Atherosclerosis 281 (2019) 31–37

Contents lists available at ScienceDirect

Atherosclerosis

journal homepage: www.elsevier.com/locate/atherosclerosis

Adipose tissue macrophages do not affect atherosclerosis development in mice

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HIGHLIGHTS

- Adipose tissue macrophages affect circulating triglycerides and immune cells.
- Transplantation of obese adipose tissue affects circulating cytokines.
- Obese adipose tissue transplantation does not affect atherogenesis.
- Depleting adipose tissue macrophages does not prevent atherosclerosis development.

ARTICLE INFO

Keywords:
Adipose tissue macrophages
Atherosclerosis
Obesity
Adipose tissue inflammation

ABSTRACT

Background and aims: Obese individuals have a higher risk of developing atherosclerosis, possibly driven by adipose tissue (AT) inflammation. We recently showed that AT macrophages (ATMs), which accumulate in the expanding obese AT, produce mediators causing immune cell recruitment from the bone marrow. In the current study, we evaluated whether ATMs are directly involved in atherosclerotic plaque development.

Methods: Lean *ldlr*$^{-/-}$ acceptor mice received visceral AT (vAT) from lean, obese, or ATM-depleted obese *ldlr*$^{-/-}$ mice. Acceptor mice were fed high cholesterol diet (HCD) for 4 weeks before and 8 weeks after AT transplantation to induce atherosclerosis. Atherosclerotic plaque development was studied 8 weeks after transplantation.

Results: Transplanting donor vAT from obese mice increased circulating triglycerides and B-cells, but decreased Ly6c$^-$ monocytes. Plasma cholesterol, Ly6c$^+$ monocytes, T-cells, NK-cells and eosinophils were unaffected. Depleting ATMs from obese AT using clodronate liposomes prior to vAT transplantation prevented the increase in triglycerides and B-cells and decrease in Ly6c$^-$ monocytes, but did increase eosinophils. Circulating Cxcl1 was reduced by obese AT transplantation and Ifn-γ tended to be increased while Tnf and Il-1β were unaffected. ATM-depleted obese AT transplantation also reduced Cxcl1, but increased circulating Tnf levels. However, obese AT transplantation with or without depletion of ATMs did not influence atherosclerotic plaque size, phenotype, or stability.

Conclusions: ATMs from obese vAT do not affect atherosclerotic plaque development or phenotype.

Abbreviations: AT, adipose tissue; ATM, adipose tissue macrophage; Cd, cluster of differentiation; CVD, cardiovascular disease; Cxcl, chemokine (C-X-C motif) ligand; DOTA, depleted obese transplanted acceptor; FACS, fluorescence-activated cell sorting; HCD, high cholesterol diet; HE, Haematoxylin and Eosin; HFD, high fat diet; Ifn, interferon; Il, interleukin; LFD, low fat diet; LTA, lean transplanted acceptor; MCP1, monocyte chemoattractant protein 1; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; OTA, obese transplanted acceptor; scAT, subcutaneous AT; SEM, standard error of the mean; Tnf, tumor necrosis factor; vAT, visceral adipose tissue

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https://doi.org/10.1016/j.atherosclerosis.2018.12.010
Received 9 July 2018; Received in revised form 6 December 2018; Accepted 7 December 2018
Available online 19 December 2018

1. Introduction

Over the last few decades, the prevalence of obesity has nearly tripled. In 2016, 39% of the global adult population was overweight and 13% was obese [1]. Obesity has been associated with diabetes, non-alcoholic fatty liver disease (NAFLD) and cardiovascular disease (CVD). Several studies, including the Framingham heart study, have connected increasing degrees of obesity with increasing rates of coronary heart disease including atherosclerosis, an important cause of CVD [2,3].

Atherosclerosis is a progressive disease in which lipids, fibrotic proteins, and immune cells accumulate in the vessel wall of large arteries forming an atherosclerotic plaque. When this plaque ruptures, the vessel is promptly occluded and can cause a myocardial infarction or stroke [4]. Immune cell recruitment and accumulation plays a key role in plaque development. At first, hyperlipidemia, shear stress and circulating cytokines all contribute to endothelial dysfunction resulting in endothelial expression of adhesion molecules. In parallel, cholesterol loaded LDL particles accumulate in the vessel wall, are modified and trigger the endothelium to produce chemokines [5]. Next, circulating neutrophils and monocytes will bind to the endothelial adhesion molecules and are attracted to modified LDL induced chemokines allowing migration into the vessel wall. Neutrophils can there aggravate endothelial dysfunction and attract more monocytes, while monocytes differentiate into macrophages [6]. In the vessel wall, these macrophages take up (modified *i.e.* oxidized) lipids, recruit more immune cells to the vessel wall and produce extracellular matrix degrading enzymes that increase the risk of plaque rupture [7]. Neutrophils also further stimulate lipid uptake by plaque macrophages and produce both proteases and reactive oxygen species contributing to plaque instability [6].

Both the expansion of the adipose tissue (AT) as well as the accompanying AT inflammation has been suggested to be responsible for the increased risk of atherosclerosis [8,9]. The expansion of adipose tissue and the accompanying increase in circulating lipids (including cholesterol loaded LDL) is being tackled by lowering lipids and, indeed, this reduces mortality rates [10]. However, simply lowering lipids is not effective enough with two thirds of cardiovascular events still occurring [10]. Therefore, it is necessary to investigate the link between AT inflammation and atherosclerosis.

An important driver of AT inflammation are the AT macrophages (ATMs) which accumulate in the visceral AT (vAT) of obese humans and mice [11,12]. In addition to higher macrophage numbers in the obese AT, obese ATMs also differ in phenotype when compared to ATMs in lean AT. The obese ATMs are considered to be more proinflammatory as they produce Tnf and iNOS and are typically characterized by expression of Cd11c. ATMs found in lean AT produce anti-inflammatory factors such as arginase-1 and Il-10 and are identified based on expression of MRC1 [12]. Recently, we and others demonstrated that the proinflammatory $Cd11c^+$ ATMs express genes involved in myelopoiesis and immune cell recruitment, thereby affecting circulating monocyte and neutrophil levels [13,14].

Öhman et al. have shown that vAT inflammation accelerates atherosclerosis in $ApoE^{-/-}$ mice and that monocyte chemoattractant protein (Mcp1) plays a key role in this vAT stimulated atherogenesis [15,16]. These findings strongly suggest a key role of ATMs in atherosclerosis. Recently, our group transplanted lean, obese or ATM-depleted obese AT to acceptor mice. We demonstrated that inflammatory $Cd11c^+$ ATMs directly cause an increase in circulating neutrophils and monocytes, potentially by expression of cytokines such as Cxcls, Csfs and S100a8/a9. This elevated immune cell recruitment resulted in hepatic neutrophil and macrophage accumulation and consequently liver damage [13]. Due to these effects of ATMs on circulating immune cells and systemic inflammation, we investigated whether ATMs directly cause atherosclerotic plaque development.

2. Materials and methods

2.1. Adipose tissue transplantation

Eight-week-old male C57BL/6 $ldlr^{-/-}$ mice were fed a low fat (LFD) or high fat diet (HFD) for 12 weeks before acting as an AT donor. HFD-feeding induced AT inflammation in these donor mice was characterized and is described elsewhere [13]. One group of HFD-fed donor mice was injected with clodronate liposomes (i.p. 115 mg/kg) two days prior to sacrifice to deplete ATMs. The effectiveness of ATM depletion in the used donor mice has been previously described [13]. Visceral AT (epididymal fat; ± 250 mg) derived from LFD-fed, HFD-fed or ATM depleted HFD-fed mice was transplanted to the peritoneal cavity of eighteen-week-old male C57BL/6 $ldlr^{-/-}$ mice resulting in the following three groups: lean-transplanted acceptors (LTA), obese-transplanted acceptors (OTA) and ATM-depleted obese-transplanted acceptors (DOTA). Acceptor mice received a high cholesterol diet (HCD) for 4 weeks before and 8 weeks after AT transplantation to induce atherosclerosis. Mice with signs of infection or peritonitis based on weight of the transplanted AT and visual inspection at the end of the experiment were excluded from all data. An extensive description of all AT transplantation procedures used in this experiment has been previously reported [13]. All performed experiments were approved by the Animal Experiments Committee of Maastricht University.

2.2. Histology

Aortic roots were cryosectioned (7 μm) and stained for Haematoxylin and Eosin (HE; Sigma-Aldrich), Sirius red (stains collagen) or Oil Red O (stains lipids). An experienced mouse pathologist scored plaque phenotype from 0 to 5 based on fibroblasts, necrosis, foam cells, general inflammation, endothelial adhesion, granulocytes, adventitia influx and calcification using the HE sections. Photographs were taken using a Jenoptik camera and progress capture pro 2.8.8 software. Photos of HE (40x magnification), Sirius red (40x magnification) and Oil Red O (100x magnification) sections were analysed blindly using computerized morphometry (Leica QWin V3, Cambridge, UK) to quantify plaque size, necrotic area and collagen and lipid content. For each mouse, the average plaque size of all three plaques was quantified based on four sections and then summed to determine the total plaque size. Two sections of each plaque were quantified per mouse to calculate an average percentage of plaque collagen content. Cryosections were stained using antibodies against neutrophils (custom antibody; clone NIMP-R14) and macrophages (custom antibody; clone Moma-2) as previously described [17]. Neutrophils were counted in three sections per mouse (40x magnification) and an average was calculated. Plaque macrophage content was quantified in a blinded manner using computerized morphometry.

2.3. Cytokine levels

Circulating cytokine levels of Il-6, Cxcl1, Ifn-γ, Il-1β, Il-2, Il-4, Il-5, Il-10, Il-12P70, and Tnf were measured using a V-plex Mouse Cytokine Assay kit (Meso Scale Discovery), according to the manufacturer's instructions.

2.4. Flow cytometry

Blood was collected at the end of the study by cardiac puncture. FC-receptor block (anti-CD16/CD32) was added to the blood. Next, a cocktail of antibodies was added (Supplementary Table 1). Erythrocytes were lysed by adding lysisbuffer (8.4 g/L NH_4CL and 0.84 g/L $NaHCO_3$ in H_2O, pH 7.4) before measurement. Flow cytometry was performed using a BD FACSCANTO II running FACS Diva 8.0.1 software, which was used for analysis of all flow cytometry data. The FACS gating strategy can be found in the supplementary materials (Supplementary

Fig. 1).

2.5. RNA isolation, cDNA synthesis and qRT-PCR

RNA was isolated from aortic arch tissue using Trizol reagent (Ambion) before cDNA synthesis using the iScript cDNA synthesis kit (170–8891; Bio-Rad, Hercules, USA) following manufacturer's instructions. Gene expression was determined using IQ SensiMix SYBR master mix (Bioline, London, UK) on a CFX96 Touch with CFX manager software (Biorad). The geometric mean of two reference genes, Cyclophillin and Beta2-microglobulin, was used as reference and the ΔΔCT method was employed to calculate expression levels [18]. Primer sequences are given in Supplementary Table 2.

2.6. Plasma cholesterol and triglyceride measurements

Plasma levels of cholesterol and triglycerides were measured using a colorimetric test (Cholesterol FS′10 and Triglycerides FS 5‘ ecoline, Diagnostic System GmbH, Holzheim, Germany) as previously described [19].

2.7. Statistical analysis

A one-way ANOVA with a Tukey's multiple comparison post-hoc test was performed to analyze all data using GraphPad Prism 5. All data are expressed as the mean ± SEM and were considered statistically significant at $p \leq 0.05$.

3. Results

3.1. ATMs affect circulating triglycerides, Ly6c$^-$ monocytes, B-cells and eosinophils, but not cholesterol, Ly6c$^+$ monocytes, T-cells or NK-cells

The transplanted AT from the donor mice was characterized extensively to confirm diet-induced AT inflammation and effective depletion of ATMs in clodronate-liposome injected mice, these data are described elsewhere [13]. HCD feeding of the acceptor mice resulted in comparable weight gain in all groups both before and after transplantation indicating similar recovery from the AT transplantation surgery (Supplementary Fig. 2). While plasma total cholesterol was unaffected by the type of transplanted vAT, triglycerides were increased in OTA mice. Depleting ATMs from donor obese AT prior to transplantation prevented this increase in triglycerides in acceptor mice (Supplementary Figs. 3A and B). Free fatty acids (FFA) levels and measures of lipolysis gene expression levels have been previously reported and were comparable between groups [13]. Total circulating monocytes were decreased in the OTA mice, but not in the DOTA mice when compared to LTA mice (previously published data [13]). These effects were mainly due to lower patrolling Ly6c$^-$ monocytes in the OTA mice (Fig. 1A). Circulating proinflammatory Ly6c$^+$ monocytes were unchanged and comparable between all groups after 8 weeks of HCD (Fig. 1B). Blood neutrophils, T-cells and NK-cells were unaffected by transplantation of obese AT with or without ATMs (Fig. 1C and D and previously published data [13]). The OTA mice had increased circulating B-cells when compared to LTA or DOTA mice (Fig. 1E). Eosinophil levels were similar between LTA and OTA mice, but elevated in the DOTA mice when compared to the OTA mice (Fig. 1F). It is important to note that immune cell levels were already high before the transplantation since the HCD, given from 4 weeks prior to transplantation, rapidly induces immune cell recruitment [20,21]. In addition, at the end of the experiment, the transplanted AT was well vascularized and had a comparable weight and macrophage content in all groups (previously reported data [13]).

3.2. Transplantation of obese AT affects circulating cytokines

To investigate whether AT macrophages affect systemic inflammation, we measured circulating cytokine levels. LTA and OTA mice exhibited similar circulating levels of Tnf (proinflammatory cytokine), but the DOTA mice displayed increased circulating Tnf levels compared to LTA and OTA mice (Fig. 2A). Circulating levels of Il-1β (mediator inflammatory response) did not differ significantly between groups (Fig. 2B). Cxcl1 (chemotaxis protein) plasma levels were decreased after obese AT transplantation, but not affected by ATMs (Fig. 2C). The macrophage activating cytokine, Ifn-γ, tended to be increased in OTA, but not in DOTA mice when compared to LTA mice (Fig. 2D).

3.3. Depletion of ATMs does not prevent atherosclerotic plaque development

Atherosclerotic plaques were characterized 8 weeks after AT transplantation (*i.e.* after 12 weeks of HCD). Plaque phenotype was determined by scoring plaque severity and complexity and was similar in all groups (Fig. 3A). In addition, plaque size, collagen content, necrotic plaque area and plaque lipid content did also not differ between the groups (Fig. 3B and C). Plaque size was determined in a small sham-operated group as well and was comparable to the other groups (data not shown). As we previously reported strong effects of ATMs on circulating and hepatic neutrophil levels [13], we quantified plaque neutrophils. However, neutrophil cell count was similar between all groups 8 weeks after AT transplantation (Fig. 3D) and plaque macrophage content was comparable as well (Fig. 3E). Aortic arch gene expression analysis was similar in all groups, supporting that there is no difference in plaque inflammation (Fig. 3F).

4. Discussion

The current study showed neither effect of obese AT nor a key role of ATMs on the development of atherosclerosis despite effects on circulating lipids, immune cells and cytokines.

Transplanting obese AT resulted in increased circulating triglycerides and this effect was dependent on ATMs. As both the OTA and DOTA group received similar amounts of adipose tissue and lipolysis gene expression in the donor AT was comparable [13], the change in triglycerides is likely not due to lipolysis of the donor AT. In addition, the FFA levels were comparable in the donor mice [13]. Potentially, the increase in circulating triglycerides is due to hepatic dysfunction caused by liver damage present in the obese transplanted group. Previously, we observed liver damage in this group, which was reduced when ATMs were depleted before transplantation [13]. This is in line with the effects on plasma triglycerides. Indeed, Alwahsh et al. showed in rats that diet-induced liver damage coincides with increased plasma triglycerides [22]. Furthermore, Corey et al. showed an association of non-alcoholic steatohepatitis (NASH) resolution with reduced triglyceride levels, again demonstrating a link between liver inflammation and damage with triglyceride levels [23]. Interestingly, this increase in circulating triglycerides did not affect atherosclerotic plaque size, phenotype or lipid content. However, if the observed difference in triglycerides is due to liver damage, only present at a late stage of NASH, it is possible that these changes only occurred at a later stage of atherosclerotic plaque development. Alternatively, the observed difference in total lipid levels may not be large enough to impact plaque development or lipid accumulation. Moreover, plasma cholesterol, the major driver of experimental atherosclerosis, was comparable between all groups.

The levels of most immune cells were not different between the groups. Despite an initial effect of ATMs on neutrophil recruitment from the bone marrow, neutrophils were comparable between all groups 8 weeks after AT transplantation [13]. Total monocytes were lower (most likely due to reduced patrolling Ly6c$^-$ monocytes) after transplantation of AT from obese mice and this was not observed when transplanting

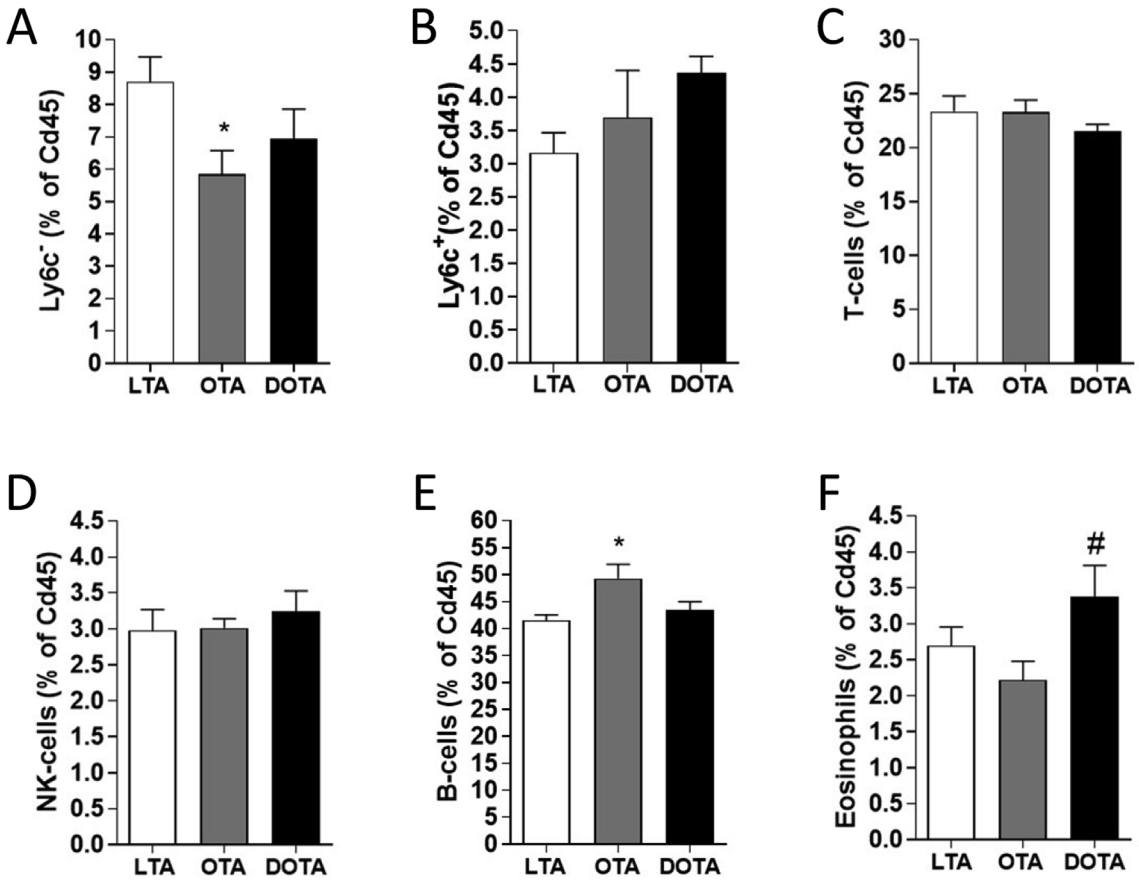

Fig. 1. Effects of adipose tissue macrophages on circulating immune cell levels.
Ly6c$^+$ monocytes (A), Ly6c$^-$ monocytes (B), T-cells (C), B-cells (D), NK-cells (E) and eosinophils (F) in LTA, OTA and DOTA mice 8 weeks after AT transplantation (*i.e.* after 12 weeks of HCD feeding) presented as percentage of CD45$^+$ (immune) cells. All data are means ± SEM. *$p \leq 0.05$ *vs.* LTA. #$p \leq 0.05$ *vs.* OTA.

ATM-depleted AT. The reduction in circulating monocytes might indicate elevated recruitment of monocytes to tissues as we reported higher levels of hepatic macrophages in the obese transplanted group, which are likely monocyte-derived [13,24]. Importantly, the hypercholesterolemia induced by HCD-feeding triggers monocytosis resulting in elevated monocyte levels in all groups even before transplantation [20]. This strong induction of monocytosis by the diet might have overshadowed the effects of the ATMs on circulating immune cells. In line, greater effects of ATMs on circulating cells were observed in chow-fed mice, albeit immune cell levels were analysed two weeks and not 8 weeks after transplantion [13].

Notably, circulating B-cells were slightly increased by obese AT transplantation. Current literature supports both an atherogenic and atheroprotective role for B-cells in atherosclerosis [25]. However, as we did not observe any effects on atherosclerosis in our study, the increase in B-cells might not have been sufficiently high to affect the atherosclerotic plaque. Eosinophils were increased only when transplanting ATM-depleted obese AT. Zhang et al. described that eosinophils can promote M2 macrophage polarization in AT, thereby reducing chronic inflammation [26]. Moreover, M2 macrophages are known to recruit eosinophils by expressing chemokines such as Ccl17, Ccl22 and Ccl24 [27]. To our knowledge, effects of specifically ATMs on eosinophils have not been described, but based on the data from Zhang et al., it is conceivable that this interaction does exist and should be further explored. It should be noted that transplanted AT vascularization, morphology, necrosis and macrophage content was comparable in all groups after 8 weeks (previously reported data [13]). Therefore, it is expected that the transplanted AT no longer has a direct effect on circulating cells at the end of the experiment.

To assess systemic inflammation, we measured several circulating cytokines. However, 8 weeks after transplantation, obese AT only tended to increase circulating Ifn-γ, an important proinflammatory cytokine important in tissue macrophage differentiation. Ifn-γ is expressed at high levels in the atherosclerotic plaque and its role in atherosclerosis is a topic of ongoing discussion. Likely, it has a proatherogenic role when produced in the plaque [28], but we found no difference between groups in plaque size or phenotype. However, we only measured Ifn-γ in the circulation and not in the plaque itself. Furthermore, the effects on circulating levels are subtle and might be transient, therefore not impacting plaque development over time. Levels of Tnf, a major proinflammatory cytokine both produced by adipocytes and macrophages, were elevated in the acceptor mice that received ATM-depleted obese AT. In AT, macrophages are the main producers of Tnf [29]. As macrophages were depleted from the obese AT, the elevated Tnf plasma levels are therefore most likely caused by increased Tnf production in other tissues. Cxcl1 (also known as CXCL8 in humans), was measured and found to be decreased by transplantation of obese AT, independent of ATM depletion. Cxcl1 is implicated in both neutrophil chemotaxis and monocyte mobilization [30,31], but lower levels in the obese transplanted groups did not impact atherosclerosis. Collectively, these data show that ATMs impact systemic inflammation, but that these changes are not sufficient to alter atherosclerosis development. This is in line with work by Dalmas et al., which showed that intima-media thickness (marker of subclinical atherosclerosis) was unaffected by systemic inflammation or ATM accumulation in obese humans [32].

We previously showed that the increase in circulating neutrophils and monocytes caused by ATMs resulted in hepatic neutrophil and macrophage accumulation worsening NASH. Interestingly, NASH is associated with cardiovascular disease and over five times as many

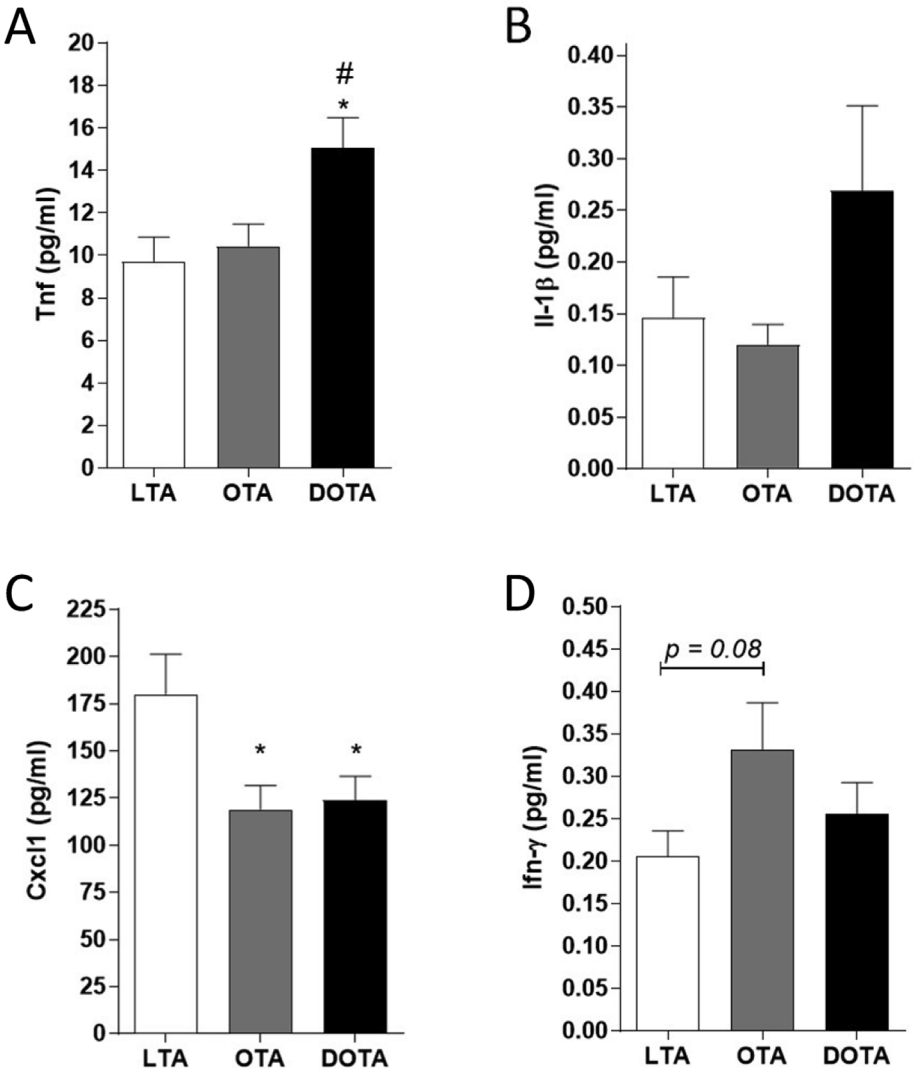

Fig. 2. Circulating inflammatory cytokines are influenced by adipose tissue macrophages.
Plasma levels of Tnf (A), Il-1β (B), Cxcl1 (C) and Ifn-γ (D) in LTA, OTA and DOTA mice after AT transplantation and 12 weeks of HCD feeding. All data are means ± SEM. *$p \leq 0.05$ *vs*. LTA. #$p \leq 0.05$ *vs*. OTA.

NASH patients die from CVD than from liver-related causes. Furthermore, a long-term follow up study predicted lower survival of CVD-related diseases when a patient had NASH and this lower survival was not present when a patient had only liver steatosis in which there is very little inflammation, suggesting that hepatic inflammation plays a key role in modulating this risk [33]. We observed no effects of ATMs on atherosclerotic plaque size, phenotype, composition or inflammation despite our previously reported effects on hepatic inflammation in the same model [13]. Possibly, the early changes in hepatic neutrophils and later effects on hepatic macrophage accumulation and damage are not sufficient to impact atherosclerotic plaque development. However, a necessary specific sequence of events might also explain the lack of effects of ATMs on atherosclerosis in our experiments. The observed differences in hepatic inflammation and especially liver damage take time to develop and are therefore absent in the early stages of atherosclerotic plaque development. Moreover, the strongest effects on circulating cells and hepatic inflammation are observed early as ATMs recruit neutrophils from the bone marrow and cause neutrophilia followed by hepatic neutrophil infiltration. At a later time point, this early neutrophil infiltration caused elevated hepatic macrophage accumulation and liver damage [13]. However, during the initial and relatively short neutrophilia phase caused by the ATMs, a plaque has not yet fully developed and therefore neutrophils could only impact the early stage of plaque development. Therefore, even though neutrophils have been described to affect atherosclerosis [6], the transient effect of ATMs on neutrophils might not have impacted atherosclerosis in our model. Indeed, when the plaque has fully developed 8 weeks after transplantation, circulating and plaque neutrophil levels as well as plaque macrophage content were comparable between groups. This potential explanation is supported by the similar inflammatory state of the transplanted AT in all groups at this time point, which implies the obese AT is no longer inflammatory.

Our results are not in line with the work of Öhman et al., which showed effects of vAT transplantation on atherosclerosis. However, there are crucial differences in the methods employed between these studies. In the study of Öhman et al., vAT was transplanted subcutaneously into 4 dorsal (backside) incisions while in the current study the vAT was transplanted into the peritoneal space [15]. Besides the location of the AT transplant, the control group was different as we used a lean transplanted mouse as control while Öhman et al. used a sham operated control group [15]. Lastly, the atherosclerosis model used differed between studies with Öhman et al. employing the non-diet dependent *ApoE*$^{-/-}$ model compared to the HCD fed *ldlr*$^{-/-}$ used in the current study. Indeed, different results have been obtained

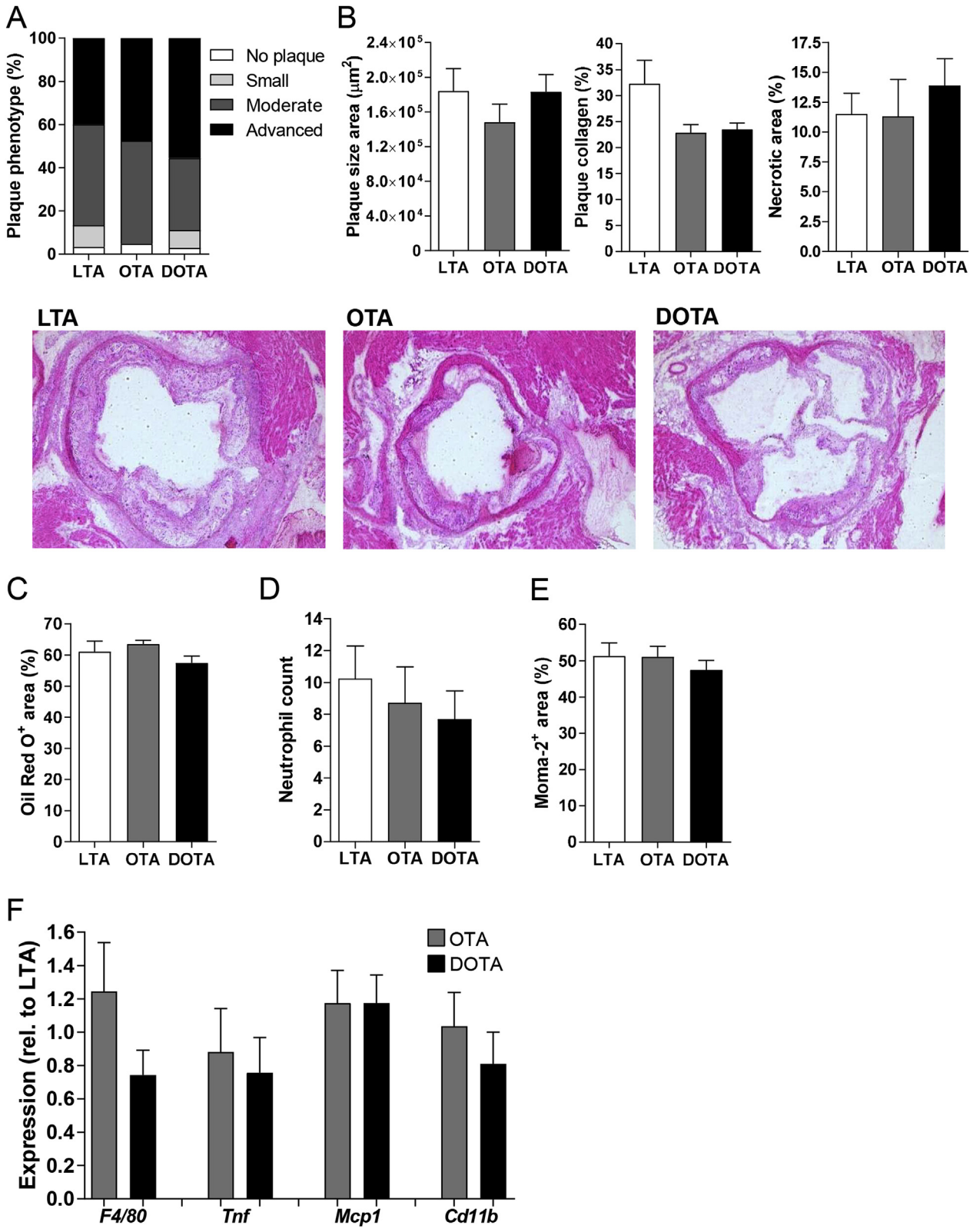

Fig. 3. The atherosclerotic plaque is unaffected by adipose tissue macrophages.
(A) Plaque phenotype graded from 0 to 5 by pathologist. (B, C) Atherosclerotic plaque size, collagen content, necrotic core size and representative images (40x magnification) of HE stained aortic roots (B) and plaque lipid content (C; Oil Red O^+ plaque area) in LTA, OTA and DOTA mice 8 weeks after AT transplantation (*i.e.* after 12 weeks of HCD feeding). (D–E) Neutrophil cell count (D) and macrophage content (E; Moma-2^+ plaque area) of the atherosclerotic plaque in LTA, OTA and DOTA mice after AT transplantation and 12 weeks of HCD feeding. (F) Aortic arch gene expression levels of inflammatory markers. All data are means ± SEM.

depending on which model was used when studying atherosclerosis [34].

In conclusion, proinflammatory ATMs in obese vAT do not affect atherosclerotic plaque development or phenotype despite effects of these ATMs on hepatic inflammation in our model of atherosclerosis.

Conflicts of interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

Financial support

This study was financed by The Netherlands Organization for Scientific Research (NWO) (Veni 916.12.056), The Netherlands Heart Foundation (2013T143) and a Seventh Framework Program (FP7) Grant (CIG 322070) to Kristiaan Wouters.

Author contributions

MB performed experiments, analysed the data, and wrote the manuscript, JvdG performed experiments, MV performed experiments, MJG scored atherosclerotic plaques and performed data analysis, MdW designed the study and supervised experiments, CGS supervised experiments and revised manuscript, KW designed the study, performed experiments, analysed the data, supervised experiments and wrote the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.atherosclerosis.2018.12.010.

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