat diet (HFD) (21, 22). However, neither the underlying mechanisms, nor the metabolic and inflammatory consequences of Mac-1 deficiency were addressed in these studies. In the present study, we explored gain- and loss-of-function of Mac-1 in a mouse model of diet-induced obesity (DIO).

Materials and methods

Animal protocols

Mac-1 deficient mice (Mac-$1^{-/-}$) on a C57BL/6 background and aged-matched wild-type mice (WT) were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). Animals received HFD (45% Kcal from fat) or a standard chow diet for 20 weeks. As indicated, mice were treated with intraperitoneal injections of the anti-Mac-1 antibody clone M1/70 or a corresponding isotype control three times a week (100 µg per injection) for a total duration of 12 weeks. Alternatively, the peptide inhibitor cM7 (100 µg diluted in 100 µl saline) was injected daily by intraperitoneal injections. Injections with saline (100 µl) or of the peptide scM7 (100 µg diluted in 100 µl saline) served as controls. Injections were carried out for 20 weeks until the end of the study. All experimental protocols were approved by the animal ethics committee of the Alfred Medical Research and Education Precinct (AMREP), Melbourne, Australia and the University Hospital Freiburg, Germany. All procedures were carried out in accordance to local guidelines.

Body composition analysis

Body composition including total fat, lean and water mass was assessed in living animals by MRI-based body composition analysis (EchoMRI, Echo Medical Systems, Houston, TX, USA) on a basis of a four-weekly follow-up. Data are presented as percentage of total body weight.

Intraperitoneal (i.p.) glucose and insulin tolerance testing (GTT and ITT)

Mice were deprived of food 4 hours (h) prior to the procedures and received intraperitoneal glucose injections (0.25–1 g/kg lean body mass as assessed by body composition analysis) for GTT. For insulin tolerance tests (ITT), mice were injected with human insulin i.p. (0.1–0.5 U/kg, Actrapid Insulin, Novo Nordisk, Bagsvaerd, Denmark). Plasma glucose was determined at the indicated time points. Fasting plasma glucose levels at the end of the study were after food deprivation for 12 h.

Euglycemic-hyperinsulinaemic clamps (EHC)

Four days prior to EHC mice were anaesthetised and an indwelling catheter was placed into the right internal jugular vein. On the day of the clamp, mice were deprived of food for 5 h followed by a continuous infusion of [3–3H] glucose (5 µCi bolus and 0.05 µCi/minute [min]) to measure basal glucose turnover. After infusion of the tracer for 2 h EHC was performed. A continuous infusion of insulin (4 mU/kg/min) was started and euglycaemia was maintained using a variable infusion of 30% glucose. To measure the tissue glucose uptake, a bolus of 2[14C] deoxyglucose (12 µCi) was injected. Blood concentrations of [3–3H] glucose and 2[14C] deoxyglucose were determined after deproteinisation with BaOH and $ZnSO_4$. Tissue glucose uptake was assessed by determining the tissue content of 2 [14C] deoxyglucose-6-phosphate and the plasma 2 [14C] deoxyglucose profile.

Metabolic caging

Animals were allowed to acclimatize to metabolic cages (Oxymax/CLAMS, Columbus Instruments, USA) for 24 hours prior to the observation period. Ambulatory movement and heat production were assessed and recorded during the 24-hour observation period. Normalized heat production was expressed as energy expenditure/total body weight.

Preparation of the stromal vascular fraction (SVF)

Murine epididymal fat pads were minced and digested with collagenase for 30 minutes at 37 °C (0.5 mg/ml, Collagenase B, Roche, Switzerland). Digested tissue was then filtered through a nylon

mesh (100 µm). The stromal vascular fraction (SVF) and adipocyte fraction were obtained from the resulting pellet and supernatant, respectively. As indicated, adipose tissue macrophages were isolated from SVF preparations by magnetic bead isolation (Stem Cell Technologies).

Flow cytometry

Cells were washed in PBS, Fc-Receptors were blocked by anti-CD16/CD32 (eBioscience, USA), and cells were labeled with the indicated antibodies before quantification with a flow cytometer (BD FACS Canto II, BD, USA). All antibodies were obtained from eBioscience, USA. A representative gating strategy is shown in Suppl. Figure 1 (available online at www.thrombosis-online.com).

Isolation of total RNA and quantitative real-time PCR

Isolation of RNA and generation of cDNA was performed as previously described (23). Quantitative real-time PCR was performed with a LightCycler 480 System with the LightCycler 480 SYBR Green I Master (Roche, Basel, Switzerland) detection format. Primer sequences can be provided upon request. Murine GAPDH served as control.

Histology, immunohistochemistry (IHC)

Adipose tissue was fixed for 24 h with 10% paraformaldehyde solution (Sigma-Aldrich, St. Louis, MO, USA) at 4°C, dehydrated, embedded in paraffin and cut into 6 µm sections. Sections were mounted on glass slides, depleted of paraffin and rehydrated. Sections were then placed in pre-heated Target Retrieval Solution (TRS, Dako, Glostrup, Denmark) and rinsed with phosphate-buffered saline (PBS). For IHC staining sections were blocked with 4% rabbit serum, incubated for 1.5 h at room temperature (RT) with anti-F4/80 or anti-CD8 (Abcam, Cambridge, MA, USA) and detected by a secondary antibody followed by detection with AEC+ substrate (Dako). Livers were embedded in Tissue-Tek compound (Sakura Finetek, Tokyo, Japan). For Oil-Red-O staining liver sections were fixed in 10% neutral buffered formalin (Harleco, Merck Corp., Kenilworth, NJ, USA) for 10 min, rinsed with water, submerged in 100% polypropylene glycol (Fischer Chemicals, Zurich, Switzerland) for 2 min, incubated in Oil-Red-O staining solution (Sigma-Aldrich) for 25 min at 60°C, washed in Millipore water and 0.05% ammonia water. Pancreatic islets were identified in pancreas sections. Expression of insulin was visualised by staining with an anti-mouse insulin antibody.

Liver homogenates

Liver samples were homogenised in 200 µl PBS using a dispergator (IKA, Königswinter, Germany) and normalised to a protein concentration of 2.5 mg/ml. Lipids were extracted as previously described (23).

Analysis of murine plasma samples

Plasma levels of insulin, leptin, and adiponectin were measured by ELISA, according to the manufacturers' protocols (Mercodia, Uppsala, Sweden; R&D Systems, Minneapolis, MN, USA; Cusabio, College Park, MD, USA; Uscn Life Science Inc, Wuhan, China). Triglyceride and cholesterol levels of murine plasma samples and in liver homogenates were assessed by an enzymatic assay. Plasma cytokines were determined by cytometric bead arrays (CBA, BD Biosciences, Franklin Lakes, NJ, USA), according to the manufacturer's protocol. Lipid fractions (LDL, HDL, total cholesterol, triglycerides) were quantified by the Roche COBAS system in fresh plasma samples.

Liquid chromatography-mass spectrometry (LC-MS) of adipocyte lipids

Adipocytes were isolated by digestion of visceral adipose tissue and separation from the stromal-vascular fraction. Cell lysates were run on an Applied Biosystems 4000 QTrap instrument (Applied Biosystems, Foster City, CA, USA) for quantification of the indicated lipid species.

Statistical analysis

Data are presented as mean ± SEM. Statistical testing employed Student's unpaired t-test, analysis of variance (ANOVA), or Mann-Whitney test as indicated. Normal distribution was tested by Shapiro-Wilk test. P-values < 0.05 were considered significant.

Results

During diet-induced obesity, Mac-1 is preferentially expressed by adipose tissue macrophages.

Mac-1 is a classic adhesion receptor expressed by a variety of leukocyte subsets (10–12). To decipher which specific leukocyte subset expresses Mac-1 in obese visceral adipose tissue (VAT), C57Bl/6 mice were fed HFD for 20 weeks and the percentage of Mac-1$^+$ leukocytes resident in the stromal vascular fraction (SVF) of VAT was quantified by flow cytometry (►Figure 1A and Suppl. Figure 1, available online at www.thrombosis-online.com). We observed that the fraction of Mac-1 expressing cells was highest among F4/80$^+$-macrophages (►Figure 1B). 99.0 ± 0.2% of CD11c$^+$ M1-like macrophages and 96.2 ± 0.5% CD11c$^-$ M2-like macrophages expressed Mac-1, whereas 52.1 ± 2.5% of all CD11c$^+$ F4/80$^-$ dendritic cells were positive for Mac-1. Only 13.3 ± 1.7% of Gr-1$^+$ granulocytes in the stromal vascular fraction expressed Mac-1. Lymphocytes were positive for Mac-1 in only 1.4 ± 0.2% (CD4$^+$ T cells), 0.8 ± 0.2% (CD8$^+$ T cells), and 14.8 ± 3.2% (CD19$^+$ B cells). These data establish that preferentially adipose tissue macrophages express Mac-1 in obese adipose tissue.

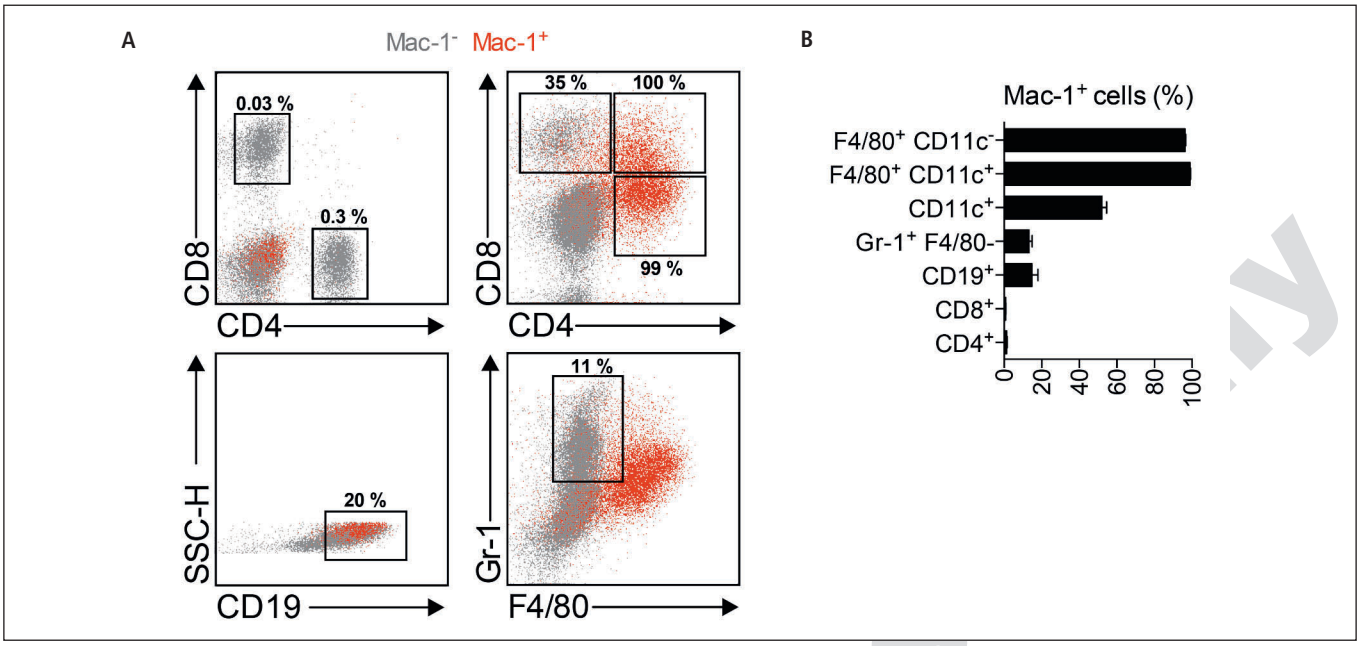

Figure 1: Mac-1 is preferentially expressed by adipose tissue macrophages during diet-induced obesity. C57Bl/6 mice consumed a high-fat diet (HFD) for 20 weeks. Leukocytes resident in visceral adipose tissue (VAT) were extracted after enzymatic digestion of epididymal fat pads and stained for flow cytometry with antibodies for specific detection of the indicated leukocyte subsets. Mac-1 positive cells were identified by specific antibody binding and are indicated in red within each leukocyte subset in representative dot blots (A). Quantification is shown as Mac-1 expressing leukocyte subsets as percentage of all leukocytes within this subset (B). Data are presented as mean ± SEM of at least four animals.

Genetic deficiency of Mac-1 causes excessive weight gain, hyperlipidaemia, insulin-secretion deficiency, and selective insulin resistance in insulin-clamps.

The MS is defined by the presence of visceral obesity. To address whether genetic deficiency of Mac-1 affects DIO, eight-week-old, male WT and Mac-1-deficient (Mac-$1^{-/-}$) mice on a C57Bl/6J background consumed HFD for 20 weeks. Mac-$1^{-/-}$ mice gained significantly more relative and absolute weight during the later stages of obesity (an increase of 18.7 ± 7.4% in Mac-$1^{-/-}$ mice at week 20 of HFD, n≥34 per group, p=0.0172, ▶Figure 2A). Interestingly, the increase in weight was not associated with an increase of adiposity, since relative body fat mass in living animals did not differ as assessed by EchoMRI body composition analysis (▶Figure 2B, C). Accordingly, no differences in the weight of several organs, including peripheral fat pads, heart, spleen, kidney, and liver could be detected (Suppl. Figure 2, available online at www.thrombosis-online.com), indicating that Mac-1 contributes to increased, but balanced body weight. In addition, Mac-1 deficiency had no effect on weight and body composition of mice consuming a standard chow diet (Suppl. Figure 2, available online at www.thrombosis-online.com), indicating that the changes in weight were diet-induced. Furthermore, the increase in weight in Mac-$1^{-/-}$ mice consuming HFD was not caused by decreased physical activity or impaired thermogenesis as assessed by metabolic caging (Suppl. Figure 3, available online at www.thrombosis-online.com). In addition, we did not detect changes in systolic blood pressure in Mac-1-deficient mice (▶Figure 2D).

Obesity is a likely cause of insulin resistance (5). Mac-$1^{-/-}$ mice demonstrated increased fasting plasma glucose levels after overnight starvation (▶Figure 2E), indicating that Mac-1 contributes to insulin sensitivity or glucose utilisation. We detected reduced insulin levels in fasting Mac-$1^{-/-}$ mice (▶Figure 2I), which coincided with a reduced size of pancreatic islets and reduced insulin expression in the pancreas, indicative of an insulin-secretion deficiency (▶Figure 2I, J). We observed that glucose disposal rate (GDR) tended to increase at the baseline measurement of a hyperinsulinaemic-euglycemic clamp, suggestive of enhanced endogenous glucose production (EGP) in the liver. The hepatic insulin resistance index (GDR x insulin) decreased in Mac-$1^{-/-}$ mice (Suppl. Figure 4, available online at www.thrombosis-online.com), indicating that under baseline conditions, reduced insulin levels may be causal for enhanced EGP and glucose levels. However, under clamp conditions, EGP and GDR rose in Mac-$1^{-/-}$ mice, while GIR tended to slightly decrease, indicating an additional hepatic insulin resistance in Mac-$1^{-/-}$ mice under clamp conditions (Suppl. Figure 4, available online at www.thrombosis-online.com). During insulin clamp, glucose tracer uptake into some tested muscles, but not adipose tissue was lower in Mac-$1^{-/-}$ animals, indicating that presence of Mac-1 is partially involved in insulin signalling in muscle tissue (▶Figure 2G). However, this localised insulin resistance in some muscle tissue and in the liver during the clamp did not impact on global glucose utilisation and insulin responsiveness in an intraperitoneal glucose (GTT) – and insulin challenge (ITT) (▶Figure 3H and Suppl. Figure 4, available online at www.thrombosis-online.com). Conversely, Mac-$1^{-/-}$ mice on a standard low fat

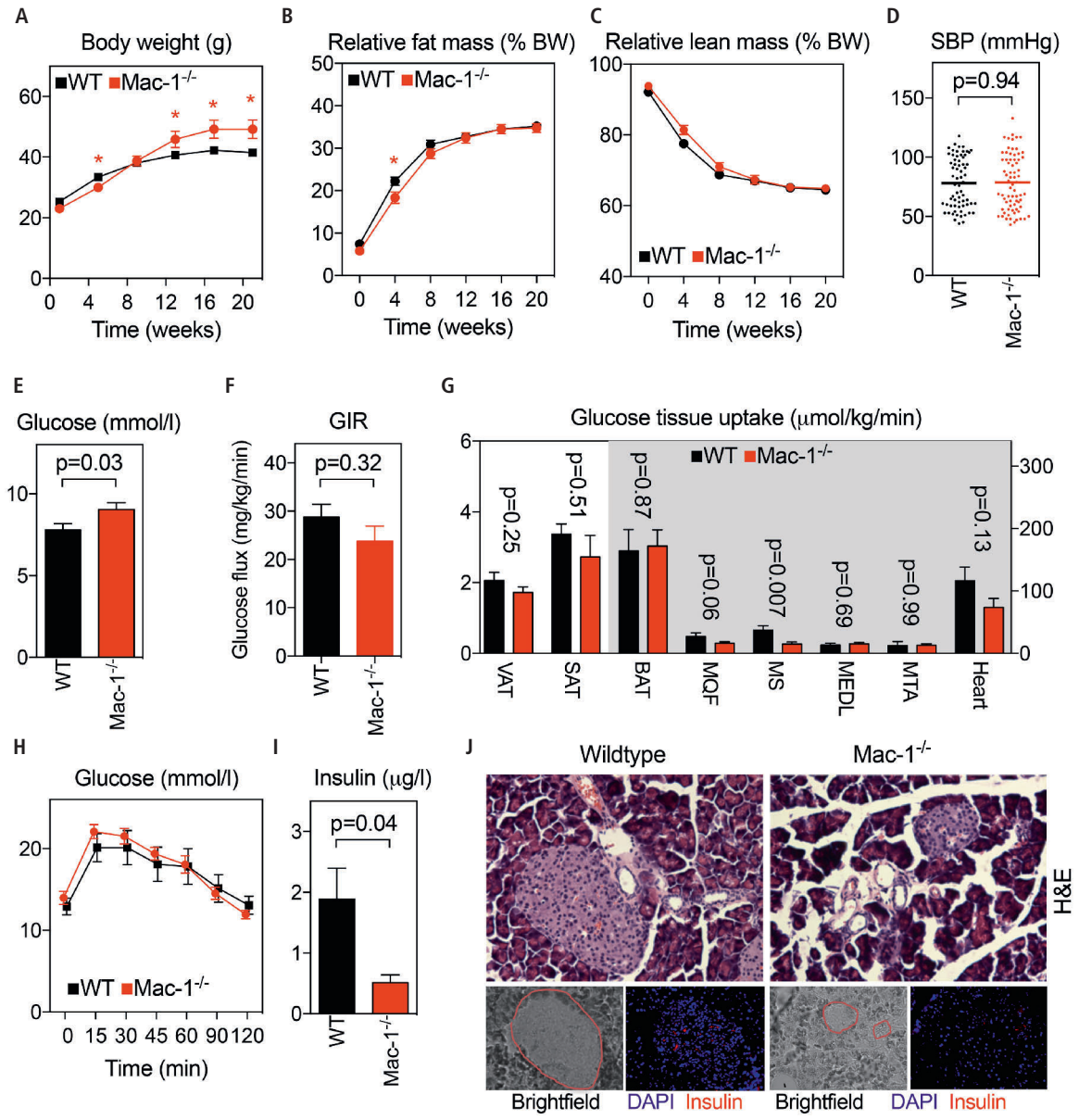

Figure 2: Genetic deficiency of Mac-1 induces an overweight and hyperlipidaemic phenotype with a partial insulin resistance in skeletal muscle tissue in mice. Male, eight-week-old C57Bl/6 or Mac-1 deficient mice on a C57Bl/6 background consumed a high-fat diet (HFD) for 20 weeks. Diet-induced weight gain is shown at the indicated time points (A). Relative body fat mass (B) and lean mass (C) was assessed by EchoMRI body composition analysis in living animals at the indicated time points (expressed as % of total body weight). Systolic blood pressure (SBP) was quantified by non-invasive measurement after 20 weeks of HFD (D). Glucose and insulin were analysed in fasting animals at the end of the feeding period (E, I). To assess insulin and glucose utilisation, hyperinsulinaemic-euglycaemic clamp analysis was performed by infusion of radioactively labelled glucose (glucose infusion rate, GIR) to maintain euglycaemic steady state under continuous insulin infusion (F). Glucose tracer uptake was quantified in subcutaneous (SAT), visceral (VAT), and brown adipose tissue (BAT), *M. quadriceps femoris* (MQF), *M. soleus* (MS), *M. extensor digitorum longus* (MEDL), *M. tibialis anterior* (MTA), and in the heart. SAT and VAT refer to the left y-axis (G). Glucose tolerance test (GTT) after intraperitoneal injection of glucose (H). Microscopy of pancreatic islets and visualisation of insulin-expression in IHC (J). Data are presented as mean ± SEM of at least 34 (A, D), 15 (B, C), and eight animals (E-I) per group. Statistical significance was calculated by unpaired, two-tailed Student's t-test at each time point between both groups. P-values are indicated within each graph or indicated by asterisks (* =p<0.05).

diet (chow) presented with ameliorated insulin resistance and lowered fasting glucose levels (Suppl. Figure 5, available online at www.thrombosis-online.com), indicating that HFD-consumption is causal for this phenotype.

Hepatic steatosis is a well-established feature of the metabolic syndrome (24). Plasma cholesterol as well as LDL- and HDL-fractions, and triglycerides tended to rise in Mac-1$^{-/-}$ mice (Suppl. Figure 6 and Suppl. Table 1, available online at www.thrombosis-online.com). In liver homogenates, cholesterol and triglyceride levels as well as lipid depositions in ORO-stained liver sections were reduced (Suppl. Figure 6, available online at www.thrombosis-online.com). Correspondingly, we found decreased expression of fatty acid synthase (FAS) in liver tissue, indicating that Mac-1 deficiency either protects from hepatic steatosis or induces the efflux of lipids from the liver.

Taken together, these findings unravel a complex metabolic phenotype in Mac-1$^{-/-}$ mice with enhanced body weight, dyslipidaemia, insulin-secretion deficiency, and selective insulin-resistance in skeletal muscle tissue and in the liver under insulin-clamp conditions.

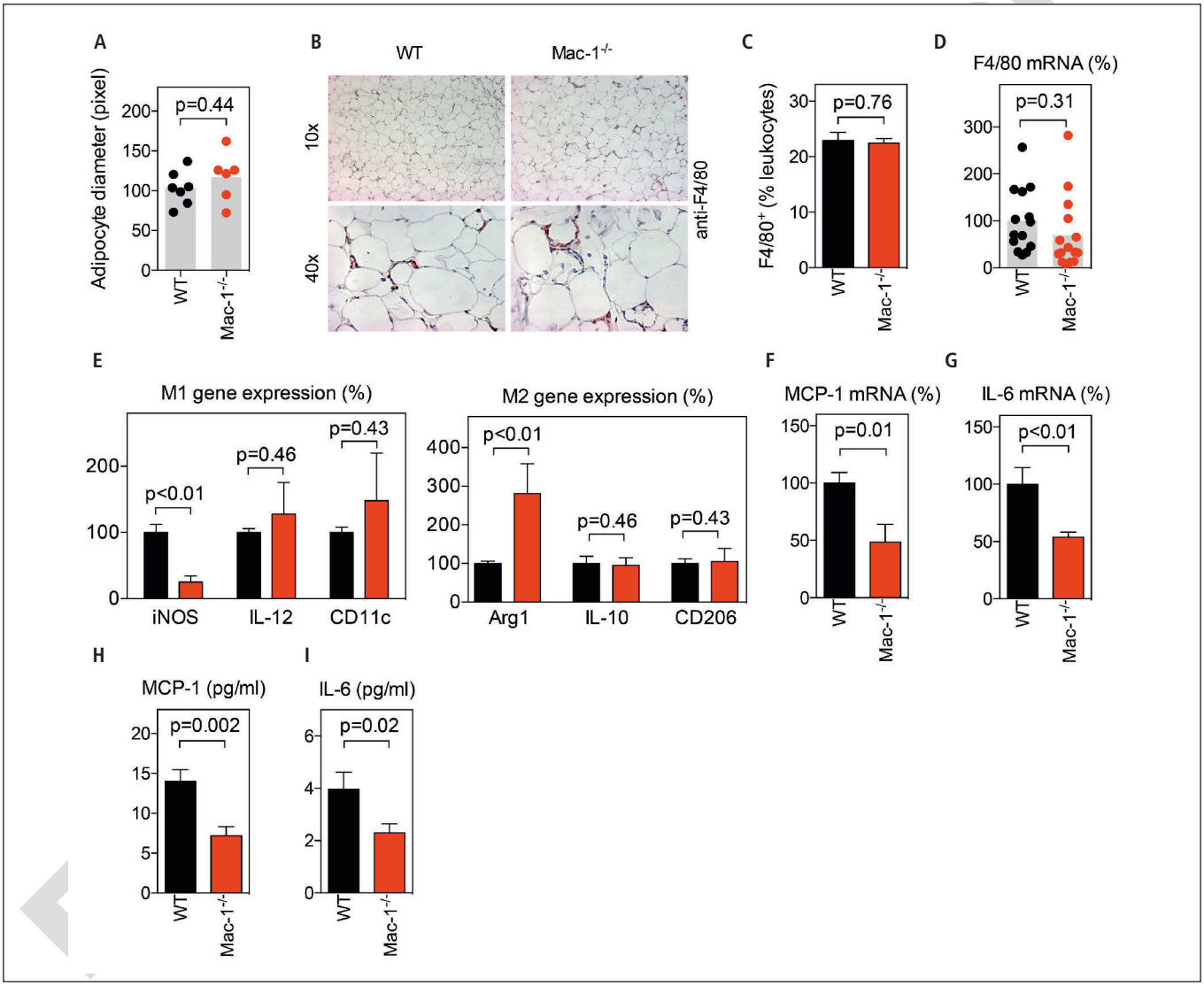

Figure 3: Genetic deficiency of Mac-1 does not alter accumulation of adipose tissue macrophages, but ameliorates local and systemic cytokine expression. C57Bl/6 (WT) and Mac-1$^{-/-}$ mice consumed a high-fat diet for 20 weeks. Mean adipocyte diameter was quantified in sections of visceral adipose tissue (VAT) by image processing software (A) or stained for the macrophage antigen F4/80 in immunohistochemistry (B). Infiltration of macrophages in VAT was further quantified by flow cytometry and expressed as percentage of all leukocytes resident in VAT (C). mRNA preparations of VAT from eight animals per group were analysed for the abundance of the indicated mRNAs (D-G). Plasma levels of MCP-1 and IL-6 were quantified by ELISA (H,I). Data are presented as mean ± SEM of at least eight animals (A–D). Statistical significance was calculated by Mann-Whitney test (A), or unpaired, two-tailed Student's t-test. P-values are indicated within each graph. iNOS indicates Inducible nitric oxide synthase, Arg1 indicates Arginase-1.

Genetic deficiency of Mac-1 does not alter macrophage accumulation in adipose tissue, but ameliorates pro-inflammatory cytokine and chemokine expression.

DIO causes inflammation and remodelling of adipose tissue with a hypertrophy of adipocytes, enhanced adipocyte lipids, inflammatory cell accumulation, and expression of pro-inflammatory cytokines (4, 25). Despite increased weight gain and dyslipidaemia in Mac-1$^{-/-}$ mice, we observed unchanged adipocyte size and adipocyte cholesterol content as well as no relevant changes in the plasma levels of the two adipokines adiponectin and leptin (►Figure 3A and Suppl. Figure 7, available online at www.thrombosis-online.com). Conversely, mean adipocyte diameter and overall size distribution of adipocytes was smaller in Mac-1$^{-/-}$ mice under a chow diet (Suppl. Figure 8, available online at www.thrombosis-online.com), indicating that a genetic loss of Mac-1 does not directly impact on adipocyte metabolism, but rather reflects diet-induced changes.

In this context, it has been proposed that inflammation of adipose tissue requires recruitment of bone marrow-derived monocytes, and their differentiation towards macrophages (6). Adhesion and transmigration requires efficient integrin function, such as of Mac-1 (26). We therefore hypothesised that Mac-1$^{-/-}$ mice fail to recruit macrophages into adipose tissue during DIO. While Mac-1$^{-/-}$ mice contained lower numbers of circulating leukocytes before and during the study (Suppl. Table 2, available online at www.thrombosis-online.com), total numbers of VAT-resident leukocytes were equal in WT and Mac-1$^{-/-}$ mice (Suppl. Table 3, available online at www.thrombosis-online.com), indicating that Mac-1 deficiency does not cause major changes in the cellular composition in VAT. Indeed, we observed a high percentage of macrophages in both genotypes (~22.4% of all VAT leukocytes). Histological sections of visceral adipose tissue revealed that F4/80^{+} macrophages did not differ between Mac-1$^{-/-}$ and WT mice (►Figure 3B). Accordingly, analysis of VAT cells in flow cytometry (►Figure 3C) and gene expression of the macrophage marker F4/80 in qPCR (►Figure 3D) confirmed similar macrophage content in Mac-1$^{-/-}$ and WT mice. In addition, VAT cell suspensions from Mac-1$^{-/-}$ mice consuming a chow diet contained equal numbers of macrophages compared with WT mice (Suppl. Table 4, available online at www.thrombosis-online.com). Also, CD19^{+} B cells, CD8^{+} T cells, and regulatory T cell accumulation in VAT of Mac-1$^{-/-}$ mice was not changed, while we observed a slight increase of anti-inflammatory CD4^{+} T-helper cells (27) in VAT of Mac-1-deficient animals (Suppl. Table 5, available online at www.thrombosis-online.com).

Beyond its function in cell adhesion, binding of ligands to Mac-1 can induce cell activation by induction of pro-inflammatory signalling pathways, referred to as integrin outside-in signalling (28). Adipose tissue macrophages acquire pro-inflammatory gene expression similar to that of M1-macrophages during DIO (7). Interestingly, we found that among several M1/M2-markers, the expression of iNOS, indicative of M1, was highly reduced, while that of arginase-1, indicative of M2, increased in whole tissue mRNA isolated from epididymidal adipose tissue of Mac-1$^{-/-}$ mice (►Figure 3E). Also, the abundance of IL-6 and MCP-1 mRNA were lower in Mac-1$^{-/-}$ mice (►Figure 3F, G). In accord, plasma levels of both, IL-6 and MCP-1, decreased in Mac-1$^{-/-}$ mice compared with WT mice (►Figure 3H, I), indicating that a lack of Mac-1 reduces macrophage effector function and inflammatory signalling in adipose tissue. These findings coincided with enhanced gene expression of the anti-inflammatory nuclear receptor peroxisome proliferator-activated receptor (PPAR) –γ and the lipid master regulator sterol regulatory element-binding protein (SREBP) -1c (Suppl. Figure 9, available online at www.thrombosis-online.com), emphasising that Mac-1-deficiency protects from adipose tissue inflammation. Notably, this phenotype was limited to adipose tissue, since inflammatory gene expression at distant locations, such as in skeletal muscle tissue was not altered in Mac-1$^{-/-}$ mice (Suppl. Figure 10, available online at www.thrombosis-online.com). Collectively, data establish that the absence of Mac-1 does not affect the accumulation of macrophages, while it improves adipose tissue inflammation.

Stimulation with the agonistic anti-Mac-1 antibody M1/70 aggravates immune cell accumulation and pro-inflammatory cytokine expression in visceral adipose tissue (VAT).

To decipher whether lowered cytokine expression in VAT of Mac-1-deficient mice was caused by absence of integrin outside-in signalling, we aimed for an experimental strategy, by which blockade and agonistic activation of Mac-1 can be tested simultaneously. Interestingly, particular monoclonal antibody clones targeting Mac-1, such as the clone M1/70, are efficient blockers of cell adhesion in neutrophils, but exhibit strong intrinsic activation of Mac-1 particularly in macrophages (29). Indeed, co-incubation of M1/70 with isolated macrophages from obese adipose tissue (ATMs) increased intracellular expression of MCP-1 in ATMs after pre-stimulation with LPS to allow for integrin activation and switching into the active conformation open for ligand-binding (18) (►Figure 4). We next addressed whether treatment of mice with M1/70 or a corresponding isotype control during a 12-week course of HFD would induce pro-inflammatory gene expression in adipose tissue macrophages or block cell infiltration. Interestingly, neither relative weight gain (►Figure 5A), nor plasma lipids were affected by M1/70 treatment (►Figure 5B, C). Also, fasting glucose levels ($p=0.96$, $n\geq10$ per group) and functional assessment of insulin sensitivity and glucose utilisation in intraperitoneal glucose (GTT) and insulin-tolerance testing (ITT) did not show relevant differences (►Figure 5D-F). Interestingly, infiltration of F4/80^{+} macrophages was not affected by Mac-1 engagement (►Figure 5G, H). However, inflammatory cell accumulation of CD4^{+} CD3^{+} and CD8^{+} CD3^{+} T-cells, but not of CD19^{+} B-cells, was increased in M1/70 treated mice (►Figure 5I and Suppl. Figure 11, available online at www.thrombosis-online.com). In accord, percentages of anti-inflammatory T-regulatory (T_{reg}) cells, defined by co-expression of CD3, CD4, CD25, and FoxP3, decreased after M1/70 treatment (32.8 ± 4.9 vs 8.9 ± 4.6% of CD4^{+} T-helper cells, $n\geq10$

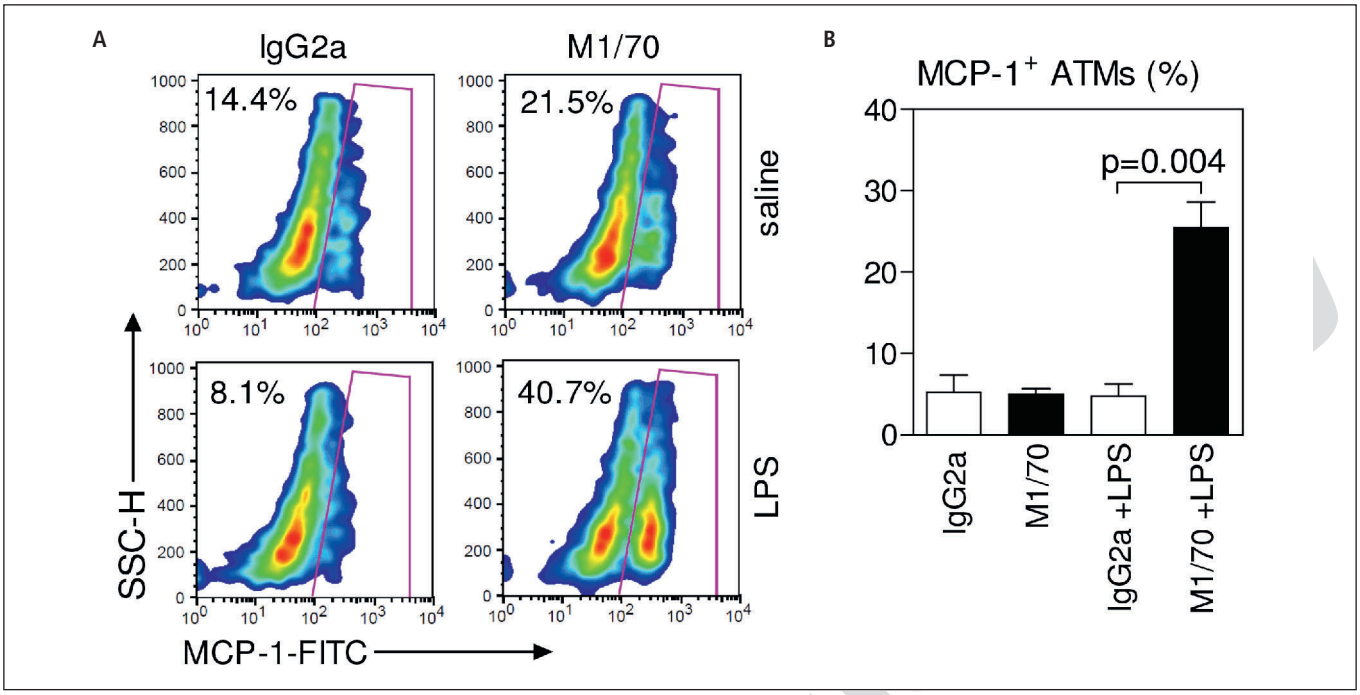

Figure 4: Stimulation with the agonistic anti-Mac-1 antibody M1/70 induces pro-inflammatory gene expression by adipose tissue macrophages. Adipose tissue macrophages (ATMs) were isolated from C57Bl/6 mice consuming high-fat diet (HFD) for 20 weeks by digestion of visceral adipose tissue and magnetic bead separation. ATMs were subsequently cultured *in vitro* in the presence of an activating anti-Mac-1 antibody (clone M1/70), a corresponding isotype control (IgG2a), or with or without LPS. Intracellular expression of the pro-inflammatory cytokine MCP-1 was detected by flow cytometry (A) and quantified as percentage of MCP-1 expressing cells (B). Data are presented as mean ± SEM of at least three independent samples. Statistical significance was calculated by unpaired, two-tailed Student's t-test. P-values are indicated within each graph.

per group, p=0.002, ▶Figure 5I). Notably, gene expression of IL-6 increased by 546 ± 91% (n≥9 per group, p<0.0001), while MCP-1 showed a strong tendency to increase (▶Figure 5J). These data indicate that the relative outcome of simultaneously blocking adhesive properties and activating Mac-1-dependent signalling pathways results in clear shifts towards a hyper-inflammatory phenotype with increased T cell accumulation and pro-inflammatory gene expression, while not affecting accumulation of ATMs.

Specific inhibition of the CD40L/Mac-1 interaction by the peptide inhibitor cM7 does not protect from diet-induced obesity in mice.

Interaction of the biased agonist CD40L with Mac-1 potently regulates cellular adhesion of myeloid cells, while not inducing integrin outside-in signalling (17, 30). To selectively assess the functional consequences of Mac-1's adhesive properties, we tested the specific peptide inhibitor of the CD40L/Mac-1 interaction, cM7 (17), in a therapeutic intervention study. Male C57Bl/6 mice were treated with daily i.p. injections of 100 µg cM7 or its scrambled peptide control scM7 or an equal volume of saline (n≥10 per group). Mice consumed HFD for 20 weeks. Inhibition of the CD40L/Mac-1 interaction did not affect body weights between the groups (▶Figure 6A). Furthermore, treatment with cM7 did not change parameters of glucose or lipid metabolism as demonstrated by equal glucose levels after fasting (▶Figure 6B) or GTT and ITT (data not shown), as well as by plasma cholesterol and triglyceride levels (▶Figure 6C, D). Accordingly, histological signs of hepatic steatosis were detected to a similar degree in all groups (▶Figure 6E). Previous evidence suggests that CD40L deficiency attenuates the accumulation of macrophages in obese VAT (23). In our study, flow cytometric analysis of VAT stromal vascular fraction did not reveal any differences in total number of macrophages in the cM7-treatment group (▶Figure 6F). Interestingly, immunohistochemistry and flow cytometric cell phenotyping of leukocytes resident in VAT revealed lowered infiltration of $CD8^+$ T-effector memory (T_{EM}) cell infiltration in animals treated with cM7 (a decrease by 39 ± 22.8%, p=0.047, ▶Figure 6G, H). These data suggest that the adhesive interaction of Mac-1 to its biased agonist CD40L does not regulate myeloid cell recruitment into adipose tissue, but instead limits conversion of naïve T cells into their effector phenotype.

Discussion

Accumulation of inflammatory cells, in particular of adipose tissue macrophages (ATMs), is a hallmark of adipose tissue inflammation (6). It has been previously proposed that macrophage progenitor cells, such as monocytes, migrate into adipose tissue, differentiate and interact with resident cells, such as adipocytes and other leukocytes (4). ATMs secrete the pro-inflammatory cyto-

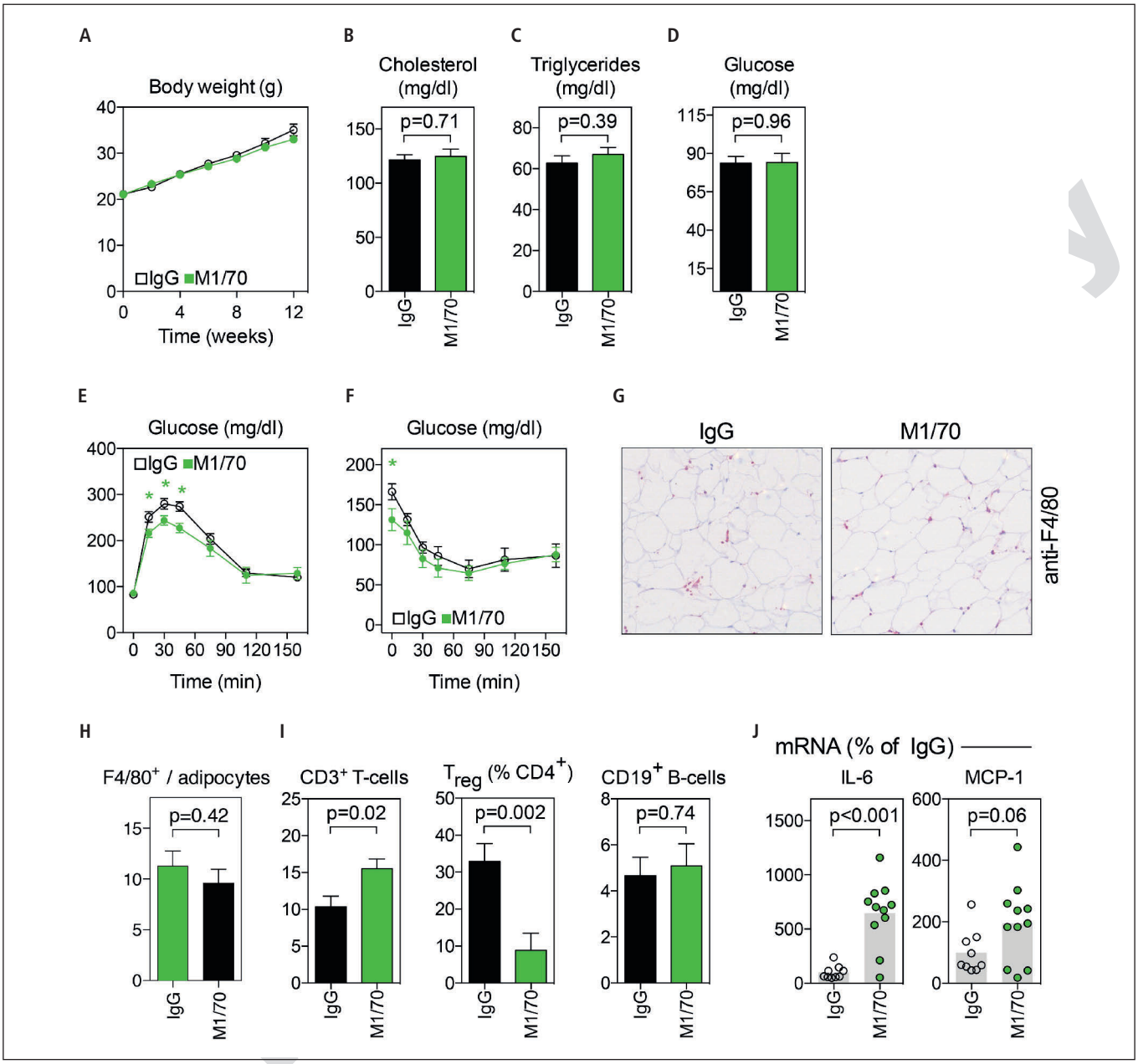

Figure 5: Stimulation with the agonistic anti-Mac-1 antibody M1/70 aggravates immune cell accumulation and pro-inflammatory cytokine expression in visceral adipose tissue (VAT). Male C57Bl/6 mice consumed a high-fat diet (HFD) for 12 weeks and were treated with intraperitoneal injections of the agonistic antibody clone M1/70 or a corresponding isotype antibody (IgG). Diet-induced weight gain is shown at the indicated time points (A). Glucose and serum lipids were analyzed in fasting animals at the end of the feeding period (B-D). Glucose and insulin utilisation were quantified by intraperitoneal glucose (GTT, E) and insulin tolerance tests (ITT, F). Accumulation of F4/80+ macrophages was determined in immunohistochemistry and quantified as macrophages/adipocytes ratio (G, H). T and B cell infiltration into VAT was determined by flow cytometry (I). Abundance of mRNA in VAT was normalised for GAPDH (J). Data are presented as mean ± SEM of at least 10 animals (A-D, G-I) or seven animals (E, F) per group. Statistical significance was calculated by unpaired, two-tailed Student's t-test. P-values are indicated within each graph or indicated by asterisks (*=p<0.05).

and chemokines IL-6, MCP-1, and TNFα, which orchestrate inflammatory remodelling of adipocytes as well as recruitment and activation of further myeloid cells. It has been well established that macrophage inflammation represents a powerful link between obesity, adipose tissue inflammation, and its metabolic complications, such as insulin resistance (5).

Mechanistically, it was proposed, but functionally never proven, that recruitment of monocytes precedes accumulation of and differentiation into adipose tissue macrophages. Sequential engage-

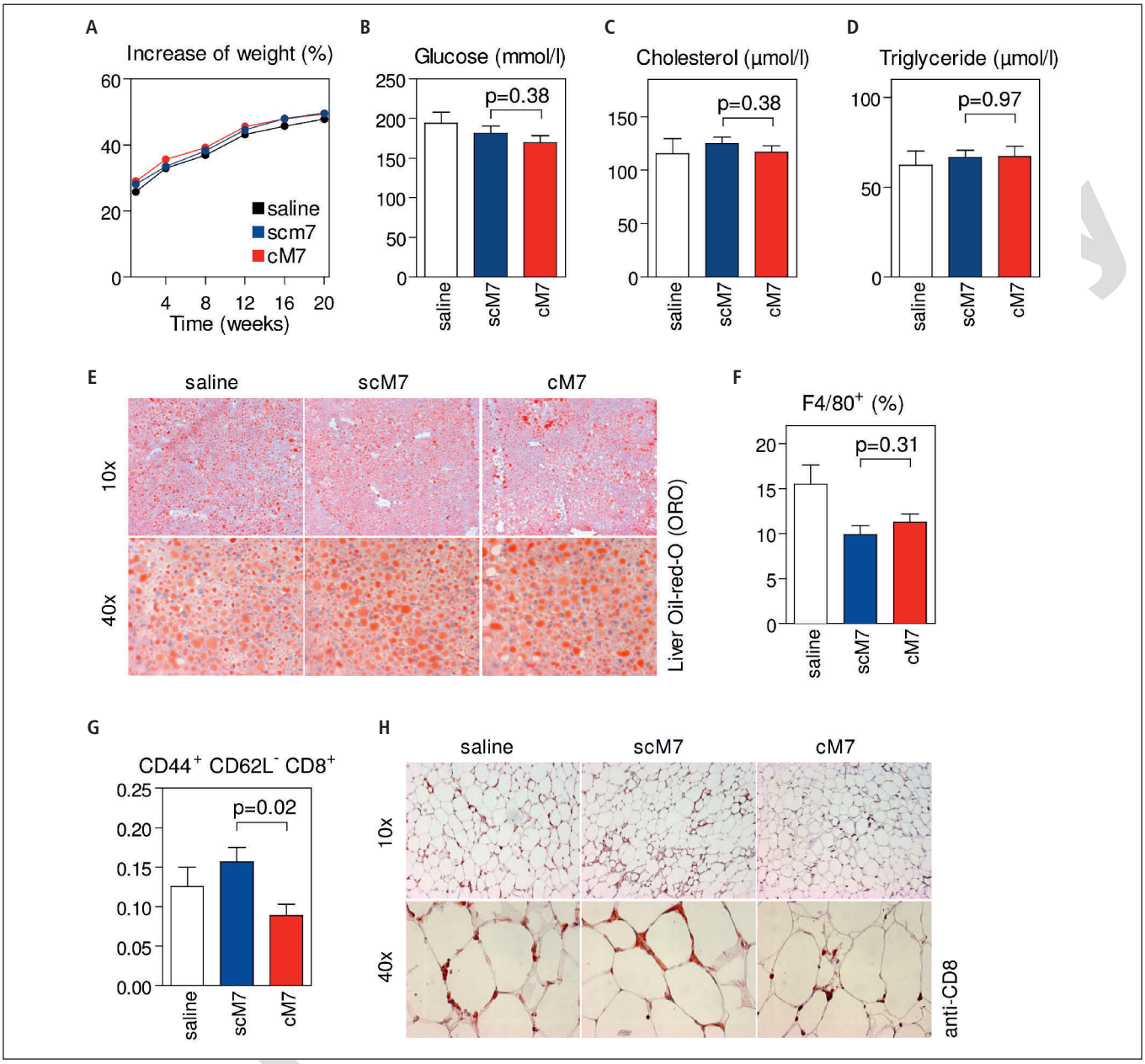

Figure 6: Selective disruption of Mac-1's pro-adhesive interaction to CD40L selectively prevents T cell activation in diet-induced obesity. To test the selective impact of Mac-1's interaction to CD40L during the metabolic syndrome, male C57Bl/6 mice consumed a high-fat diet (HFD) for 20 weeks and were treated with daily intraperitoneal injections of the specific peptide inhibitor cM7, its control peptide scM7, or saline. Peptides were dosed at 100 µg per mouse. Weight increase was monitored at the indicated time points (A). Glucose and serum lipids were analysed in fasting animals at the end of the feeding period (B-D). Liver lipids were detected by Oil-red-O staining of liver sections (E). Infiltration of F4/80$^+$ macrophages (F) and activated CD44$^+$CD62L$^-$ T effector memory cells (G) in VAT was assessed by flow cytometry or in sections of visceral adipose tissue (H). Data are presented as mean ± SEM of at least 11 animals per group. Statistical significance was calculated by unpaired, two-tailed Student's t-test at each time point between both groups. P-values are indicated within each graph.

ment of the leukocyte integrins Mac-1 and LFA-1, along with selectins and the cell adhesion molecules ICAM-1 and VCAM-1, is required to facilitate transmigration of pro-inflammatory leukocytes into target tissues (26). In line, binding of Mac-1 to ICAM-1 and CD40L expressed on the endothelium is required for efficient recruitment of inflammatory cells into the peritoneal cavity and the atherosclerotic plaque (11, 17). Here, we present the surprising finding that a genetic deficiency of the adhesion receptor Mac-1 is not required for recruitment of adipose tissue macrophages during obesity. Our results confirm previous reports that macrophage numbers in adipose tissue are reduced in mice deficient for ICAM-1, but not in mice deficient for Mac-1 (21). Consistently, in-

hibition of ICAM-1 blocked cellular adhesion in mesenteric venules in intravital microscopy studies in obese mice (31). These results suggest a strong role for ICAM-1 in leukocyte recruitment in adipose tissue, and support a role for an interaction with the alternative β_2-integrin LFA-1 (CD11a/CD18), rather than an interaction with Mac-1. Of note, it has recently been demonstrated that deficiency of LFA-1 protects against DIO (32). In this regard it is important to note that Mac-1 preferably regulates adhesion and recruitment of Gr-1$^+$ (Ly6C$^+$) pro-inflammatory monocytes, but not of 'patrolling' Ly6c$^-$ monocytes, which are recruited by engagement of LFA-1 (33). These findings raise the interesting question whether distinct monocyte subsets give rise to adipose tissue macrophages, while others do not. Notably, anti-inflammatory M2-like macrophages are already present in lean adipose tissue. Their origin has been linked to LFA-1-dependent Ly6c$^-$ monocytes. During obesity, ATMs convert into classically activated pro-inflammatory macrophages, which at least partially account for the increase in obesity-associated macrophages (7). This helps to understand how the pool of ATMs may be replenished in the absence of Mac-1-dependent cell recruitment. The concept that tissue macrophages are at least partially of embryonic origin rather than dependent on constant monocyte influx has recently been verified for cardiac and arterial macrophages (34, 35). Finally, our results also do not rule out the possibility that macrophage accumulation in adipose tissue is at least partially independent of continuous cell recruitment. Recent reports suggest that local proliferation and differentiation of tissue-resident macrophages may be sufficient for maintaining tissue macrophage populations in certain stages of atherosclerotic disease (36). Our finding that Mac-1 deficient mice had reduced numbers of circulating leukocytes at every stage of disease and independently of the diet regime without affecting macrophage content in VAT may support this finding.

Our results indicate that the overall net effect of Mac-1 deficiency does not impede the accumulation of ATMs. Instead, we observed that gene expression of the pro-inflammatory mediators IL-6 and MCP-1 decreased in the absence of Mac-1, indicating that ligation of Mac-1 by outside-in signalling is needed for pro-inflammatory gene expression as suggested in previous studies (28). For further proof of concept and to outweigh the differential effects on myeloid cell recruitment versus integrin agonism, we tested the blocking and agonistic anti-Mac-1 antibody clone M1/70 in a second animal study. Notably, M1/70 blocks infiltration of leukocytes, particularly of neutrophils, in a variety of models including peritoneal inflammation and atherosclerosis (16), while inducing cell activation by outside-in signalling in macrophages (29). Our results that M1/70 does not affect macrophage accumulation appear contradictive to previous results from our group and others, demonstrating attenuated atherosclerotic plaque size with reduced macrophage content after treatment with M1/70 (16). A possible explanation is that monocyte/macrophage infiltration in atherosclerosis might primarily be caused by attenuated neutrophil infiltration as recently suggested by several studies, which propose that neutrophils precede monocytes in atherosclerosis (37, 38). Interestingly, Yakubenko et al. proposed that Mac-1 agonism inhibited inflammatory gene expression and foam cell formation in macrophages (39). These data indicate a protective effect for Mac-1 in lesional macrophages (16). This idea is also consistent with previous reports proposing activation of Mac-1 by small molecules as a novel anti-inflammatory strategy (40). Correspondingly, genetic lack of Mac-1 on bone marrow-derived cells did not protect from atherosclerosis in an LDLR$^{-/-}$-model, which likely represents the net effect of lowered monocyte recruitment and missing protection by protective Mac-1 agonism (41).

In contrast to plaque macrophages, we observed that Mac-1 agonism in ATMs is pro-inflammatory. Talukdar et al. recently suggested that mice with a genetic deficiency for neutrophil elastase are protected from insulin resistance while they show no effect on obesity or overall macrophage infiltration (42). In accord, we show that mice treated with the neutrophil-blocking, but macrophage-activating antibody M1/70 exhibited slightly ameliorated insulin sensitivity and glucose utilisation, but showed no difference in macrophage accumulation. Moreover, the specific inhibitor of Mac-1's interaction to its biased, non-agonistic ligand CD40L (17, 30) was shown to be effective in atherosclerosis, but had no effect on adipose tissue inflammation in this study. Of note, we show that Mac-1 is also expressed on antigen-presenting

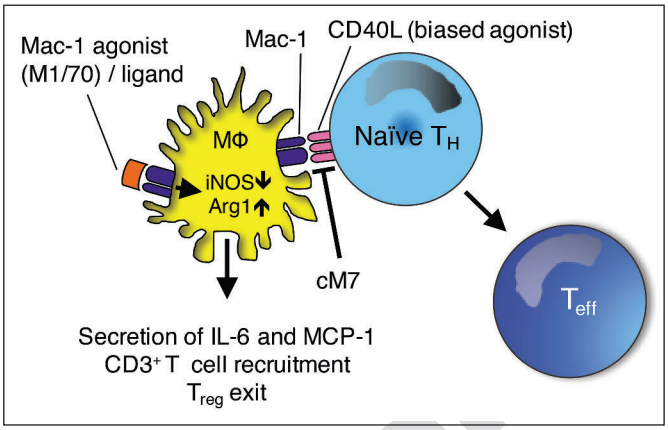

Figure 7: Proposed model of Mac-1's effector pathways in adipose tissue macrophages (ATMs). Macrophages resident in adipose tissue (MΦ) express Mac-1 in high quantities. While Mac-1 is not required for accumulation of macrophages in adipose tissue, activation with agonists, such as with the activating antibody clone M1/70, induces secretion of the pro-inflammatory mediators MCP-1 and IL-6 and initiate chemotactic recruitment of inflammatory cells or induce tissue exit (or apoptosis) or anti-inflammatory leukocyte species, e.g. T-regulatory cells. If Mac-1 is not expressed on macrophages, expression of the M1-marker iNOS in ATMs decreases, while expression of the M2-marker Arginase-1 (Arg1) increases, indicative of a skew towards a M2-phenotype. In contrast to Mac-1's (agonistic) ligands, which bind and activate Mac-1 in an outside-in signalling dependent fashion, CD40L describes a new class of integrin binding partners not inducing integrin activation, so called biased agonists. In line with this concept, interaction of CD40L with Mac-1, as tested by inhibition studies with the specific peptide inhibitor cM7, does neither modulate cytokine expression in adipose tissue, nor myeloid cell recruitment. Instead, cM7 inhibits T-cell activation and conversion to CD44$^+$CD62L$^-$ T-cells, indicating that macrophages employ CD40L/Mac-1 binding during adipose tissue inflammation to induce T-cell activation.

What is known about this topic?

- Cardio-metabolic disease, such as the metabolic syndrome, is driven by a low-grade inflammatory response in obese visceral adipose tissue.
- Local inflammation is critically dependent on the accumulation and functioning of inflammatory leukocytes and myeloid cell adhesion factors, such as the leukocyte integrin Mac-1 (CD11b/CD18).
- The exact role of Mac-1 in adipose tissue inflammation is not known.

What does this paper add?

- Both, genetic deficiency and therapeutic inhibition of Mac-1's adhesive properties by an antibody and the novel peptide inhibitor, cM7, did not affect myeloid cell infiltration in adipose tissue.
- Instead, activation of Mac-1's outside-in signalling by an agonistic antibody induced a hyper-inflammatory phenotype in adipose tissue. Consistently, Mac-1 deficient mice show attenuated inflammatory gene expression.
- These data demonstrate that Mac-1 figures potently in inflammatory gene expression, but does not regulate leukocyte infiltration. Thus, conventional strategies to inhibit Mac-1 by blocking antibodies have to be evaluated re-considered in cardio-metabolic disease.

dendritic cells. Furthermore, monocytes were recently shown to function as antigen-presenting cells (43). It was proposed that T cells invading adipose tissue may respond to particular antigens and thereby induce specific immunity in adipose tissue likely being dependent on co-stimulatory pathways (27, 44). Given our findings that the peptide inhibitor cM7 reduced conversion of naïve T cells into activated T-effector-memory cells, it is possible that the interaction of antigen-presenting monocytes, macrophages, or dendritic cells employ the CD40L/Mac-1 interaction to boost T cell activation in a co-stimulatory like manner. This is further supported by the fact that genetic deficiency of 4–1BB and CD40L attenuated T cell responses and propagation of specific T cell subsets (23, 45, 46), while engagement of CD40 receptor in DIO rather induces a protective phenotype in T cells (47, 48). Moreover, genetic deficiency of the β_2-integrin LFA-1 (CD11a/CD18) potently attenuated T cell activation in DIO (32). Interestingly, Mac-1-deficiency resulted in increased numbers of VAT $CD4^+$ T-helper cells that may protect from adipose tissue inflammation (27).

Surprisingly, we observed that Mac-1-deficiency broadly impacts on the metabolic phenotype. Some facets of this complex phenotype remain unresolved in the current study. In particular, we observed that Mac-1-deficiency causes an insulin-secretion deficiency that may be causal for increased fasting glucose levels and a higher EGP under fasting conditions. On the other hand, reduced glucose uptake in some muscles and an impaired suppression of EGP under clamp conditions suggest tissue-specific insulin resistance. Also, it is not clear whether reduced triglyceride accumulation in the liver is a result of improved hepatic steatosis mediated by dampened triglyceride synthesis or caused by the increased lipid efflux, e.g. of very low-density lipoprotein. It is also to speculate whether some of these metabolic derangements could be secondary to altered macrophage and myeloid cell infiltration in distinct tissues. Mac-1 is a potent regulator of macrophage and monocyte infiltration in some tissues and diseases, albeit it did not regulate macrophage accumulation in adipose tissue in the current study. Notably, depletion of macrophage-like Kupffer cells in the liver prevents diet-induced hepatic steatosis (49), while local macrophages in the pancreas protect from losing pancreatic islets during inflammation (50). Similarly, muscle tissue homeostasis is maintained by local macrophages (51). In accord, the presence and phenotype of macrophages in adipose tissue will likely affect adipocyte function and tissue remodelling (25). Such tissue-attributed effects could be caused by a lack of myeloid cells or the impaired ability of Mac-$1^{-/-}$ leukocytes to exit tissue and resolve inflammation (52).

It is also important to note the limitation of complex studies such as ours that the causal relationship of our findings may be multi-dimensional. For example, adipocyte size was decreased in Mac-$1^{-/-}$ mice under a standard chow diet, which could be caused by altered macrophage polarisation in adipose tissue at baseline. On the other hand, despite the consideration that smaller adipocytes are better protected from metabolic derangements, a smaller adipocyte size at baseline favours a more insulin resistant phenotype after DIO (53). Also, a lack of inflammatory pathways may to some degree be needed to restore the adipocyte's ability to maintain adequate adipose tissue remodelling and expansion (54). In accord, some pro-inflammatory cytokines may partially be needed to limit inflammation in a counter-regulatory manner, as recently shown for IL-6 (55).

In conclusion, we propose a model in which Mac-1 is dispensable for macrophage accumulation, but required for pro-inflammatory gene expression by macrophages in adipose tissue inflammation (►Figure 7). Overall, our findings provide a novel insight into Mac-1-dependent signalling pathways and question the net effect of therapeutic strategies to inhibit integrin-mediated cell adhesion in cardio-metabolic disease.

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Conflicts of interest
None declared.

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