A NOVEL SUBSET OF PRO-INFLAMMATORY MACROPHAGES SERVES AS A SWITCH FROM METABOLICALLY **HEALTHY** OBESITY TO METABOLICALLY **UNHEALTHY** OBESITY

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ABSTRACT

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Candice Johnson

Doctor of Philosophy

Lewis Katz School of Medicine at Temple University, 2020

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Over the last 30 years, the United States has seen a steady increase in the obesity rate. More than a body image issue for many patients, obesity is a risk factor for several health conditions. As a result of the various concurrent morbidities, the cost to treat obesity-related complications is steep at nearly 150 billion dollars. The causes and risk factors of obesity are also varied, including certain medical conditions/medications, being at an older age, insufficient sleep, pregnancy, chronic stress, etc. However, overconsumption of low-nutrition foods and inadequate daily activity account for the sustained rise in obesity in the United States.

Metabolically healthy obesity (MHO) accounts for roughly 35 percent of all obese patients. No clear consensus has been reached on whether MHO is a stable condition or merely a transitory period between metabolically healthy lean and metabolically unhealthy

obesity (MUO). Furthermore, the mechanisms underlying MHO and any transition to MUO are not clear.

Macrophages are the most common immune cells in adipose tissue (lean and obese) as well as in atherosclerosis. There are several subsets of macrophages, of which M1 (pro-inflammatory) and M2 (anti-inflammatory) are best documented. Fas (or CD95), which is highly expressed on macrophages, is classically recognized as a pro-apoptotic cell surface receptor. However, Fas also plays a significant role as a pro-inflammatory molecule.

Previously, we established a mouse model (apo$E^{-/-}$/miR$155^{-/-}$; DKO mouse) of MHO, based on the criteria of not having metabolic syndrome (MetS) and insulin resistance (IR). In our current study, we hypothesized that MHO is a transition phase toward MUO and that inflammation driven by macrophages is a novel mechanism for this transition. We found that with extended high-fat diet (HFD), MHO mice became MUO, as judged by increased atherosclerosis. At the MHO stage, DKO mice exhibited increased pro-inflammatory markers, including CD95, in adipose tissue and serum. We found that total adipose tissue macrophages were increased and that CD95+CD86- subset of adipose tissue macrophages were increased. Moreover, we showed that human aortic endothelial cells (HAECs) were activated (as judged by ICAM1 expression) when incubated with both DKO and human macrophage-conditioned media in comparison to respective controls. To summarize, we have found a molecular mechanism explaining how MHO transition to MUO may occur. We have also found a previously unappreciated role of CD95+ macrophages as a potentially novel subset that may be utilized to assess pro-inflammatory characteristics of macrophages, specifically in adipose tissue in the absence of pro-inflammatory miR-155. As a result, we hope that these findings will propel the field of MHO forward towards greater understanding and advise clinical treatment.

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LIST OF ABBREVIATIONS

ApoE	Apolipoprotein E
AT	Adipose Tissue
ATM	Adipose Tissue Macrophage
BMDM	Bone Marrow-Derived Macrophage
BMI	Body Mass Index
CD	Cluster of Differentiation
cDNA	Complementary DNA
CLS	Crown-Like Structures
CVD	Cardiovascular Disease
DKO	Double Knockout Mouse (miR-155-/-/apoE-/-)
EDTA	Ethylenediaminetetraacetic Acid
FBS	Fetal Bovine Serum
FFA	Free Fatty Acid
GTT	Glucose Tolerance Test
gWAT	Gonadal White Adipose Tissue
HAEC	Human Aortic Endothelial Cell
HBSS	Hank's Balanced Salt Solution
HDL	High-Density Lipoprotein
HFD	High-Fat Diet
IACUC	Institutional Animal Care and Use Committee
ICAM-1	Intracellular Adhesion Molecule-1

IL-1β	Interleukin-1β
IR	Insulin Resistance
IRB	Institutional Review Board
ITT	Insulin Tolerance Test
LDL	Low-Density Lipoprotein
MCP-1	Monocyte Chemoattractant Protein-1
MetS	Metabolic Syndrome
MHO	Metabolically Healthy Obesity
miR-155	MicroRNA-155
MiRNAs	MicroRNAs
MUO	Metabolically Unhealthy Obesity
NaCl	Sodium Chloride
NAFLD	Non-Alcoholic Fatty Liver Disease
NC	Normal Chow
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
PSA	Penicillin, Streptomycin, and Amphotericin B
RT-qPCR	Reverse Transcription-Quantitative Real-Time Polymerase Chain Reaction
SAT	Subcutaneous Adipose Tissue
SDS	Sodium Dodecyl Sulfate

SVF	Stromal Vascular Fraction
T2DM	Type 2 Diabetes Mellitus
TLR4	Toll-Like Receptor 4
TNF-α	Tumor Necrosis Factor-α
VAT	Visceral Adipose Tissue
WAT	White Adipose Tissue
WT	Wild-Type

CHAPTER 1

GENERAL INTRODUCTION

Overview of Obesity

Overweight and obesity are defined as having a body mass index (BMI) that is 25 to 29.9 kg/m^2 (overweight) and 30 kg/m^2 or greater (obesity). There are several ways to measure adiposity; however, BMI [person's weight (kg) divided by square of the height (m)] is a reasonably reliable and common method for determining overweight and obesity [1]. Overweight and obesity occur when there is more caloric intake than usage over time. There are several factors that promote this surplus outcome. These factors can generally be characterized as either behavioral or genetic. For instance, behavioral factors include eating an unhealthy diet, leading a sedentary lifestyle, being part of a family that consumes unhealthy food, and not getting sufficient sleep (allowing more opportunity to eat). Genetic factors include being female, having a family history of overweight/obesity, and one's ethnic/racial background. Other factors include being in an environment where access to healthy foods is limited, taking certain medications, experiencing stress, and having certain illnesses [2].

In 2018, the worldwide incidence of obesity was almost three times the incidence in 1975. More specifically, in 2016, 1.9 billion adults were overweight or obese. In that same year, 650 million adults (13 percent) were designated obese [1]. **Figure 1** details overweight and obesity rates in various regions throughout the world since 1980 [3].

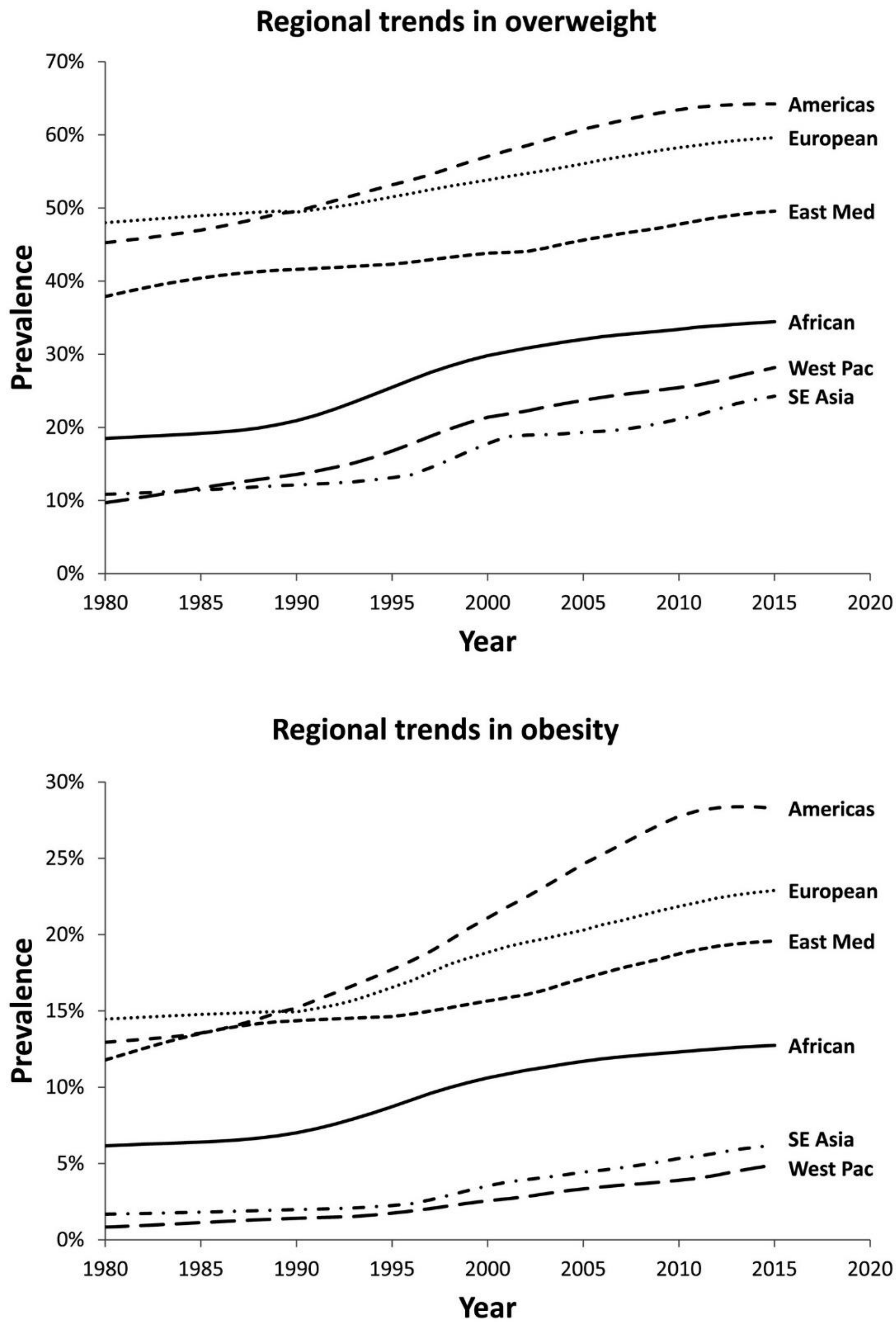


Figure 1. Prevalence of overweight and obesity in adults by region. Americas: Argentina, Brazil, Colombia, Mexico, United States of America. **European:** France, Germany, Russia, Turkey, United Kingdom. **Eastern Mediterranean:** Afghanistan,

Egypt, Iran, Iraq, Pakistan. **African:** Congo, Ethiopia, Nigeria, South Africa, Tanzania. **South East Asian:** Bangladesh, India, Indonesia, Myanmar, Thailand. **Western Pacific:** China, Japan, Philippines, South Korea, Vietnam.

From the graph, it is clear that Western regions have the highest prevalence of both conditions. In the United States, there was an estimated 39.8 percent of adults with obesity (Prevalence of Obesity among Adults and Youth: United States, 2015–2016), with all states and territories having a prevalence of adult obesity at 20 percent or greater (**Figure 2**) [4].

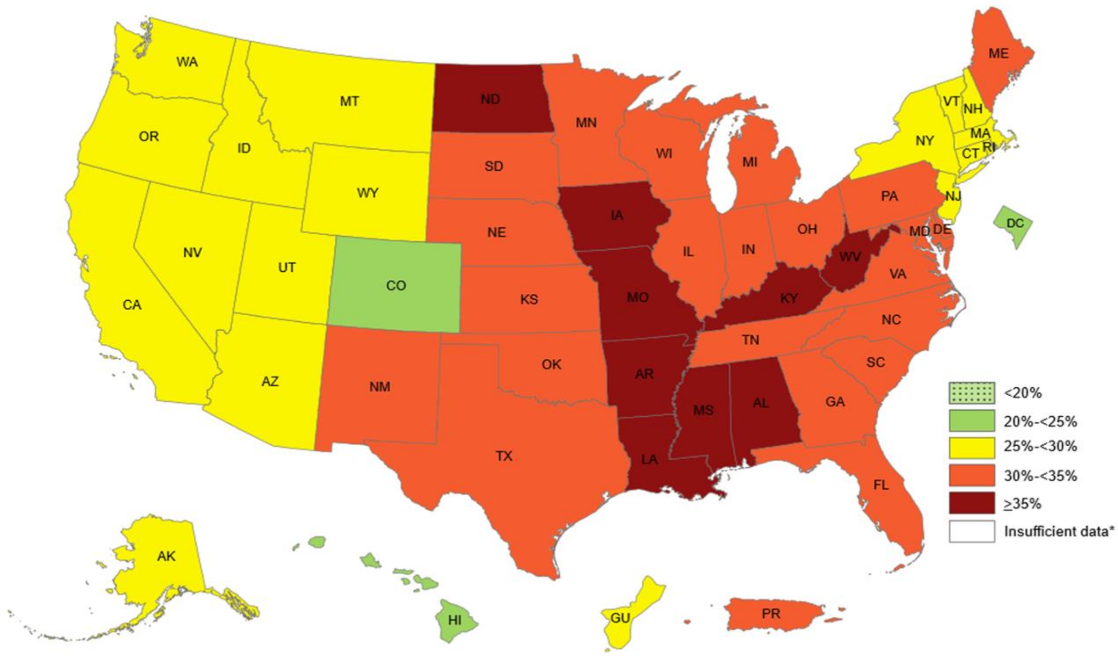


Figure 2. Prevalence of obesity in the United States by state. All states have an obesity prevalence of at least 20 percent. Nine states have a prevalence of at least 35 percent. Twenty-two states and one territory have 30 to 34.9 percent prevalence. Seventeen states and one territory have 25 to 29.9 percent prevalence. Only two states and the District of Columbia have 20 to 24.9 percent prevalence.

In 2014, 32.5 percent of US adults were overweight, while the prevalence of adults who were overweight or obese was over 70 percent [5]. While the rate of overweight in adults has remained relatively unchanged at approximately 40 percent for men and 25 percent for women, the same cannot be said for obesity. Since the mid-1970s, the United States has seen an unrelenting rise in obese men and women at approximately 35 percent and 40 percent, respectively [6].

In the face of such high obesity prevalence, it is unsurprising that the cost of obesity is precipitous. Direct and indirect medical costs of obesity ring in at 147 billion dollars [7], a large increase from the 39.3 billion dollar estimate in 1986 [8]. Moreover, work productivity has been impacted. For instance, time off from work due to obesity-associated health problems was estimated to cost up to 6.38 billion dollars in the US [7]. Behind much of the high cost of obesity are the commonly resulting ailments, such as type 2 diabetes (T2DM), coronary heart disease, hypertension, dyslipidemia, atherosclerosis, non-alcoholic fatty liver disease (NAFLD), sleep apnea [9], osteoarthritis, gallbladder disease, stroke, several forms of cancer, and depression as well as all-cause death [7].

There are several ways to approach obesity treatment. These approaches may be at the individual level as well as the societal level. At the individual level, implementing a healthy diet and exercise is common [10], supplemented in some cases with medications [11]. However, there are instances where the above approaches are inadequate; therefore more invasive methods are warranted. For example, surgeons may employ weight-loss devices, such as the gastric balloon system and electrical stimulation system, which promote satiety. Bariatric surgery is another option, particularly for sleep apneic or type 2 diabetic patients [12]. At the societal level, laws that require food manufacturers to reduce/report unhealthy food components are one way to combat the problem. Community health programs that promote healthy school lunches and that educate families on proper diet and exercise are another tactic [13]. Especially for those whom exercise, diet and current methods are insufficient, increasing biomedical research to identify new therapeutic targets is another necessary approach.

Overview of Metabolic Healthy Obesity

Defining metabolically healthy obesity (MHO) has proven challenging due to a lack of consensus in the field [14] [15]. For instance, studies have used criteria based on metabolic syndrome (MetS), which is defined as having at least three of the following five risk factors: hypertension, enlarged waistline, low HDL level, high triglyceride levels, and high fasting blood glucose level [14] [16]. As a common outcome of obesity, having MetS raises a person's risk for developing ischemic heart disease, T2DM and stroke [17]. More specifically, MHO has been defined as obesity without one or more of the aforementioned MetS characteristics. Additionally, MHO has been defined as obesity without insulin resistance (IR) and/or increased C-reactive protein level [18] [19] [20] [21] [22] [23] [24]. Moreover, the cut-off values vary for the same criteria [25] [26]. For this reason, several studies have estimated widely disparate percentages for MHO—anywhere from 6 to 75 percent [27]. In 2017, Lin *et al*. conducted a meta-analysis of 40 population-based MHO studies and found that the global rate was an estimated 35 percent of obese individuals [24]. Another aspect of obesity that determines metabolic health is adipose tissue location. Lipid storage in organs, such as the liver, is associated with adverse effects on health compared with storage in legs [28] [29].

While several studies agree that, compared with metabolically unhealthy obesity (MUO), MHO is associated with a reduced risk for developing cardiovascular diseases (CVDs) and T2DM [30] [31], MHO has an increased risk compared with MH lean. This remains the case, no matter the stringency of criteria applied [32]. In addition to lower risk for developing CVD and T2DM, MHO patients experience lower mortality compared with

MUO counterparts [33] [34]. It is important to note that just as MHO is an exception to obesity, leanness does not always correlate with metabolic health [35].

In a three to ten-year follow-up of a meta-analysis of 12 MHO studies, Lin *et al*. reported that 49 percent had developed at least one metabolic abnormality [24]. Another study found that approximately 30 percent of MHO patients become MUO during a 5.5 to 10.3-year follow-up [36]. After six years, Soriguer *et al*. discovered that between 30 and 47.8 percent of MHO patients had switched to MUO, depending on the criteria for MHO used [37]. Several correlations have been determined to help explain what prompts MHO switch to MUO. For example, Schröder *et al*. reported that increasing waist-to-hip ratio, waist circumference and BMI are factors [38], in addition to increasing age [14]. Moreover, MHO patients more likely to transition were women [39]. Although a molecular mechanism for MHO switch to MUO status has not been discovered, genetic mechanisms have been proposed, since MUO has been linked to genes [40].

Likewise, a number of mechanisms have been suggested to explain how obesity can be MH. Some include reduced immune cell infiltration in adipose tissue; conserved insulin sensitivity; MHO patients' proclivity to deposit lipids in subcutaneous adipose tissue (SAT) depot along with lower visceral adipose tissue (VAT) and lower ectopic fat (in skeletal muscle, liver). Additionally, greater level of fitness and exercise have been proposed [34]. Furthermore, lower levels of C-reactive protein, tumor necrosis factor-α (TNFα), and interleukin-6 (IL-6) were reported compared with MUO individuals [16] [41] [42] [43] [44] [45]. Additionally, MHO individuals had higher adiponectin (anti-inflammatory adipokine) level [16] [46], lower white blood cell count, and lower plasminogen activator inhibitor-1 (PAI-1) level [16].

Like MUO, MHO occurs more often in women; but unlike MUO, MHO more often presents in younger individuals [47]. One study found that retained MHO was associated with patients less than 40 years old [36]. In addition, MHO more commonly occurs in Caucasians and in individuals with BMI less than 35 kg/m^2 [14] as well as with a smaller waist circumference compared with MUO individuals [36]. Furthermore, MHO individuals tend to have less intra-abdominal fat compared with MUO counterparts and have the fat concentrated in their legs [14] [44]. Given what is already known, it is important to improve our understanding on MHO and the underlying mechanisms of its transition to MUO.

Overview of Macrophages in Adipose Tissue

Adipose tissue is the primary storage site of lipids. There are three main types of adipose tissue; white, brown, and brite (beige or brown-in-white). White adipose tissue (WAT) can be further classified as subcutaneous and visceral [48] [49]. Virtue and Vidal-Puig proposed that adipocytes, as the primary site for lipid storage, possess a limit in storage capacity. Beyond this limit, lipids will accumulate in ectopic sites, such as the liver, leading to metabolic disturbances within those organs. This idea is supported by the understanding that expansion of SAT is less metabolically damaging than expansion of VAT, which surrounds organs [50] [51].

It is well-documented that obesity is characterized by chronic, low-grade, sterile inflammation, and that macrophages are the predominant immune cell in adipose tissue

[14] [52] [53] [54]. In fact, human adipose tissue macrophages (ATMs) increase from between five and ten percent in lean adipose tissue to between 40 and 50 percent in obese adipose tissue. Macrophages play a key role in maintaining adipocyte health [52] [53] [55]. For instance, during lipolysis, macrophages serve to take up lipids in order to mitigate inflammation. During lipolysis in obesity, however, lipid release becomes chronic, promoting macrophage inflammation. Additionally, crown-like structures (CLS), which are macrophages surrounding a dying adipocyte, is a key histological feature of obesity [56] [57]. Several papers document a link between ATM accumulation and IR. As an example, deletion of CCL2 (macrophage-recruiting chemokine) and blocking its receptor (CCR2) led to reduction in macrophages and improved insulin sensitivity; whereas overexpression of CCL2 resulted in macrophage increase and IR [58] [59] [60].

Adipokines are cytokines, chemokines and hormones secreted by adipose tissue [61] [62]. Many adipokines are secreted by ATMs. One such cytokine is TNFα. Seminal studies of obesity have helped us to understand that free fatty acids (FFAs), released from dying adipocytes, stimulate macrophages via toll-like receptor 4 (TLR4) signaling. In response, macrophages secrete TNFα, which further enhances lipolysis, thereby driving metabolic dysfunction. Moreover, collateral damage to nearby organs, such as liver and pancreas, when this occurs in the VAT can lead to metabolic dysfunction of these organs [53] [63] [64]. On the other hand, ATM-secreted cytokine, IL-10, which has an anti-inflammatory role, helps to maintain insulin sensitivity [65] [66]. Klöting *et al*. and Esser *et al*. report that ATMs, especially pro-inflammatory macrophages, are significantly increased in MUO subjects compared with MHO subjects [42] [67]. Spiegelman *et al*.

demonstrated in the early 90s that obese adipose tissue, via pro-inflammatory cytokine secretion, contributes to systemic inflammation [68]. However, several questions remain regarding the macrophage subtypes and by what mechanisms the ATMs contribute to systemic inflammation.

Overview of Fas in Obesity, MHO and Atherosclerosis

Fas, or CD95, is a member of the death receptor family, which also includes TNFα's cognate receptor, TNF receptor type 1 (TNFR1). As the family name suggests, Fas was initially discovered as a death receptor, specifically inducing apoptosis. However, its role in non-apoptotic inflammation has also been demonstrated [69] [70] [71] [72]. In fact, several papers document pro-inflammatory cytokine production (IL-1β, IL-6, IL-8, TNFα) via Fas signaling in adipocytes and monocytes/monocyte-derived macrophages [73] [74] [75] [76]. Moreover, Wueest *et al*. reported that Fas mRNA level is significantly greater in circulating blood monocytes of obese subjects compared with lean subjects. When obese subjects were stratified into those with normal glucose tolerance versus those with T2DM, the latter expressed higher levels of Fas in monocytes [73]. Wueest *et al*. showed that plasma TNFα levels were significantly reduced in myeloid-specific Fas-depleted mice on high-fat diet (HFD). TNFα levels were also significantly decreased in WT mice receiving myeloid-specific Fas-deficient bone marrow transplantation. Furthermore, their human data showed a positive correlation between monocyte-derived Fas mRNA levels and circulating TNFα protein levels [73]. These data support a link between Fas in myeloid cells and increased TNFα levels. Moreover, Fas protein levels were significantly increased in adipocytes of leptin receptor-deficient (*db/db*), leptin-deficient (*ob/ob*) and HFD-fed

WT mice (models of obesity) compared with WT controls [76]. Fas protein showed a trend of increase in the adipose tissue of obese subjects compared with lean subjects, and was more highly expressed in adipose tissue of T2DM obese subjects compared with non-T2DM obese subjects [76]. These data support the idea of a pro-inflammatory role for Fas in obesity, with and without metabolic disease. However, issue of whether Fas serves as a marker for pro-inflammatory macrophages remains.

MicroRNA-155

MicroRNA-155 plays a role in several diseases, including cancer, arthritis, atherosclerosis and obesity [77] [78] [79]. Ying *et al*. found that ATMs secrete exosomes containing, among other factors, miR-155 [80]. In atherosclerosis, we and others have demonstrated that global deletion of miR-155 in atherosclerotic apoE$^{-/-}$ mice (DKO mice) resulted in a reduction of atherosclerotic plaque [79] [81]. A study by Nazari-Jahantigh *et al*. found that miR-155 targeted Bcl6, Bcl6 co-localized with plaque macrophages, and that Bcl6 levels were higher in DKO bone marrow-derived macrophages (BMDMs) compared with apoE$^{-/-}$ BMDMs. Additionally, siRNA knockdown of Bcl6 in carotid artery resulted in greater plaque burden compared with siRNA control. Taken together, these data provide supporting evidence that miR-155 in macrophages may play a significant role in the disease [81]. Moreover, in our study, we show that DKO mice presented with obesity. These findings support the idea that the physiological/pathological states of ATMs may have far-reaching effects beyond the local environment.

Preliminary Data

Our preliminary data were previously reported in our *Journal of Biological Chemistry* publication (PMID: 27856635) [79]. We found that miR-155 was significantly increased in the aortas of the well-established atherosclerotic mouse model, apoE$^{-/-}$ mouse, following 12 weeks of HFD versus normal chow. Following 12 weeks of HFD, our DKO mouse model showed significant reduction of atherosclerotic plaque deposition in aortas compared with apoE$^{-/-}$ mouse. DKO mice also presented with obesity without insulin resistance, as shown by a normal GTT and ITT (**Figure 3**).

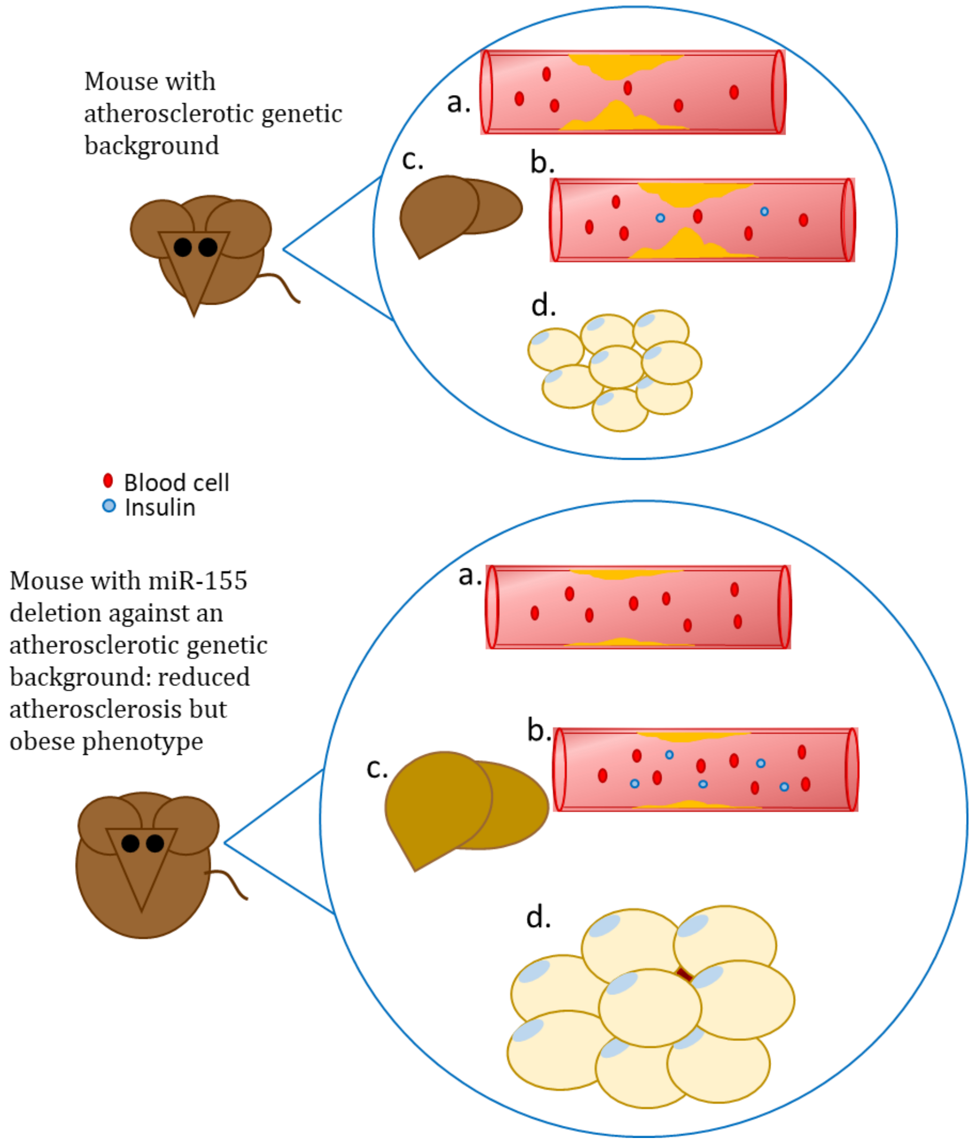


Figure 3. MicroRNA-155 deletion in atherogenic background. A. Reduced atherosclerosis and **B.** promoted obesity, **C.** non-alcoholic fatty liver disease (NAFLD), and **D.** hyperinsulinemia without insulin resistance (IR). PMID: 30369883.

In our current study, to corroborate our finding of miR-155 suppressing obesity, we found reduced miR-155 transcript levels in the classical obese model (WT mice on 12 weeks of HFD) compared with atherosclerotic mice (**Figure 4**). Furthermore, we found that human pericardial adipose tissue from obese patients with T2DM showed a trend of increased miR-155 transcripts compared with obese patients without T2DM (**Figure 5**).

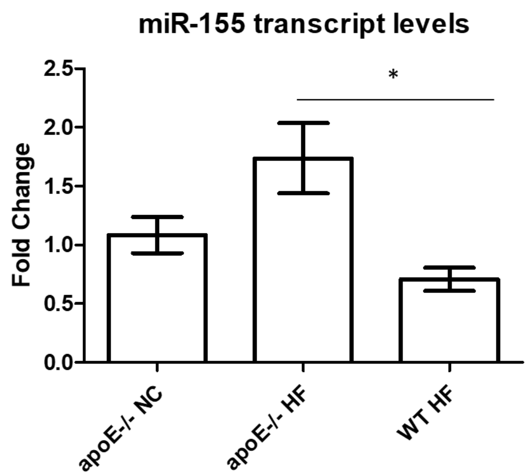


Figure 4. MicroRNA-155 transcripts are decreased in WAT of obese WT mice compared with WAT of apoE$^{-/-}$ mice. Mice were kept on either normal chow (NC) or switched to high-fat diet (HFD) at 8 weeks old and fed for an additional 12 weeks; apoE$^{-/-}$ NC (n=6), apoE$^{-/-}$ HFD (n=7), WT HFD (n=4) . *, p<0.05.

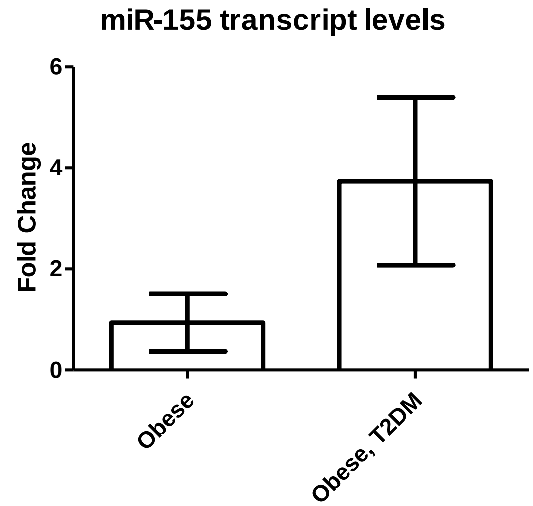


Figure 5. Obese patients with T2DM show trend of increased miR-155 transcripts. Donor information detailed in Table 3; Obese (n=2), Obese with T2DM (n=4). $p>0.05$

Knowledge Gap and Hypothesis

Several publications document the importance of macrophages in obesity and atherosclerosis. Moreover, Serbulea *et al*. and Kadl *et al*. report that Mox macrophages, a subset of macrophages that responds to oxidized phospholipids, are found in both atherosclerotic plaques and adipose tissue. Oxidized phospholipids are lipids found in both atherosclerotic plaques and adipose tissue [82] [83]. This suggests the possibility of ATMs playing an important role in atherosclerosis development/progression. As mentioned, Fas (CD95) is well-studied for its apoptotic function. However, its role in inflammation, particularly within immune cells, is gaining appreciation. We hypothesize that CD95+ macrophages are significantly increased in MHO and promote inflammation in the aortic endothelial cells potentially via secreted cytokine-dependent mechanisms, thereby encouraging atherosclerosis resurgence and switch to MUO.

CHAPTER 2

MATERIALS AND METHODS

Animal Care

All animal experiments were performed in accordance with the Institutional Animal Care and Use Committee (IACUC) Guidelines and were approved by the IACUC of Lewis Katz School of Medicine (LKSOM) at Temple University.

Apolipoprotein E (ApoE, B6.129P2-*Apoe*^{tm1Unc}/J, stock no. 002052) knockout mice, MicroRNA-155 (miR-155, B6.Cg-*Mir155*^{tm1Rsky}/J, stock no. 007745) knockout mice, and wild-type (WT) mice were of a C57BL/6J background, and were purchased from the Jackson Laboratory (Bar Harbor, ME, United States).

Mice were housed under controlled conditions in the LKSOM Animal Facility, where they had *ad libitum* access to standard chow diet/HFD and water, and were subject to a 12-hour light-dark cycle. DKO mice were generated as previously reported [79] by crossing apoE$^{-/-}$ mice with miR155$^{-/-}$ mice.

Mice were age-matched and gender-specific in all experiment groups, unless otherwise specified. At 8 weeks old, mice either remained on normal chow diet (5% fat, Labdiet 5001) or switched to HFD [0.2%(w/w) cholesterol and 20%(w/w) fat, TestDiet AIN-76A] for 12 weeks or 24 weeks, specified in each experiment.

Mouse Genotyping

Mouse genotype was confirmed using end-point polymerase chain reaction (PCR) on genomic DNA obtained from mouse tail sample. Briefly, DNA was extracted using 50µl of extraction solution on tail samples, followed by incubation at 95°C for 30 minutes. Afterwards, 50µl of stabilization solution was added (Extracta™ DNA Prep for PCR, QuantaBio, cat. no. 97065-350, VWR). PCR was then performed (**Table 1**), followed by 1.5 percent agarose gel electrophoresis. The ethidium bromide-containing gel was then imaged by ultraviolet using Foto® analyst image system (Fotodyne).

Primer	5' to 3'
miR155 WT Forward	GTGCTGCAAACCAGGAAGG
miR155 WT Reverse	CTGGTTGAATCATTGAAGATGG
miR155 Mutant	CGGCAAACGACTGTCCTGGCCG
apoE Common	GCCTAGCCGAGGGAGAGCCG
apoE WT	TGTGACTTGGGAGCTCTGCAGC
apoE Mutant	GCCGCCCCGACTGCATCT

Table 1. List of primers used in genotyping PCR.

Gonadal White Adipose Tissue (gWAT) Single Cell Suspension

Gonadal white adipose tissue (gWAT) was isolated and mechanically digested, followed by enzymatic digestion with collagenase type II (Sigma, cat. no. C6885) at 37°C. Following filtration steps and centrifugation, the remaining immune cell-containing stromal vascular fraction (SVF) was stained in preparation for flow cytometry.

Flow Cytometry

Following single cell suspension, SVF cells were stained with live/dead dye (ThermoFisher) for 30 minutes at room temperature in the dark. After washing with HBSS (Corning) supplemented with 2 percent FBS (GE Life Sciences), Fc receptor block (ThermoFisher) was added to cells. Following a five-minute incubation, cells were incubated with surface antibodies for 15 minutes at room temperature in the dark.

Intracellular markers: After washing, cells were fixed (ThermoFisher) for 30 minutes, washed and permeabilized (ThermoFisher) for 15 minutes. Cells were then incubated with antibody for 20 minutes at room temperature in the dark, followed by washing (**Table 2**). Data was collected using BD LSRII flow cytometer and DIVA software (BD). Data was analyzed using FlowJo.

A.

Marker	Fluorochrome	Catalog #
Human		
Live/Dead	UV Indo-1 (blue)	ThermoFisher (A10628)
CD45	BV421	Biolegend (368522)
CD68	PE	Biolegend (333808)
CD86	APC	Biolegend (305412)
CD95	APC	Biolegend (305612)
CD95	PE-Cy7	Biolegend (305622)
CD206	FITC	Biolegend (321104)
CD54 (ICAM1)	APC	BD Pharmingen (559771)
UltraComp eBeads	n/a	ThermoFisher (01-2222-42)
Fc Receptor block	n/a	BD Biosciences (564220)
Mouse		
Live/Dead	UV Indo-1 (blue)	ThermoFisher (A10628)
CD45	APC Cy7	Biolegend (103116)
F480	PE	ThermoFisher (12-4801-80)
CD86	PE-Cy7	Biolegend (105116)
CD206	BV421	Biolegend (141717)
CD95	APC	Biolegend (152604)
CD206	PE	Biolegend (141706)
CD11b	BV421	BD Horizon (562605)
F480	PE-Cy7	ThermoFisher (25-4801-82)
UltraComp eBeads	n/a	ThermoFisher (01-2222-42)
Fc Receptor block	n/a	BD Biosciences (553142)

B.

Human Isotype	Catalog#
APC	Biolegend (400322)
FITC	Biolegend (400108)
PE	Biolegend (401208)
APC	BD Pharmingen (555751)
BV421	Biolegend (400158)
Mouse Isotype	**Catalog#**
APC Cy7	Biolegend (400624)
PE	ThermoFisher (12-4321-81)
PE-Cy7	Biolegend (400618)
BV421	BD Horizon (562602)
APC	Biolegend (400120)
PE-Cy7	ThermoFisher (25-4321-82)
PE	Biolegend (400508)

Table 2. List of antibodies used in flow cytometry.

Cytokine Array

Mouse blood was allowed to clot for four hours at room temperature. After centrifuging for 15 minutes at 2000xg, supernatant (i.e., serum) was carefully obtained and stored at -80ºC until further use. Cytokine array experiment was conducted according to manufacturer's protocol (R&D, cat. no. ARY028). Blots were imaged via chemiluminescence method; X-ray film exposure in a dark room and development with SRX-101A medical film processor (Konica). Protein levels were quantified using ImageJ software.

Murine Aortic Single Cell Suspension

After perfusion, whole aortas were isolated and collected in DMEM-low medium (GE Life Sciences) supplemented with 20 percent FBS. Aortas were rinsed in PBS (Corning), dissected, and then enzymatically digested with cocktail consisting of FBS, HEPES (Gibco), hyaluronidase type I-S (Sigma), collagenase types I (Sigma) and XI (Sigma) at 37ºC for 30 minutes. Next, suspension was filtered, then washed and re-suspended in HBSS supplemented with 2 percent FBS, before staining for flow cytometry.

Human Aortic Endothelial Cell Culture

Human aortic endothelial cells (HAECs) (Lonza) were cultured on gelatin-coated flask in Medium 199 (GE Life Sciences) supplemented with FBS, PSA (ThermoFisher), ECGS (endothelial cell growth serum, Corning), and heparin (Sigma). Medium was changed every two days and cells were passaged at 70 percent confluency, not exceeding more than two subcultures.

Adipose Tissue Macrophage Culture

SVF cells were cultured in murine macrophage differentiation medium [RPMI 1640 medium supplemented with 25ng/mL macrophage colony-stimulating factor (R&D), HEPES (Gibco), sodium pyruvate (Sigma), non-essential amino acids (Gibco), glutamax (Gibco)] for 6 to 7 days at $5x10^5$ cells per well in 6-well plates (Falcon).

Human Adipose Tissue

We acknowledge Dr. Zsolt Bagi for generously donating IRB-approved de-identified human pericardial adipose tissue (**Table 3**).

Obese patients						
patient number	**933**	**773**	**818**	**934**	**791**	**764**
Type of surgery	LIMA SVG	MVR	LIMA SVG		AVR	LIMA SVG
age	66	48	48	73	36	69
gender	M	M	M	M	F	M
weight	92.5	98.6	111.2	96.5	154	103
height	170	170	183	175	173	175
BMI	32.01	34.12	33.20	31.51	51.46	33.63
DM	N	N	Y	Y	Y	Y

Table 3. Human pericardial adipose tissue donor information. LIMA SVG: left internal mammary artery, saphenous vein graft. MVR: mitral valve repair/replacement. AVR: aortic valve repair/replacement. BMI, body mass index. DM, type 2 diabetes mellitus.

Human PBMC Isolation and Culture

Whole blood from healthy male donors (**Table 4**) were collected in anticoagulant solution [aqueous solution of sodium citrate (Fisher Scientific), citric acid (Sigma), and dextrose (Sigma)]. Blood was then gently layered onto Histopaque-1077 (Sigma), followed by centrifugation at room temperature. PBMC-containing phase was gently isolated then washed with PBS, followed by ACK lysis. Following centrifugation, PBMCs were re-suspended in human macrophage differentiation medium [RPMI 1640 medium supplemented with 50ng/mL human macrophage colony-stimulating factor (R&D), HEPES, sodium pyruvate, non-essential amino acids, glutamax] for 6 to 7 days at $1x10^6$ cells per well in 6-well plates.

Donor Information			
Race	**Sex**	**Age**	**Medications**
White	Male	53	Not reported
White	Male	51	Not reported
White/Asian	Male	26	Not reported
South Asian	Male	27	Not reported
Black	Male	45	No meds
Black	Male	42	No meds
Black	Male	49	No meds
Asian	Male	28	No meds
White	Male	31	No meds
White	Male	23	No meds
White	Male	54	No meds
White/Asian	Male	25	No meds

Table 4. Human blood donor information.

Human PBMC Stimulation Assay

After 6 to 7 days of culture, human PBMCs remained unstimulated or were stimulated with TNFα (10ng/mL) (R&D) for 24 hours, followed by assessment of Fas expression.

HAEC Activation with CD95+ Macrophage-Conditioned Medium

HAECs were plated at $5x10^5$ cells per well in 6-well plates for 24 hours. Following, HAECs were treated with macrophage-conditioned medium, with macrophage differentiation medium, or with endothelial cell medium for 24 hours. HAECs were then assessed for EC activation via flow cytometry.

RNA Extraction and Quantification

Briefly, 100 to 200mg of liquid nitrogen-frozen adipose tissue was homogenized using mortar and pestle, followed by addition of QIAzol lysis reagent (Qiagen). After homogenate arrived at room temperature, chloroform (Sigma) was added, then solution was vigorously shaken. Following centrifugation and aqueous phase retrieval, 100 percent ethanol (PHARMACO-AAPER) was added and mixed. Next, following manufacturer's protocol (miRNeasy Mini Kit, Qiagen), ethanol-aqueous phase solution was added to RNeasy Mini column and subjected to a series of buffer washes and centrifugation steps. RNA was re-suspended in nuclease-free water. RNA quality and concentration were determined using Nanodrop 2000 (ThermoFisher).

RNA Reverse Transcription and Quantitative Real-Time PCR

Per manufacturer's instruction, total RNA was reverse transcribed to generate complementary DNA (cDNA) using the miScript II RT Kit (Qiagen). Briefly, template RNA in tubes containing buffer, Nucleics Mix, Reverse Transcriptase Mix and RNase-free water was reverse transcribed at 37^{o}C for 60 minutes and 95^{o}C for 5 minutes to generate cDNA.

Quantitative PCR was performed using the StepOnePlus PCR system (Applied Biosystems), following preparation with miScript SYBR Green PCR Kit for Use with miScript Primer Assays (Qiagen). Primers for human and mouse miRNA-155 and the housekeeping gene RNU6 (miScript Primer Assay) were purchased from Qiagen. Cycling conditions were as follows: 40 cycles at 95^{o}C for 15 minutes, 94^{o}C for 15 seconds, 55^{o}C for 30 seconds, and 70^{o}C for 30 seconds. Data was analyzed using the delta-delta Ct method.

Atherosclerotic Lesion Analysis

Following perfusion with PBS, mouse aortas were excised and fixed overnight in 4 percent paraformaldehyde (Sigma). Next, aortas were placed in 20 percent sucrose (Sigma) for 24 hours. Aortas were then stored in PBS at 4°C. For *en face* staining, aortas were stained in Sudan IV (Sigma) for 40 minutes at 37°C, followed by incubation in 70 percent isopropanol for 5 minutes. Afterwards, aortas were opened longitudinally and imaged using AxioCam camera mounted to Stemi 2000-C microscope (Carl Zeiss Inc.).

Protein Extraction and Western Blot

Adipose tissue and aortas were sonicated in an aqueous sample buffer consisting of sodium dodecyl sulfate (SDS, Sigma), Tris-hydrochloride [Tris (Fisher Scientific)-HCl (Sigma)], glycerol (ThermoFisher), PBS, EDTA, phenylmethylsulfonyl fluoride (PMSF, Sigma), cOmplete Protease Inhibitor Cocktail (Sigma). Following centrifugation and retrieval of the protein-containing supernatant, protein concentration was determined using the Pierce BCA Protein Assay Kit (ThermoFisher).

For gel electrophoresis, 15 to 30µg of protein were loaded into wells and run for 90 minutes. Next, proteins were transferred onto polyvinylidene fluoride (PVDF) membrane (Bio-Rad) for 90 minutes at 400mA. Following Ponceau S (Sigma) staining, membrane was blocked with 5 percent non-fat milk (Lab Scientific) for 1 hour at room temperature and washed. Membrane was then incubated with primary antibody in non-fat milk or bovine serum albumin (BSA, Gemini Bioproducts) overnight at 4°C (**Table 5**). Afterwards, membrane was washed and incubated with horseradish peroxidase (HRP)-linked secondary antibody at room temperature between 30 and 120 minutes (**Table 5**). Membranes were washed and incubated in enhanced chemiluminescent substrate (ThermoFisher) for 5 minutes prior to imaging. Next, protein bands were imaged on X-ray film (AGFA) after development with SRX-101A medical film processor (Konica).

Western Antibody	Catalog#
Beta Actin	Abcam (ab8227)
TNFα	Cell Signaling (7074s)
Fas	Cell Signaling (3707s)
Leptin	Abcam (ab82419)
Resistin	Abcam (ab16227)
Anti-rabbit IgG, HRP-linked	Abcam (ab119501)

Table 5. List of antibodies used in Western blot

Statistical Analysis

Statistical analyses were performed using the GraphPad Prism software. Two-tailed Student's *t*-test was used for statistical comparison between 2 groups. One-way ANOVA with Tukey Multiple Comparison test was used for 3 or more groups. Data presented as mean ± SEM (standard error of the mean). Statistical significance was defined as $p<0.05$.

CHAPTER 3

RESULTS

We have developed two aims detailing our approach to our hypothesis (**Figure 6**).

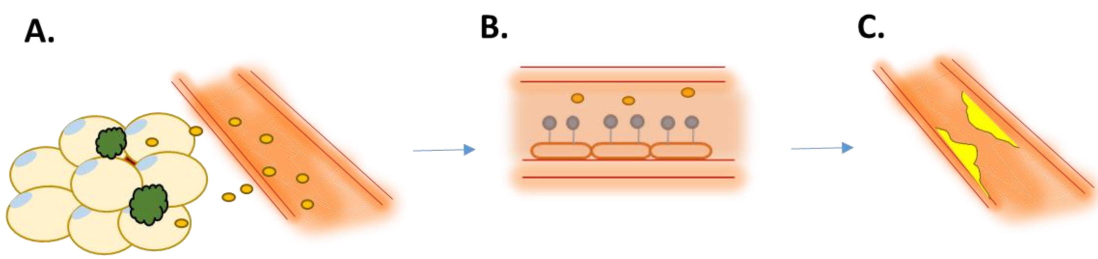


Figure 6. Hypothesis: CD95+CD86- ATM subset promotes MHO switch to MUO by promoting aortic EC activation. We hypothesize that **A.** Fas+ (CD95+) ATM subset (green) secretes pro-inflammatory cytokines/chemokines (yellow dots) into the circulation. **B.** These cytokines/chemokines induce aortic EC activation, an early step of atherosclerosis, via ICAM1 expression (gray dots). **C.** Over time, atherosclerotic plaque deposition takes place (bright yellow). In **Aim 1**, we determined whether HFD in MHO mice promotes WAT inflammation (**6A**) and whether atherosclerosis develops, following extended HFD in order to establish MHO transition to MUO in our model (**6C**). In **Aim 2**, we then examined whether CD95+ ATMs promote pro-atherogenic environment (**6B**).

1. HFD-fed DKO mice (MHO stage) exhibit WAT inflammation.

Rationale: As earlier mentioned, at least 30 percent of obese subjects eventually develop MUO [36] [37]. MUO is associated with an inflammatory environment perpetuated by ATMs. We sought to uncover whether our DKO mice developed MUO over time and whether DKO mice at the MHO stage have increased pro-inflammatory macrophages and cytokine secretion.

In order to define whether MHO mice can transition to MUO, we extended HFD from 12 to 24 weeks and determined whether MHO mice developed MUO as judged by atherosclerosis development (**Figure 7**).

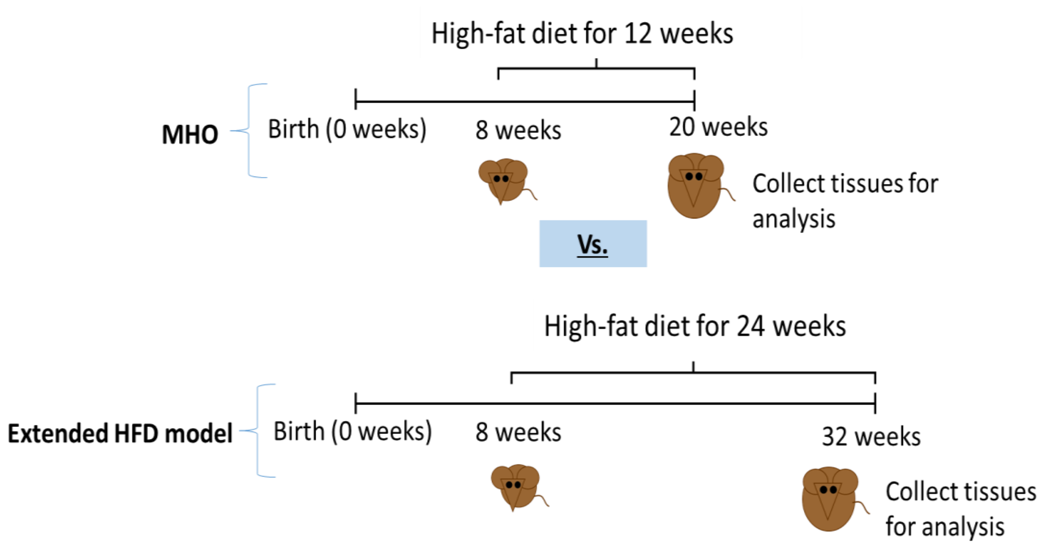


Figure 7. Extended HFD experiment design. DKO mice at MHO stage are fed on HFD for 12 weeks beginning at 8 weeks old. DKO mice at extended MHO stage (when MUO develops) are fed on HFD for 24 weeks beginning at 8 weeks old.

We found that there was no significant difference in total body weight and in gWAT weight between apo$E^{-/-}$ and DKO mice following 24 weeks of HFD (**Figure 8A-B**). However, DKO mice exhibited resurgent atherosclerosis compared with 12 weeks of HFD (**Figure 8C**). This data showed that MHO status was lost over time as mice developed MUO.

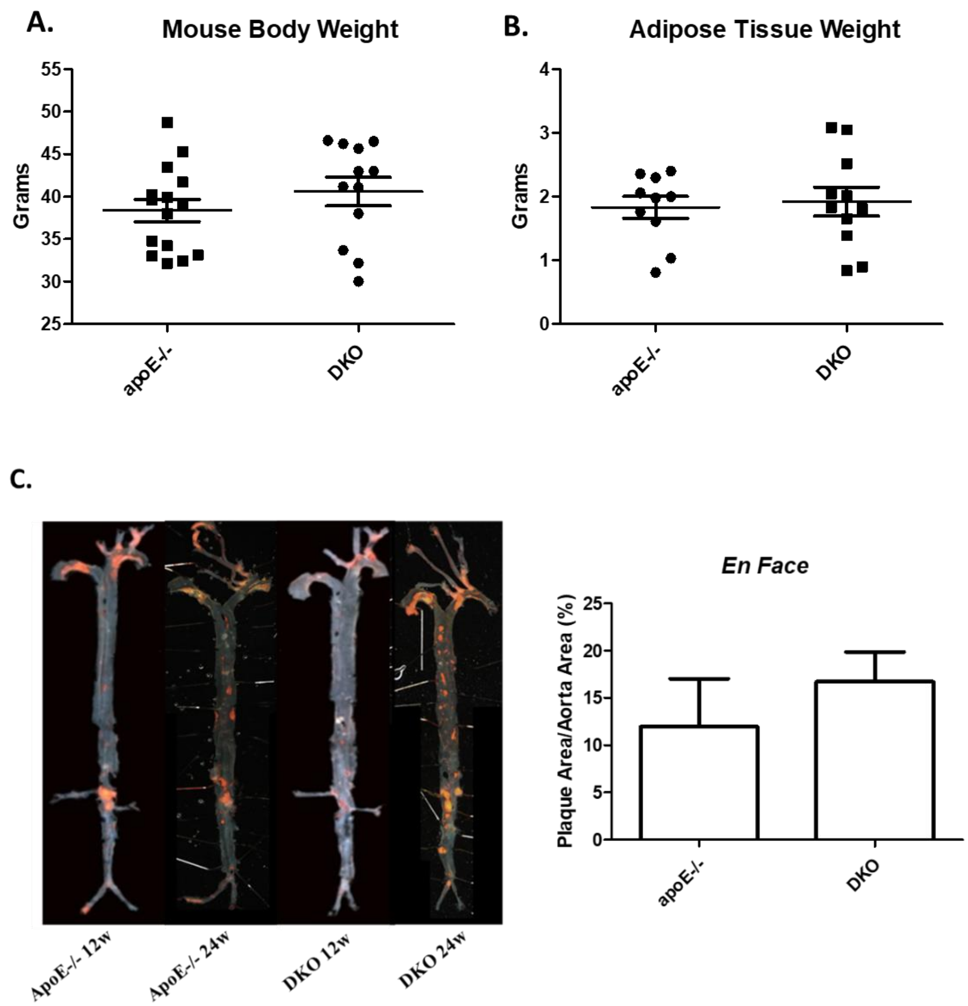


Figure 8. DKO mice exhibit resurgent atherosclerosis following extended HFD feeding. A. Total body weight (grams); apoE$^{-/-}$ (n=15), DKO (n=12). **B.** Gonadal WAT (grams); apoE$^{-/-}$ (n=10), DKO (n=11). **C.** *En Face* analysis of whole aortas; male apoE$^{-/-}$ (n=5); male DKO (n=8). *p*>0.05.

After establishing that MHO mouse on extended HFD becomes MUO and is therefore an appropriate model to study MHO switch to MUO, we assessed whether mice at MHO stage exhibited pro-inflammatory cytokine expression, which would contribute to these mice developing MUO over time. Cytokine array analysis showed that pro-inflammatory cytokine, TNFα, and EC activation markers, Icam1 and E-selectin, were induced in serum of DKO mice (**Figure 9**).

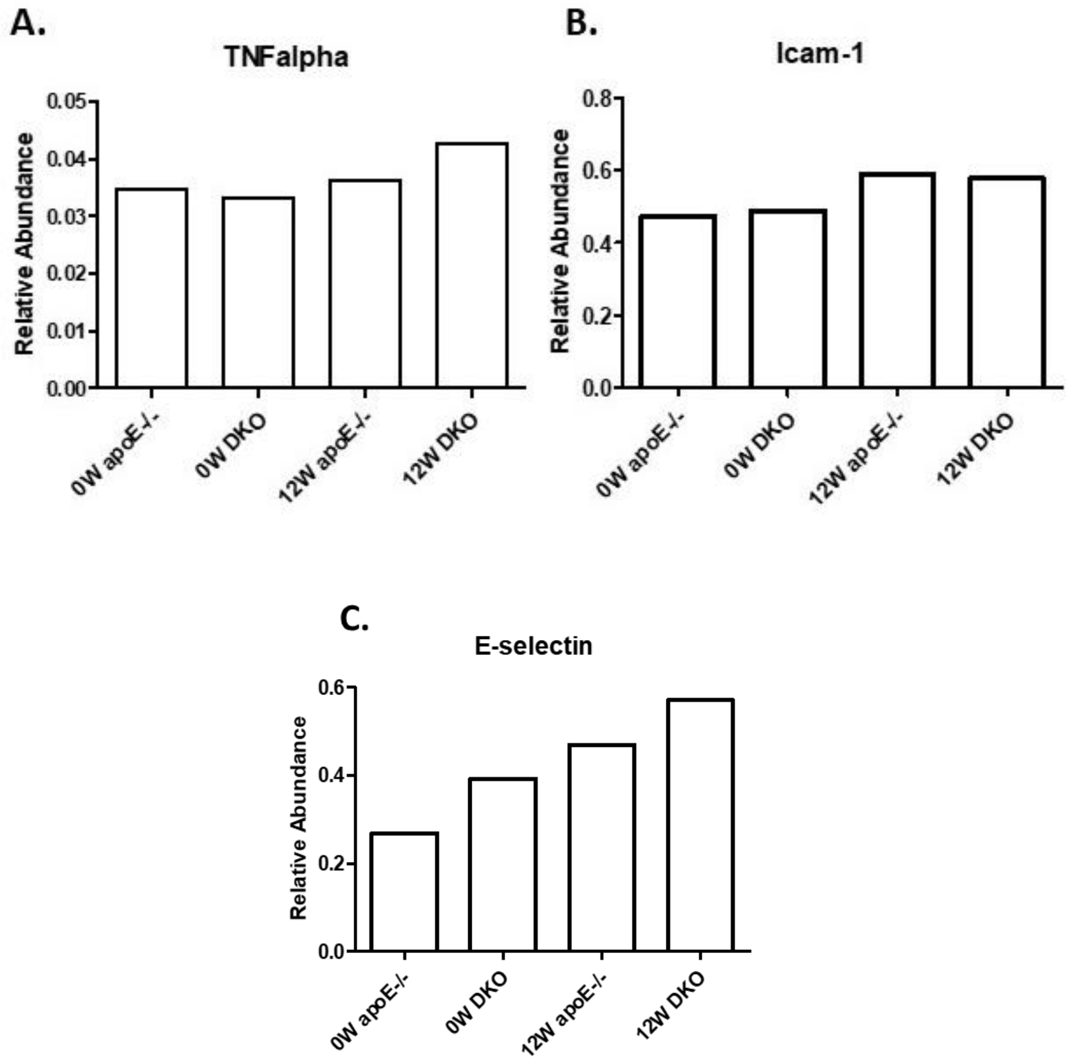


Figure 9. Pro-inflammatory cytokines are induced in serum of HFD-fed DKO mice. 0W: 8-week old male mice on normal chow (NC). 12W: 20-week old male mice that began HFD at 8 weeks old and continued for 12 weeks; n=3 per sample. Each group is composed of three mouse samples combined into one.

Previously, we reported that the pro-inflammatory adipokines, leptin and resistin, were increased in plasma of DKO mice compared with apoE$^{-/-}$ mice after 12 weeks of HFD, while anti-inflammatory adipokine, adiponectin, remained unchanged [79]. We found that while leptin was unchanged, resistin was significantly increased in DKO gWAT compared with apoE$^{-/-}$ and WT gWAT (**Figure 10**).

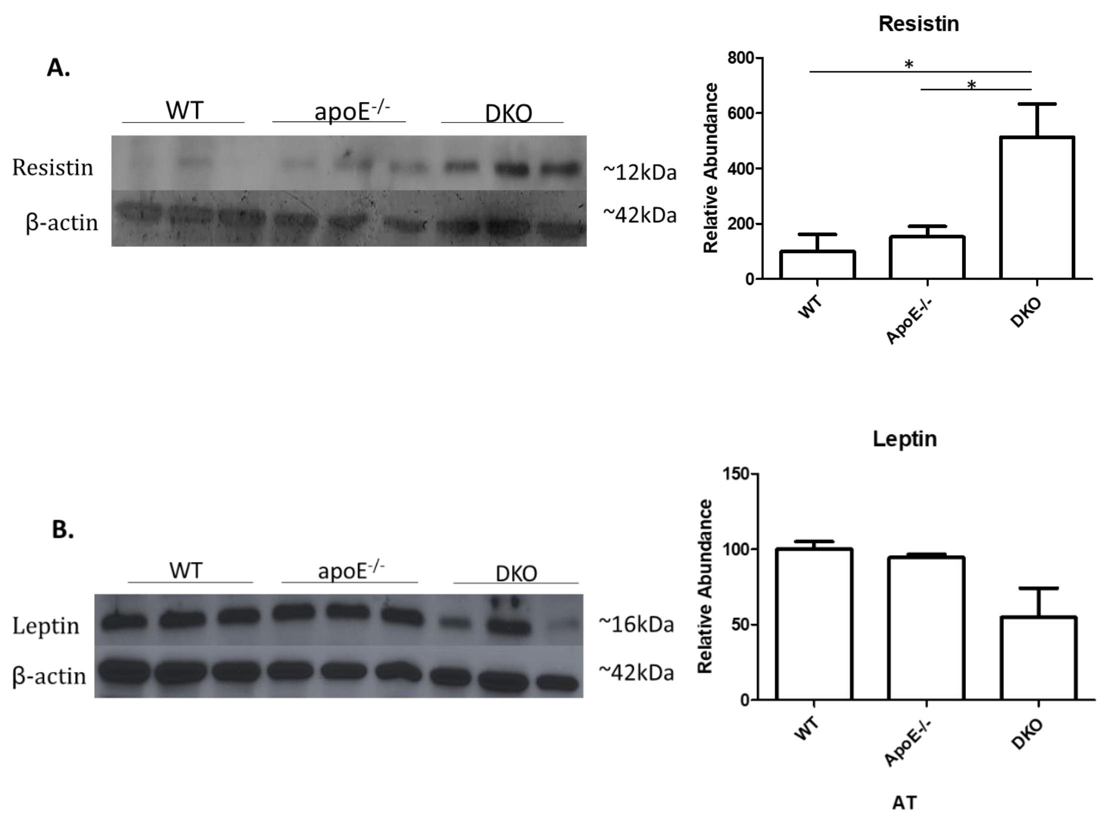


Figure 10. Pro-inflammatory adipokine, resistin, is increased in DKO WAT compared with WT and apoE$^{-/-}$ WAT. 20-week old male mice were fed on HFD for 12 weeks beginning at 8 weeks old; n=3 per group. *, *p*<0.05.

Moreover, we found that both TNFα and Fas, but not Mcp1, were significantly increased in gWAT of DKO mice following 12 weeks of HFD versus three weeks of HFD (**Figure 11**). These data indicate that although DKO mice at MHO stage are protected from atherosclerosis development, a pro-inflammatory environment is present notwithstanding.

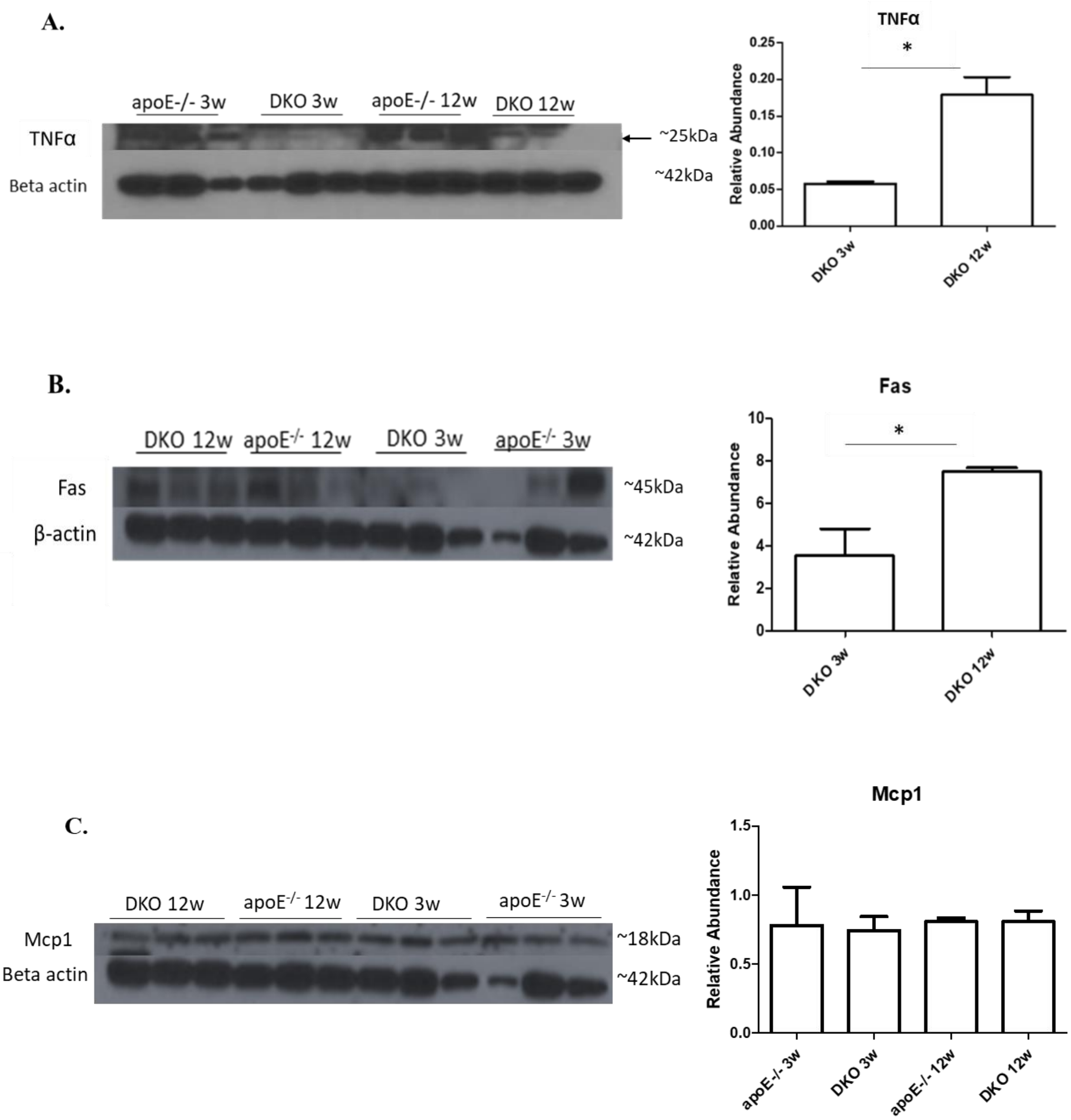


Figure 11. TNFα and Fas are increased in DKO gWAT mice after 12 weeks compared with 3 weeks of HFD. 3W: 11-week old male mice that began HFD at 8 weeks old and continued for 3 weeks. 12W: 20-week old male mice that began HFD at 8 weeks old and continued for 12 weeks; n=3 per group. *, p<0.05.

As previously mentioned, an increase in macrophage number is a hallmark of obesity [53]. We therefore examined whether HFD promoted increased macrophage presence in DKO gWAT vs. apoE$^{-/-}$ gWAT at the MHO stage. Using flow cytometry, we found that total macrophages were increased in male DKO gWAT (**Figure 12**). However, there was no difference in total macrophages when analyzing female apoE$^{-/-}$ and DKO gWAT (**Figure 13**). Total macrophages were also higher in male miR155$^{-/-}$ gWAT compared with WT gWAT (**Figure 14**), showing that miR-155 deletion supports macrophage proliferation and/or infiltration.

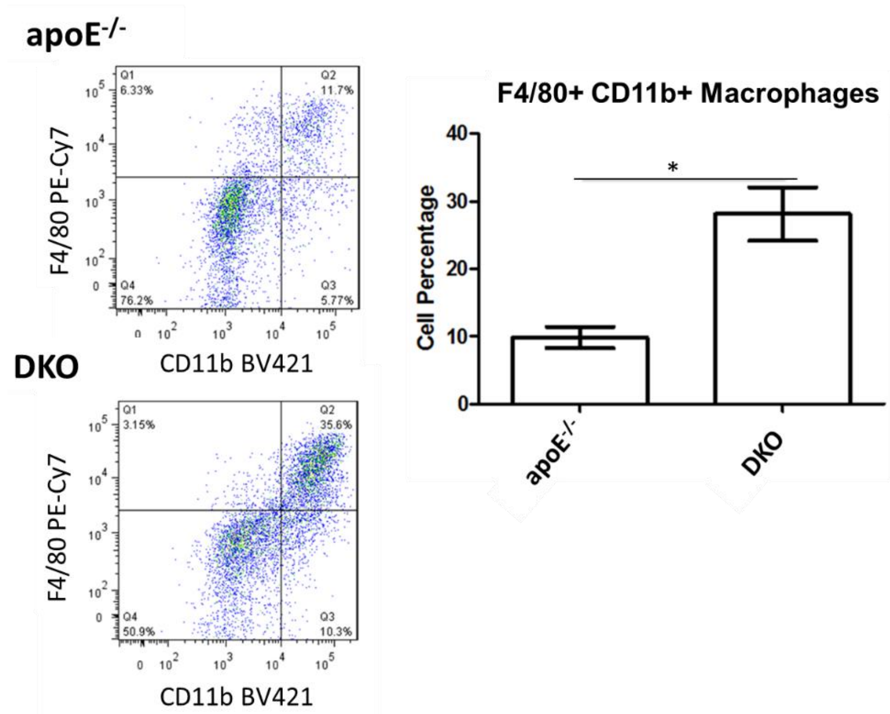


Figure 12. Macrophages are increased in male DKO gWAT compared with apoE$^{-/-}$ gWAT. Mice were fed on HFD from 8 weeks old to 20 weeks old. Macrophages were defined as CD45+F4/80+CD11b+; apoE$^{-/-}$ (n=7), DKO (n=11). *, p<0.05.

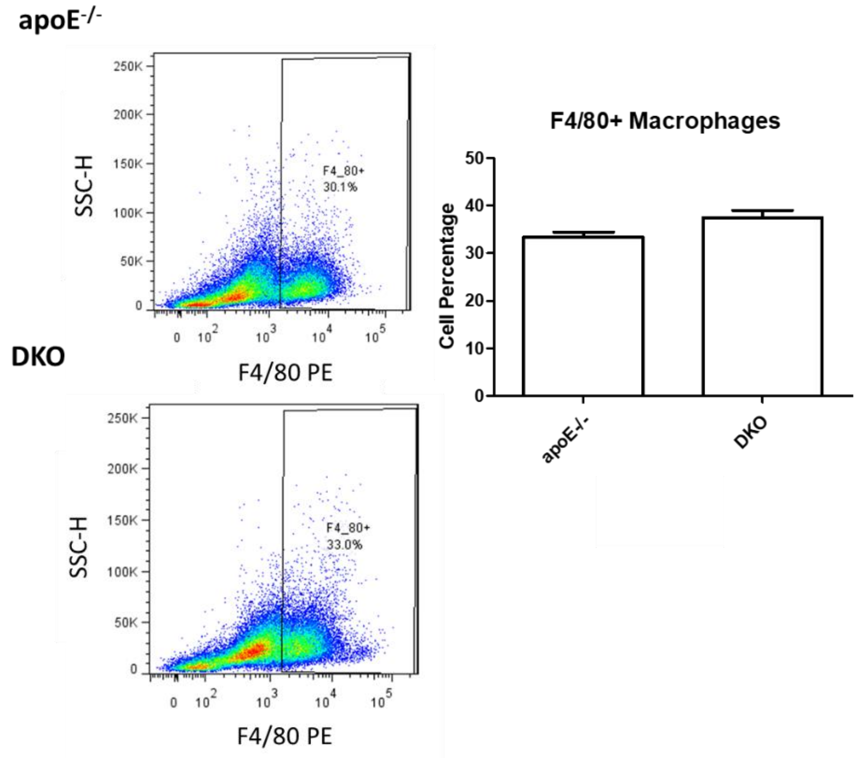


Figure 13. There is no significant difference in total macrophages between female DKO gWAT and apoE$^{-/-}$ gWAT. Mice were fed on HFD from 8 weeks old to 20 weeks old. Macrophages were defined as CD45+F4/80+; apoE$^{-/-}$ (n=5), DKO (n=4). p>0.05.

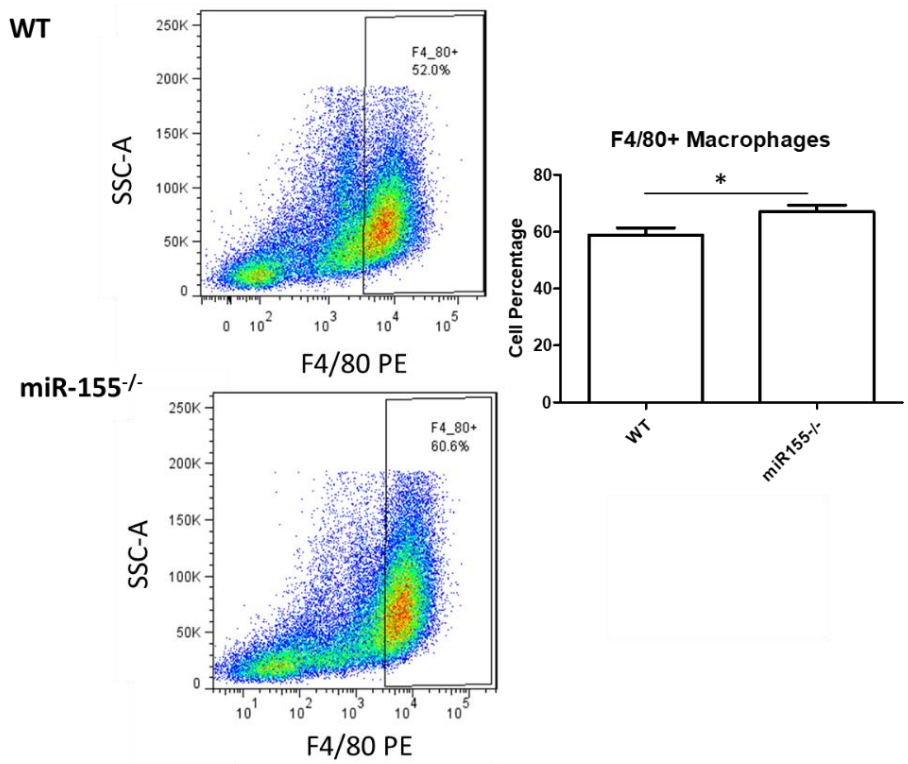


Figure 14. Macrophages are increased in male miR155$^{-/-}$ gWAT compared with WT gWAT. Mice were fed on HFD from 8 weeks old to 20 weeks old. Macrophages were defined as CD45+F4/80+; WT (n=9), miR155$^{-/-}$ (n=8). *, *p*<0.05.

We next assessed whether these macrophages could be broadly classified as pro-inflammatory or anti-inflammatory. We found that there was no significant change in pro-inflammatory (M1) ATMs nor anti-inflammatory (M2) ATMs of apoE$^{-/-}$ and DKO male mice (**Figure 15**). Interestingly, we found that the majority of the macrophages expressed both M1 and M2 markers. Recent papers have reported similar findings, where double positive macrophages are hypothesized to be pro-inflammatory [82] [84]. Similarly, there was no difference in M1 or M2 ATMs between WT and miR155$^{-/-}$ male mice (**Figure 16**).

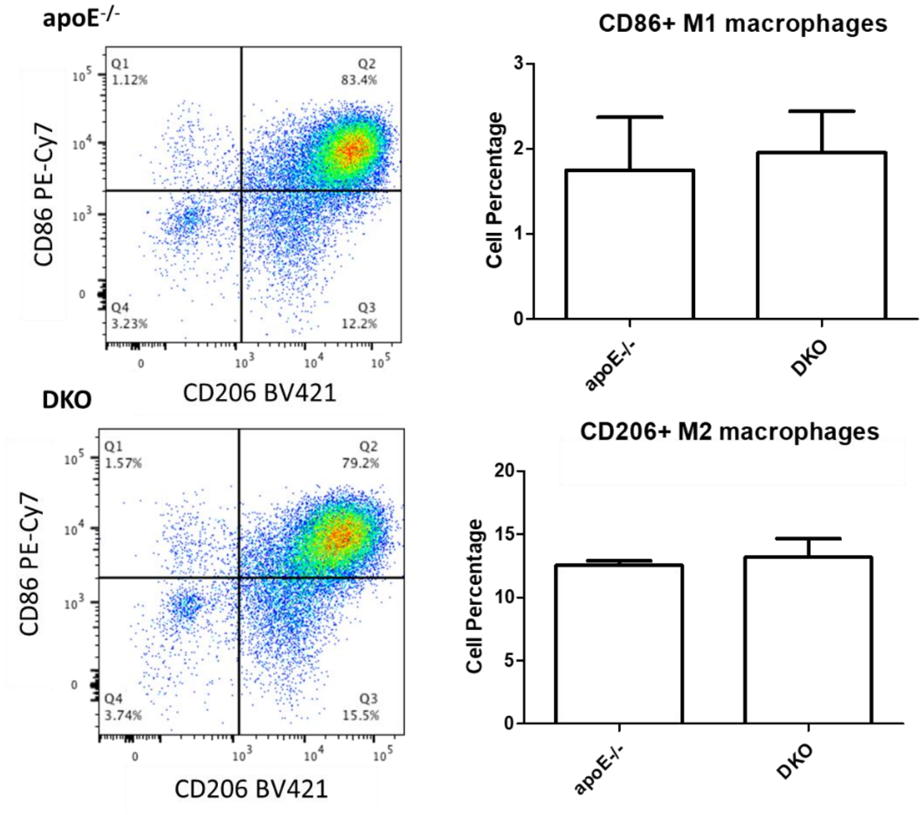


Figure 15. There is no significant difference in CD86+ (M1) or CD206+ (M2) ATMs between apoE$^{-/-}$ and DKO mice. Male mice were fed on HFD from 8 weeks old to 20 weeks old. Pro-inflammatory macrophages were defined as CD45+F4/80+CD86+. Anti-inflammatory macrophages were defined as CD45+F4/80+CD206+; apoE$^{-/-}$ (n=4), DKO (n=6). p>0.05.

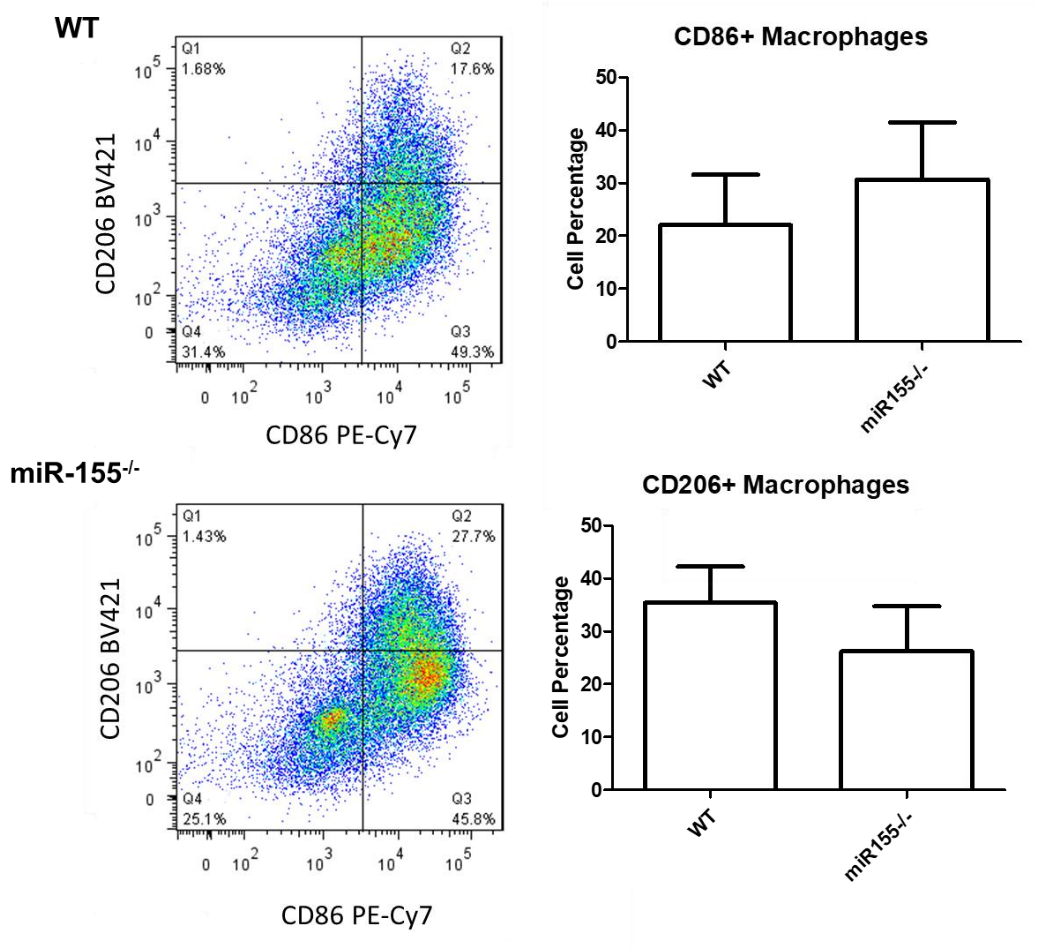


Figure 16. There is no significant difference in CD86+ (M1) or CD206+ (M2) ATMs between WT and miR155$^{-/-}$ mice. Male mice were fed on HFD from 8 weeks old to 20 weeks old. Pro-inflammatory macrophages were defined as CD45+F4/80+CD86+. Anti-inflammatory macrophages were defined as CD45+F4/80+CD206+; WT (n=13), miR155$^{-/-}$ (n=8), p>0.05.

In our previous publication, we found that pro-inflammatory macrophage markers in metabolic disease were lacking compared with markers for CVD and infectious diseases (PMID: 31824480, Table 3a in publication) [85], thereby showing the need for a novel marker. We examined whether CD95+ macrophages may be involved as a player in MHO mice. We found that while there was no significant difference in total CD95+ macrophages between apoE$^{-/-}$ and DKO male and female mice at MHO stage, there was a significant difference in a subset of CD95+ macrophages that was CD95+CD86- (**Figures 17, 18**). However, there was no difference in the CD95+CD86- macrophage population of apoE$^{-/-}$ and DKO male mice fed on NC for 20 weeks (**Figure 19**). These data identify a potential novel subset of macrophages that could play a role in MHO development, maintenance or progression.

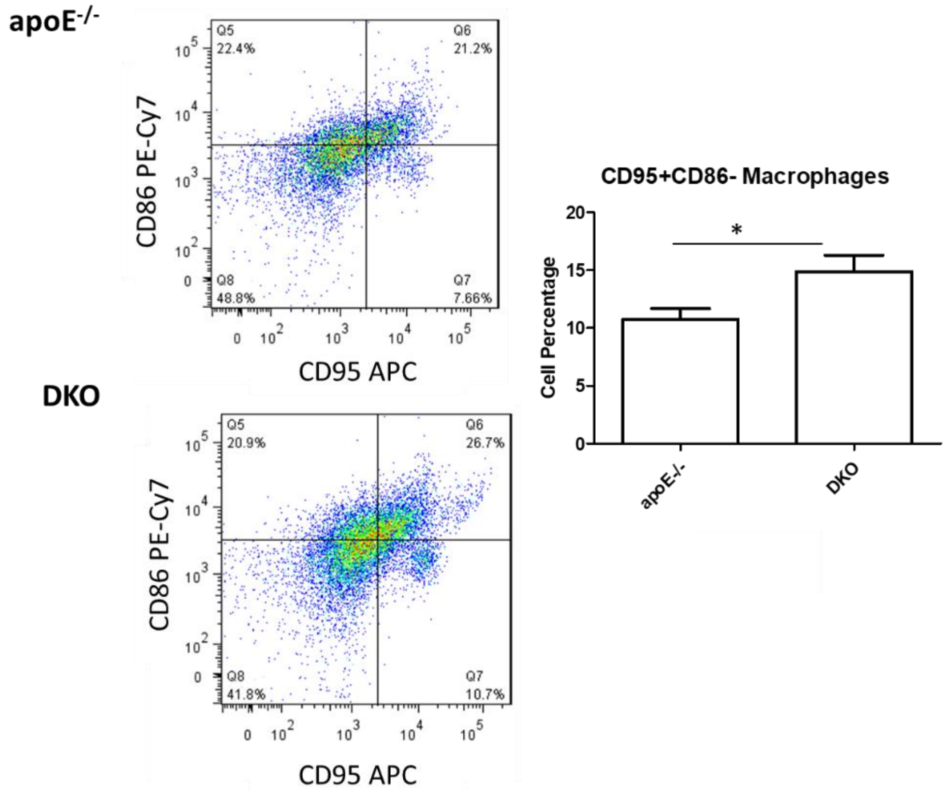
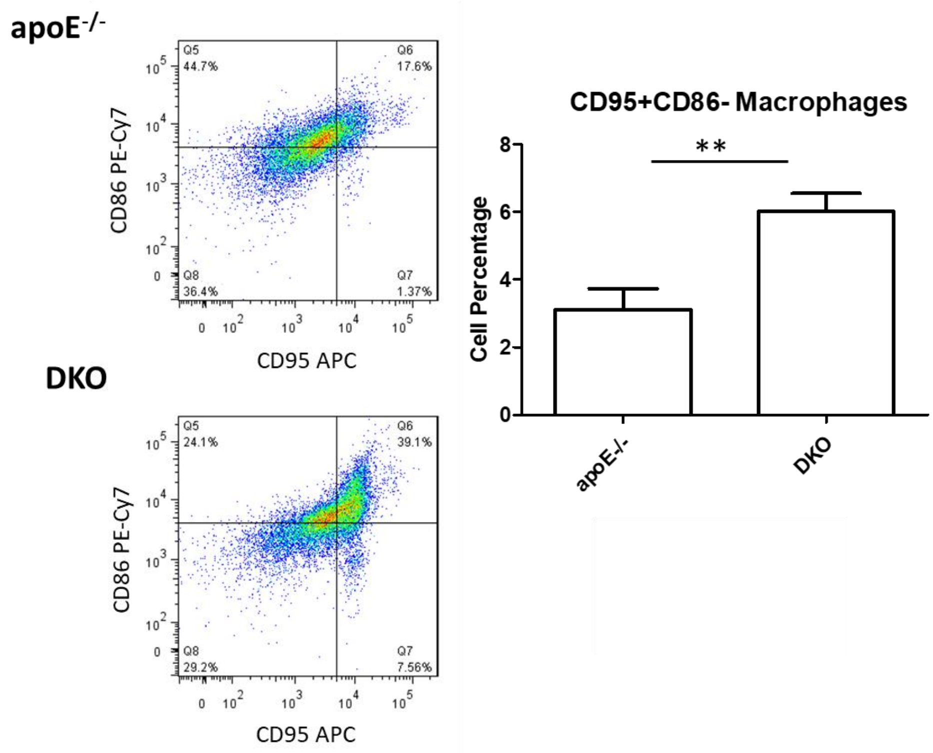


Figure 17. A novel subset of macrophages is increased in DKO vs apoE$^{-/-}$ male mice on HFD. Male mice were fed on HFD from 8 weeks old to 20 weeks old. Macrophages were defined as CD45+F4/80+CD95+CD86-; apoE$^{-/-}$ (n=6), DKO (n=7). *, p<0.05.

A.



B.

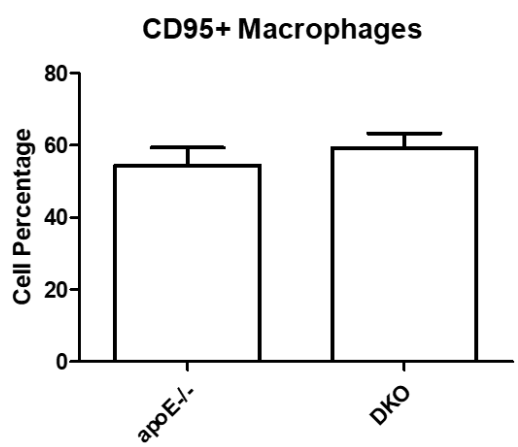


Figure 18. A novel subset of macrophages is increased in DKO vs apoE$^{-/-}$ female mice on HFD. Female mice were fed on HFD from 8 weeks old to 20 weeks old. **A.**

Macrophages were defined as CD45+F4/80+CD95+CD86-. **B.** Macrophages were defined as CD45+F4/80+CD95+; apoE$^{-/-}$ (n=5), DKO (n=4). *, $p<0.05$.

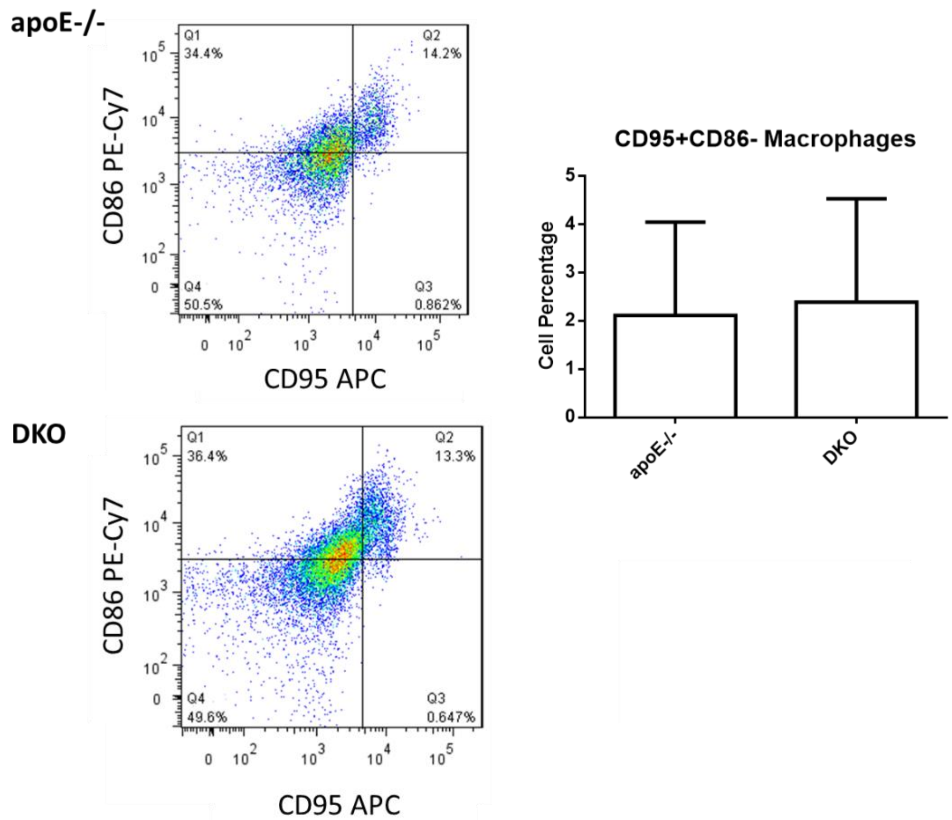
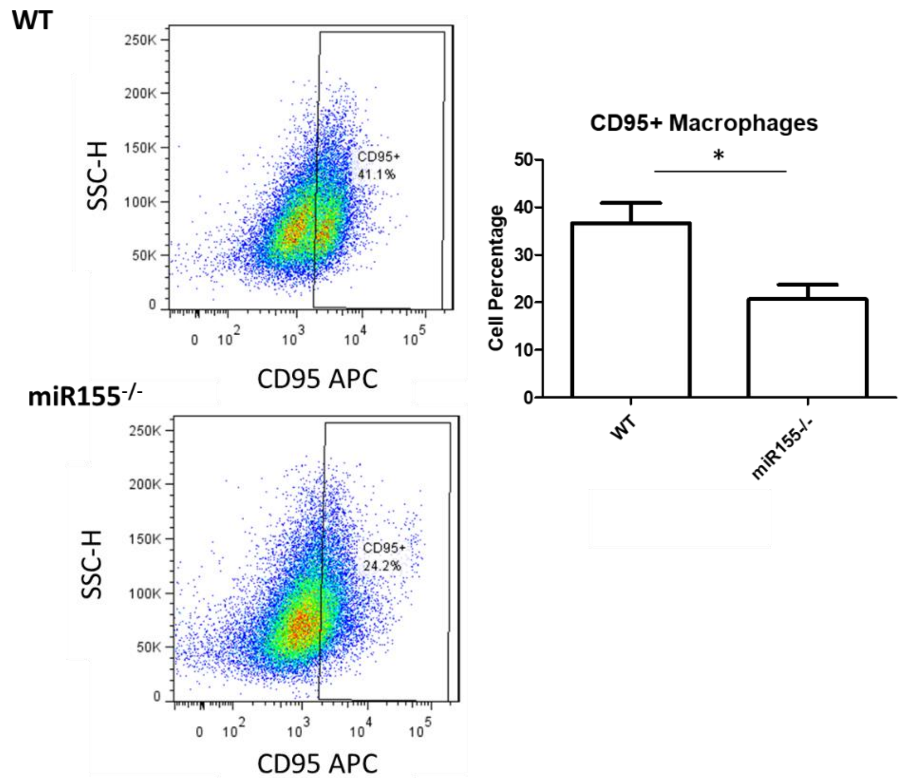


Figure 19. A novel subset of macrophages shows no significant difference in DKO vs apoE$^{-/-}$ male mice on NC. Male mice were fed on NC and analyzed at 20 weeks old. Macrophages were defined as CD45+F4/80+CD95+CD86-; apoE$^{-/-}$ (n=5), DKO (n=7). $p>0.05$.

We next assessed whether CD95+CD86- macrophage population was changed in WT versus miR155$^{-/-}$ male mice following 12 weeks of HFD. We did not find a robust population or significant difference. However, CD95+ macrophages were lower in miR155$^{-/-}$ gWAT compared with WT gWAT, showing that the WT gWAT may have a more pro-inflammatory environment than miR155$^{-/-}$ gWAT (**Figure 20**). However, in extended HFD, we see that miR155$^{-/-}$ gWAT had fewer CD95+CD86-macrophages (**Figure 21**).

A.


WT
250K
200K
150K
100K
50K
0
SSC-H
CD95+
41.1%
0 10^2 10^3 10^4 10^5
CD95 APC
miR155-/-
250K
200K
150K
100K
50K
0
SSC-H
CD95+
24.2%
0 10^2 10^3 10^4 10^5
CD95 APC
CD95+ Macrophages
*
50
40
30
20
10
0
Cell Percentage
WT
miR155-/-

B.

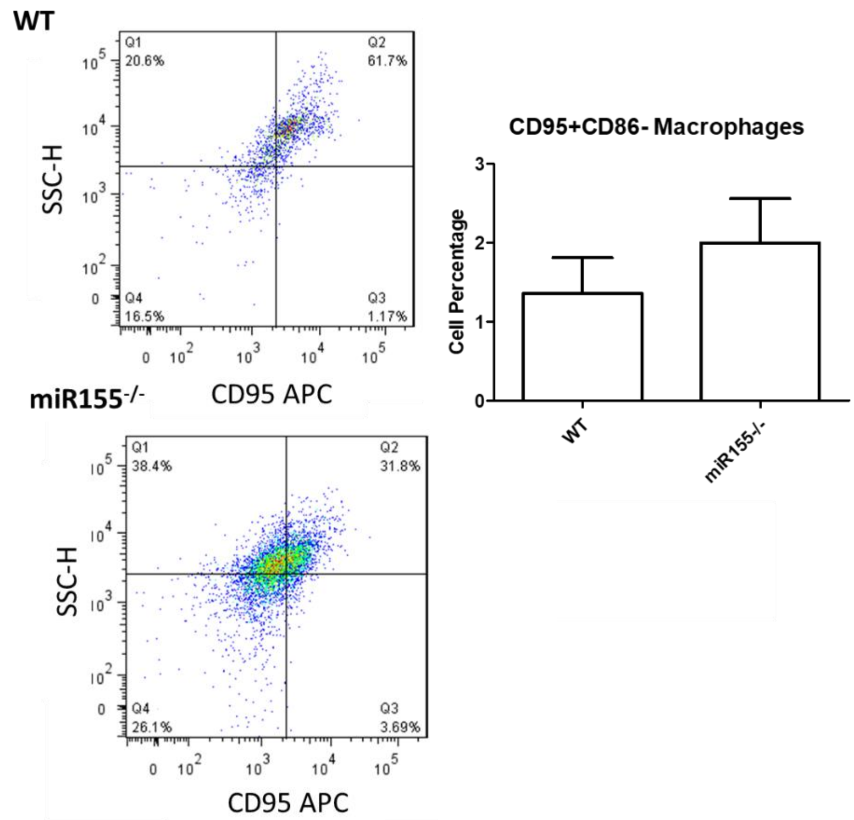
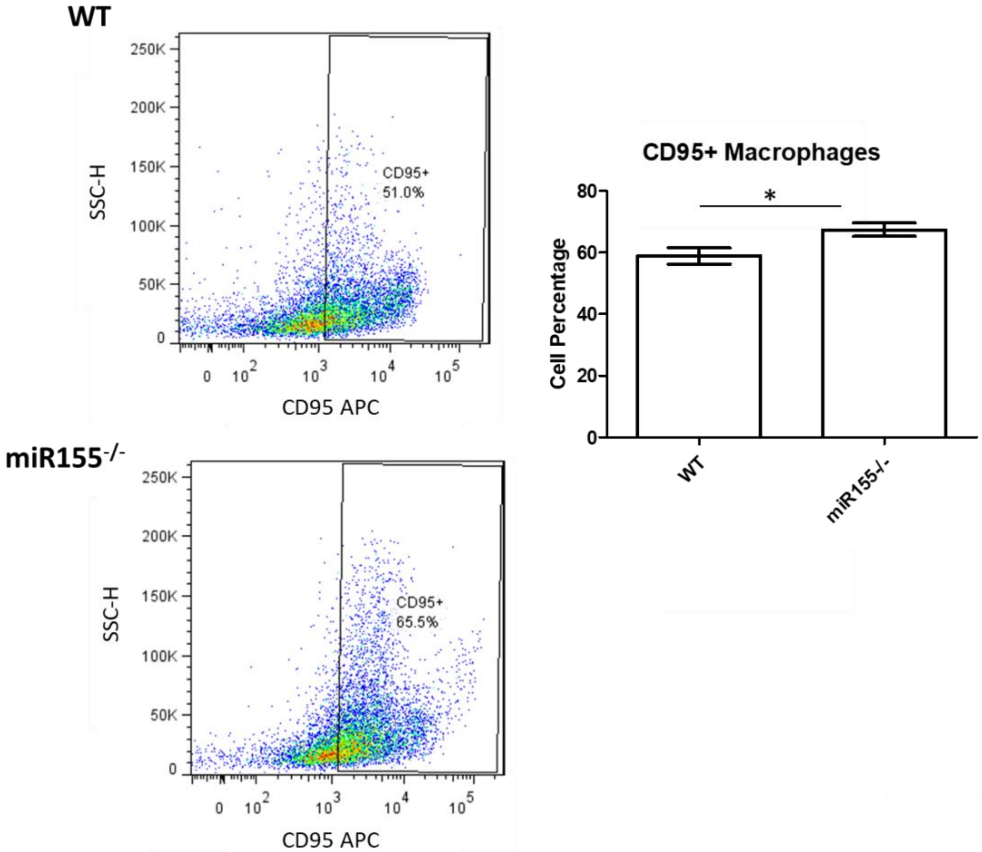


Figure 20. CD95+ macrophages are decreased in miR155$^{-/-}$ male mice after 12 weeks of HFD. Male mice were fed on HFD from 8 weeks old to 20 weeks old. **A.** Macrophages were defined as CD45+F4/80+CD95+. **B.** Macrophages were defined as CD45+F4/80+CD95+CD86-; WT (n=5), miR155$^{-/-}$ (n=6); *, p<0.05.

A.


WT
250K
200K
150K
100K
50K
0
SSC-H
CD95+
51.0%
0 10^2 10^3 10^4 10^5
CD95 APC
miR155-/-
250K
200K
150K
100K
50K
0
SSC-H
CD95+
65.5%
0 10^2 10^3 10^4 10^5
CD95 APC
CD95+ Macrophages
80
60
40
20
0
Cell Percentage
*
WT
miR155-/-

B.

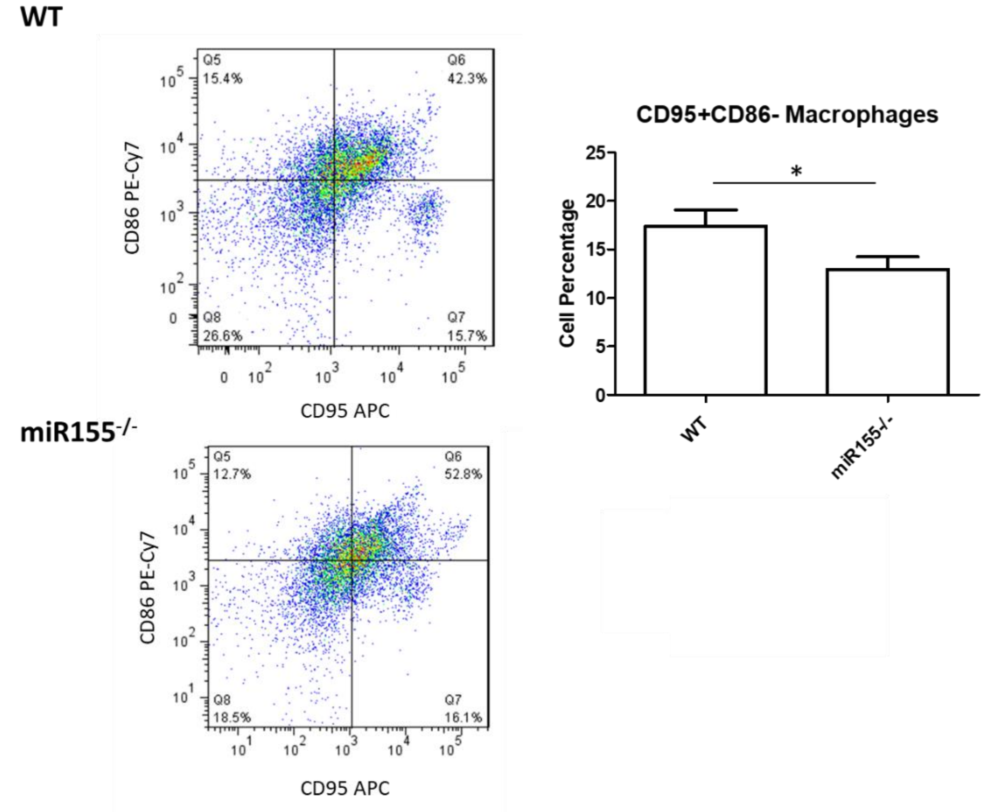
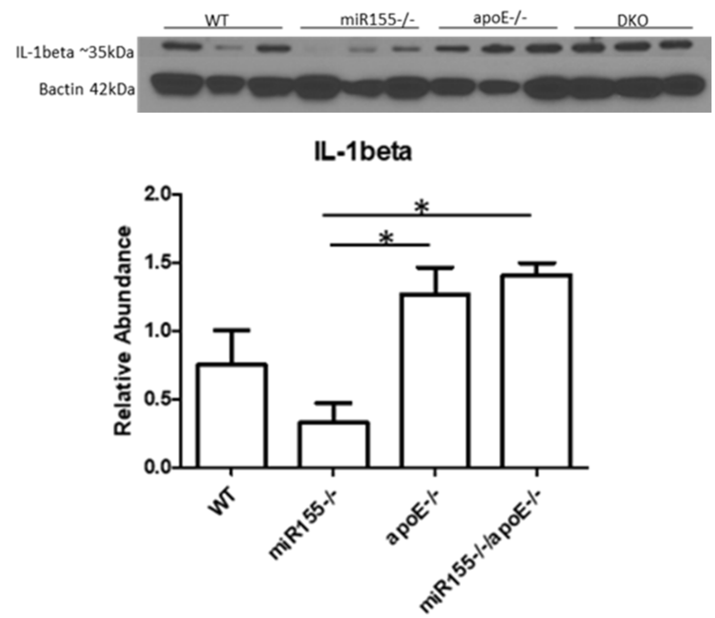


Figure 21. CD95+CD86- macrophages are decreased in male miR155$^{-/-}$ mice after extended HFD. Male mice were fed on HFD from 8 weeks old to 32 weeks old. **A.** Macrophages were defined as CD45+F4/80+CD95+. **B.** Macrophages were defined as CD45+F4/80+CD95+CD86-; WT (n=7), miR155$^{-/-}$ (n=10). *, *p*<0.05.

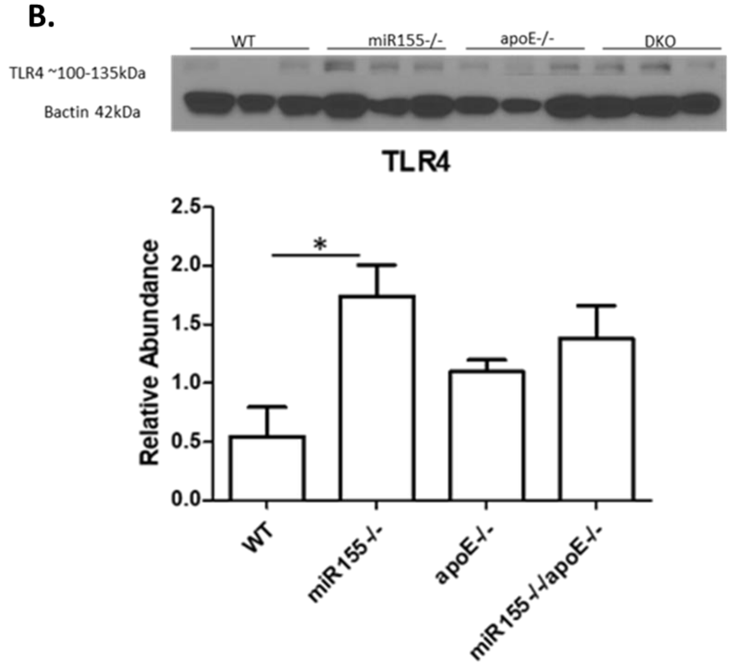
2. Fas+ (CD95+) ATMs may promote pro-atherogenic environment.

Rationale: Studies have discovered a pro-inflammatory function for Fas in both atherosclerosis and obesity [86] [76]. First, we assessed whether DKO aortas at the MHO stage exhibit a pro-inflammatory phenotype similar to apoE$^{-/-}$ aortas. We assessed the pro-inflammatory mediators, IL-1β and TLR4, since previous studies show a link with atherosclerosis [87] [88]. We found that DKO aortas showed no reduction in pro-inflammatory markers compared with apoE$^{-/-}$ aortas (**Figure 22**).

A.


WT
miR155-/-
apoE-/-
DKO
IL-1beta ~35kDa
Bactin 42kDa
IL-1beta
Relative Abundance
2.0
1.5
1.0
0.5
0.0
*
*
WT
miR155-/-
apoE-/-
miR155-/-/apoE-/-

B.


WT
miR155-/-
apoE-/-
DKO
TLR4 ~100-135kDa
Bactin 42kDa
TLR4
Relative Abundance
2.5
2.0
1.5
1.0
0.5
0.0
*
WT
miR155-/-
apoE-/-
miR155-/-/apoE-/-

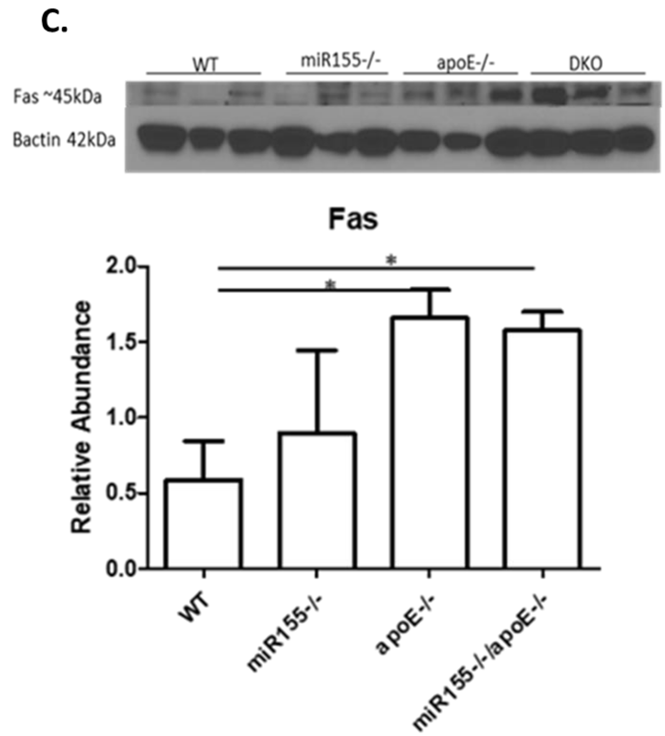


Figure 22. There is no difference in pro-inflammatory marker expression in DKO compared with apoE$^{-/-}$ aortas. Male mice were fed on HFD from 8 weeks old to 20 weeks old; n=3 per group. $p>0.05$.

After establishing that DKO aortas at MHO stage exhibit a pro-inflammatory state, we examined whether CD95+ macrophages increased in aortas of DKO mice over time. We found no difference between DKO groups (**Figure 23**).

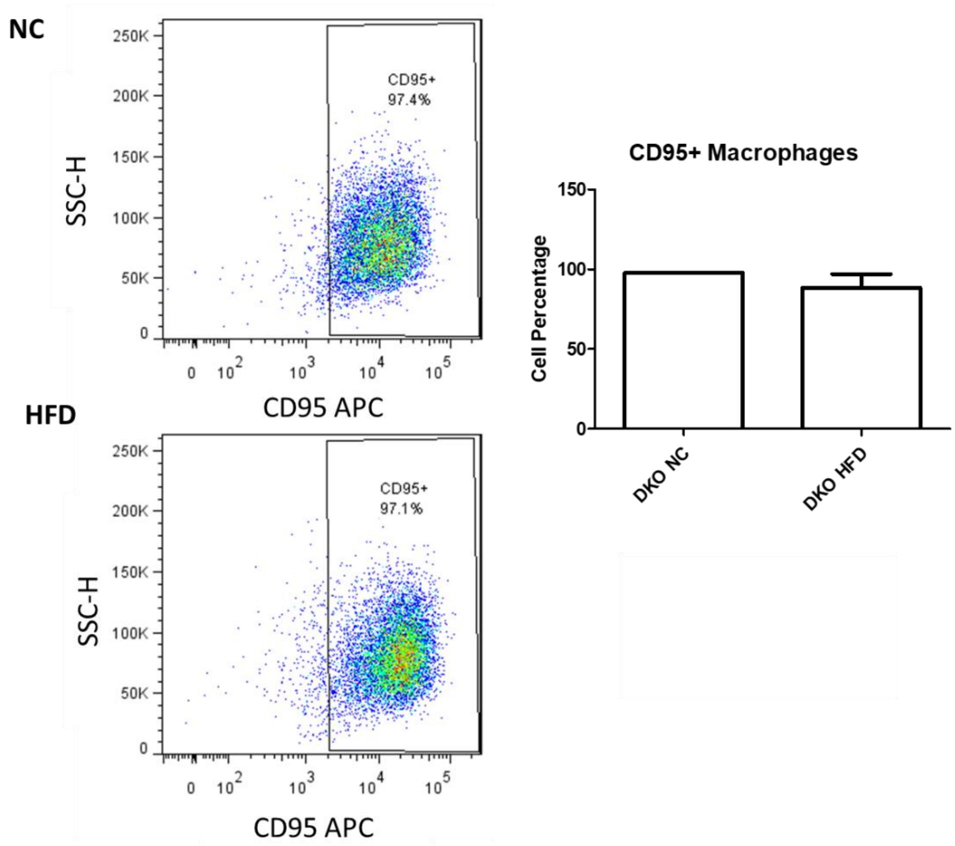


Figure 23. CD95+ macrophages show no significant difference in DKO aortas following HFD. Male mice were kept on NC or fed on HFD from 8 weeks old to 20 weeks old. Macrophages were defined as CD45+F4/80+CD95+; DKO NC (n=3), DKO HFD (n=2). $p>0.05$.

We next assessed whether CD95+ macrophages could promote EC activation. Previous studies have reported results that support a role for ATMs in atherosclerosis development [89]. Using cultured human aortic endothelial cells (HAECs), we found that HAECs incubated with DKO stromal vascular fraction (SVF)-conditioned medium (which contains CD95+ macrophages) for 24 hours resulted in increased EC activation, shown by significant increase in ICAM1 expression compared with untreated ECs (**Figure 24**). Moreover, incubating HAECs with human macrophage-conditioned medium resulted in increased ICAM1 expression compared with HAECs incubated with EC medium or macrophage differentiation medium only (**Figure 25**).

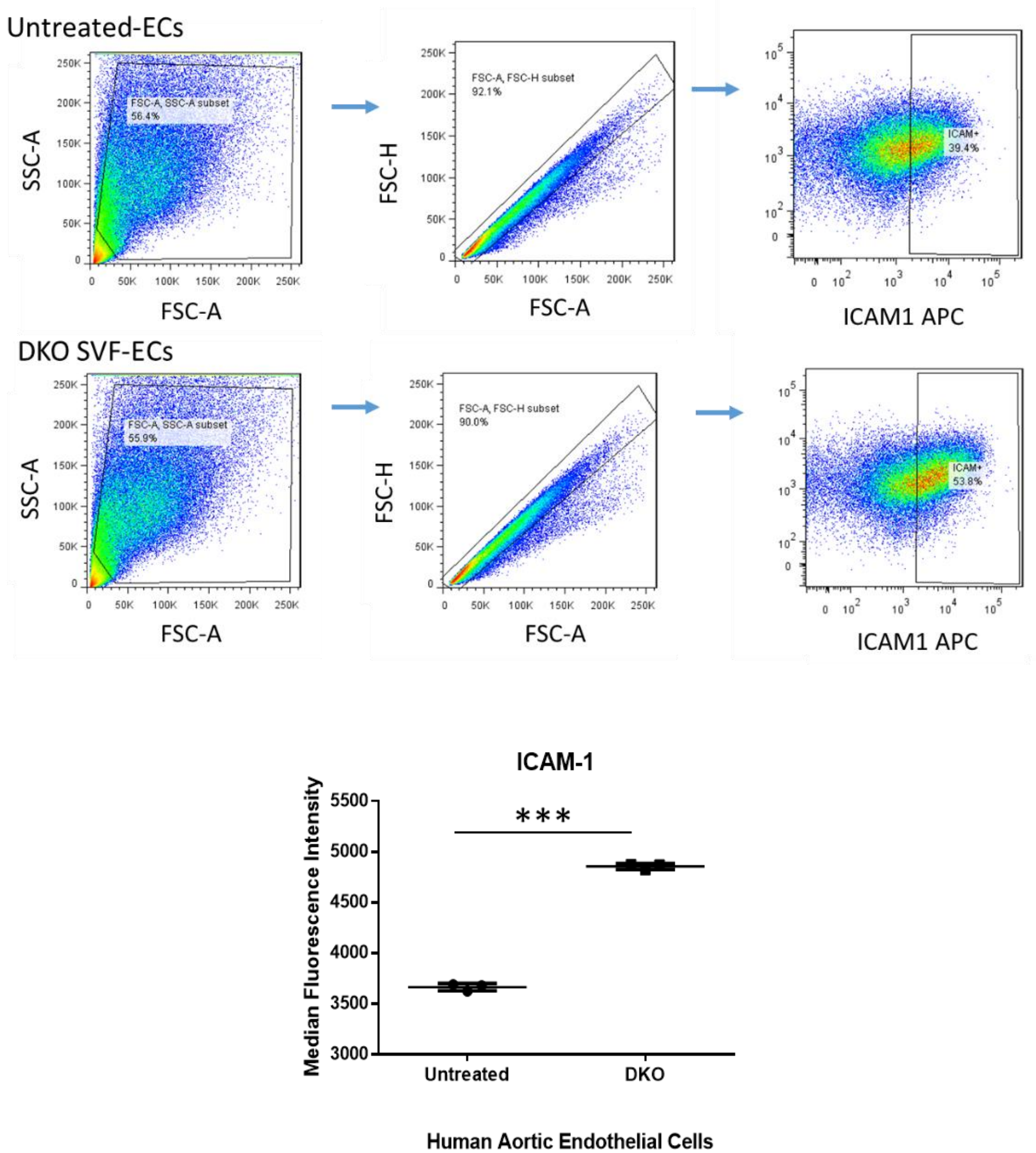


Figure 24. DKO stromal vascular cells promote HAEC activation. Stromal vascular fraction (SVF) from 20-week old DKO male mice on NC was cultured overnight in macrophage differentiation medium. Centrifuged supernatant was incubated with human aortic ECs (HAECs) for 24 hours. Untreated HAECs were incubated with EC medium; untreated (n=3), DKO-treated (n=3). ***, p<0.001.

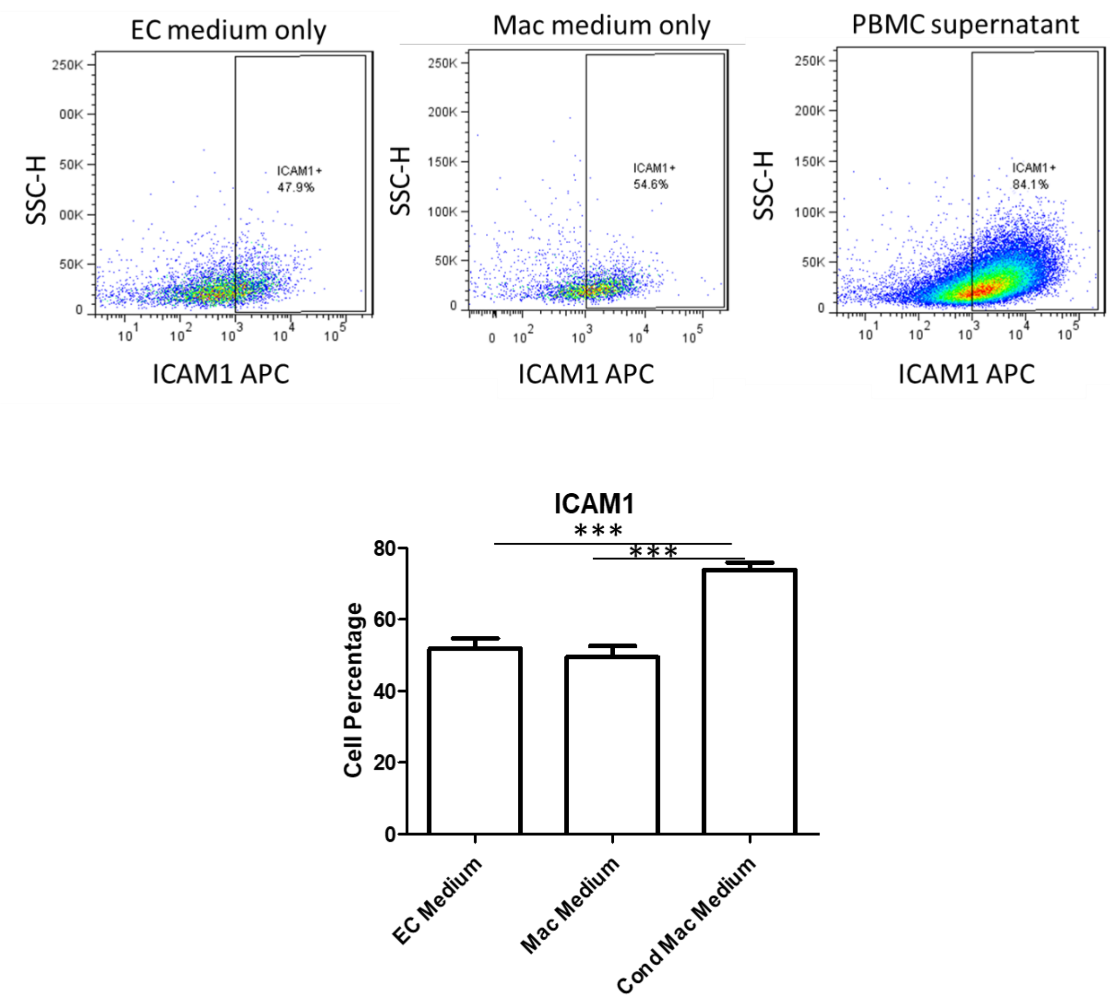


Figure 25. Human macrophage-conditioned medium promotes HAEC activation. Isolated human peripheral blood mononuclear cells (PBMCs) were grown for 7 days in macrophage differentiation medium. Afterwards, cultured HAECs were incubated with centrifuged supernatant (macrophage-conditioned medium) for 24 hours. HAECs in EC medium group were incubated with EC medium only for 24 hours and HAECs in Mac medium group were incubated with macrophage medium only for 24 hours; EC medium (n=11), Mac medium (n=11), Cond Mac medium (n=12). ***, $p<0.001$.

Regarding mechanism of MHO, a previous report showed that TNFα can increase Fas expression [90]. Additionally, we found that TNFα was significantly increased in our DKO mice at MHO stage. We found that TNFα-treated human PBMCs resulted in increased Fas detection by flow cytometry (**Figure 26**).

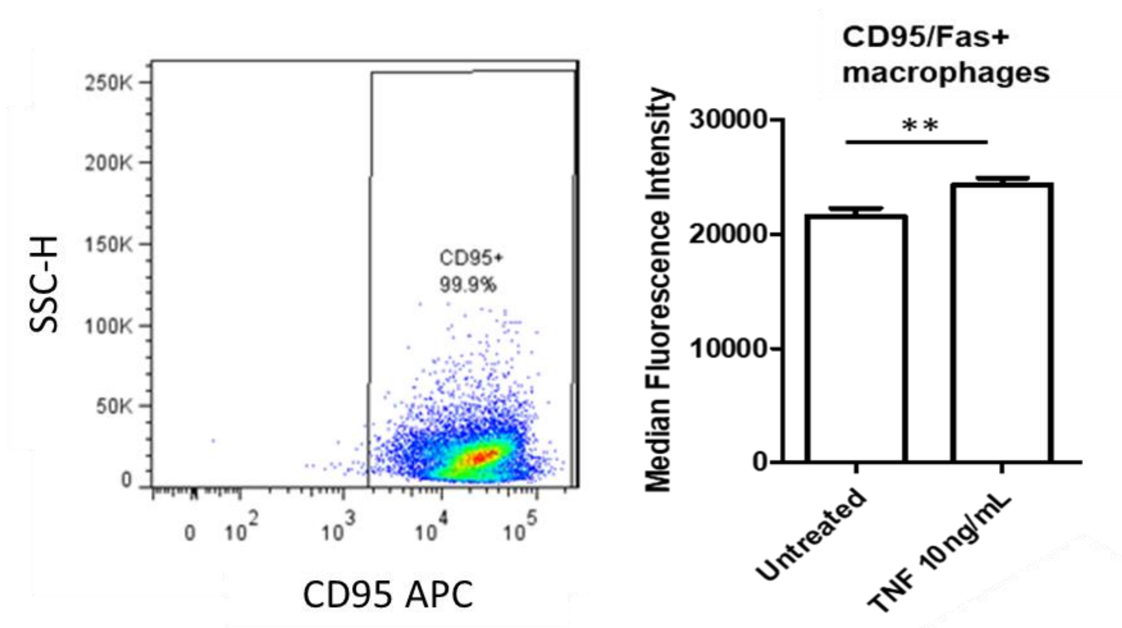


Figure 26. TNFα-treated human PBMCs show increased Fas expression. Isolated human PBMCs were grown for 7 days in macrophage differentiation medium. Cells were treated with TNFα (10ng/mL) for 24 hours. Untreated was incubated with fresh medium; Untreated (n=4), TNFα (n=4). **, p<0.01.

CHAPTER 4

DISCUSSION AND WORKING MODEL

MHO Model

Despite its recognition since the 1980s [91], the mechanisms underlying why some obese individuals enjoy MHO status as well as how MHO individuals progress towards MUO remain understudied. To this end, we have utilized our miR155$^{-/-}$/apoE$^{-/-}$ (DKO) male mice fed on HFD as a suitable MHO model. While there are several murine models to potentially study MHO [92] [93] [94] [95] [96] [97] [98] [99] [100] [101], they address one or two of the following criteria: lack of insulin resistance, increased HDL level, and normal triglyceride level. As explained in our previous publication, our MHO model maintained insulin sensitivity and did not meet the criteria for MetS, which is having at least three of five risk factors: hypertension, enlarged waistline, low HDL levels, high triglyceride levels, and high fasting blood glucose level. Our DKO mice on HFD maintained normal plasma triglyceride levels, normal fasting blood glucose level, and had increased plasma HDL level compared with apoE$^{-/-}$ mice on NC and HFD as well as with DKO mice on NC [79]. These differences highlight the significance of our MHO model. Over time, however, several studies report that approximately one-third to one-half of MHO individuals develop MUO, showing that MHO may not be a stable condition for a significant portion of obese patients. In addition, compared to MH lean individuals, MHO patients were at a higher risk of developing premalignant colorectal tumors as well as post-menopausal breast cancer. Moreover, the disparity in cut-off values further complicates the definition of when MHO transitions to MUO. It is also important to note that controversy exists in the field regarding how to interpret MHO, whether as a distinct subset of obese patients or as a continuum of obese individuals with varying degrees of preserved metabolic health [102]. Indeed, more mechanistic research is needed for clarity.

Contributions/Working Model

In light of the previously mentioned complications accompanying MHO, we aimed to better understand how MHO transitions to MUO. We studied whether HFD in MHO mice promoted WAT inflammation (**Aim 1**). We addressed Aim 1 in a series of sub-aims. We defined whether MHO mice develop MUO with extended HFD, which mimics the MHO patient continuing a HFD lifestyle. We saw that over time (more specifically, 24 weeks as opposed to 12 weeks, in our model), extended HFD-fed MHO model developed atherosclerosis. Aortic plaque deposition was not significantly different between apoE$^{-/-}$ and DKO mice at 24 weeks. However, whereas atherosclerotic plaque deposition is minimal in MHO model [79], the aortas of extended HFD-fed DKO mice exhibited extensive plaque accumulation (**Figure 8**). This demonstrated MHO transition to MUO. We next assessed the pro-inflammatory environment (if any) in DKO mice at MHO stage (i.e., 12 weeks of HFD feeding). Within gWAT, pro-inflammatory adipokine, resistin, was significantly increased in DKO mice at MHO stage compared with apoE$^{-/-}$ and WT mice. Leptin, another pro-inflammatory adipokine, showed no difference (**Figure 10**). We also evaluated TNFα and Fas expression in gWAT, where they were increased in DKO mice at MHO stage compared with DKO mice at an earlier stage of HFD feeding. The levels of Mcp1, however, were unchanged, suggesting that macrophage recruitment was not changed, at least at this stage [58] (**Figure 11**). In the circulation, we found that TNFα and soluble forms of E-selectin and Icam1 were induced in DKO mice at MHO stage. It is possible that TNFα levels may be significantly attributed to gWAT, which we showed produced increasing amounts of TNFα over time. Additionally, the soluble forms of E-selectin and ICAM1 are signatures of EC activation, suggesting that ECs at MHO stage may be activated (**Figure 9**) [103] [104].

As earlier mentioned, an increase in WAT macrophage numbers is a typical event in obesity and is characteristic of inflammation [105]. In MHO mice, total macrophage numbers (CD45+F4/80+CD11b+) were significantly increased, as assessed by flow cytometry (**Figure 12**). This was not replicated in female DKO mice (**Figure 13**). However, total macrophages (CD45+F4/80+) were significantly higher in miR155$^{-/-}$ mice compared with WT mice (**Figure 14**). Analysis of the macrophages as pro-inflammatory (CD45+F4/80+CD86+) or anti-inflammatory (CD45+F4/80+CD206+) showed that, in MHO mice, there was no significant difference compared with apoE$^{-/-}$ mice (**Figure 15**). No difference was observed between WT and miR155$^{-/-}$ mice as well (**Figure 16**). Our recent publication highlights that metabolic disease markers for pro-inflammatory macrophages are lacking, especially when compared with markers for CVD and infectious diseases (PMID: 31824480, Table 3a in publication) [85]. Therefore, novel markers are needed. Fas (or CD95) is well-known for its pro-apoptotic role, but also has a non-apoptotic function as a pro-inflammatory mediator in cells such as macrophages. We examined whether CD95+ macrophages are increased in MHO mice. We found that while total CD95+ macrophages were not increased in MHO mice compared with apoE$^{-/-}$

mice, the subset CD95+CD86- was (**Figure 17**). This was also observed in female DKO mice compared with apoE$^{-/-}$ mice (**Figure 18**). This distinction was lost in age-matched NC-fed DKO and apoE$^{-/-}$ mice, showing that the CD95+CD86- macrophage population increased due to HFD feeding (**Figure 19**). In WT and miR155$^{-/-}$, we observed that CD95+ macrophages were significantly reduced in miR155$^{-/-}$ mice following 12 weeks of HFD and that there was no difference in CD95+CD86- macrophage population (**Figure 20**). With extended HFD feeding (24 weeks HFD), we saw that CD95+ macrophages significantly increased in miR155$^{-/-}$ mice compared to WT mice (**Figure 21A**). While CD95+CD86- macrophages are significantly lower in miR155$^{-/-}$ mice compared with the population in WT mice, the overall numbers of CD95+CD86- miR155$^{-/-}$ macrophages between 12 weeks and 24 weeks have increased; from approximately 2 percent to 12.5 percent, respectively (**Figures 20B, 21B**).

Moving beyond, we sought to examine whether CD95+ ATMs promote a pro-atherogenic environment (**Aim 2**). To address this Aim, we assessed whether MHO mice exhibited a pro-inflammatory profile in aortas. We found that there was no difference in expression of pro-inflammatory IL-1β, TLR4 and Fas between the well-established atherosclerotic model, apoE$^{-/-}$ mice, and our MHO mice (**Figure 22**). In other words, MHO mice maintained a pro-inflammatory environment, despite very minimal plaque manifestation. Macrophages may exert their pro-inflammatory effects in a paracrine manner or via cell-cell contact. We examined whether CD95+ macrophages are increased in aorta of DKO mice fed on NC or HFD but found no difference, alluding to CD95+ macrophages potentially acting in a paracrine manner, rather than via cell-cell contact

(**Figure 23**). To determine if DKO SVF, which includes CD95+ macrophages, can promote aortic EC activation, we cultured SVF of DKO mice overnight in macrophage differentiation medium, then treated HAECs with the conditioned medium. We saw a significant increase in ICAM1 detection. Likewise, we isolated and cultured PBMCs from human blood in macrophage-differentiation medium. Following 24-hour incubation of conditioned medium, we found increased ICAM1 expression compared to EC medium or macrophage medium only controls (**Figures 24, 25**). As shown in Aim 1, TNFα is significantly increased in gWAT and induced in serum. We also found that TNFα treatment for 24 hours increased Fas detection in PBMCS, showing that TNFα may increase CD95+ macrophage population (**Figure 26**). Taken together, our study suggests that CD95+CD86- ATMs may represent a novel subset for MHO transition to MUO (**Figure 27**). Practically, it may represent the degree of transition to MUO in a clinical setting. Furthermore, this subset defines a previously unappreciated role for CD95+ macrophages in obesity, which may have roles in other disease conditions.

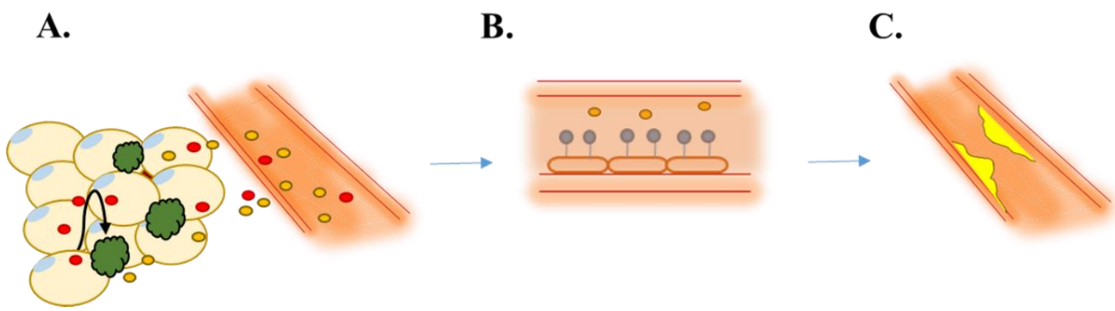


Figure 27. Working model of MHO transition to MUO. A. Adipocyte (pale yellow with light blue nuclei)-secreted and macrophage-secreted TNFα (red dots) may promote CD95+CD86- macrophage phenotype (green). This macrophage subset may secrete pro-inflammatory cytokines and chemokines into the circulation (yellow dots), which in turn, **B**. induce aortic EC activation, shown by ICAM1 upregulation (gray dots). **C.** Over time, EC activation progresses towards atherosclerosis development (bright yellow). Red dots: TNFα. Yellow dots: pro-inflammatory cytokines/chemokines/other molecules.

Future Direction

Going forward, there are several considerations to address. First, there is a need to directly assess sorted CD95+CD86- macrophages via cytokine array/ELISA to confirm their pro-inflammatory status. Additionally, knock down/genetic ablation of Fas, followed by subsequent culture of the Fas-deficient/deleted macrophages and then assessing whether supernatant no longer activates aortic ECs is a key experiment. From here, comparing the secretome of the Fas-deficient/deleted macrophages and Fas-intact macrophages would lead to identifying key secreted factors responsible for aortic EC activation. Another experiment is determining whether CD95+CD86- ATMs increase in extended HFD-fed mice (MUO phase) compared with MHO phase as well as CD95+CD86- macrophages in aorta. Moreover, increasing the sample size of a number of experiments is needed. Evaluating the presence in gWAT, serum and aortas, as well as understanding the role Fas ligand (FasL) plays in MHO transition to MUO are additional important follow-up experiments.

Potential Role for Fas in Other Frontiers

The MHO field offers several opportunities for future elucidation. One such path involves studying whether Fas and T cells play a complementary role in MHO. For instance, T cells are increased in adipose tissue during obesity, where they, in part, function to recruit and polarize ATMs [52] [106]. Additionally, CD8+ T cell subset, which expresses FasL as part of its cytotoxic function, is increased during obesity [52] [107]. In their investigation of adipocytes, Wueest *et al.* showed that induction of Fas by FasL treatment in cultured adipocytes led to increased macrophage adherence compared with untreated adipocytes, lending support to the possibility that CD95+ adipocytes may also play a role in MUO [76]. As earlier mentioned, Wueest *et al.* found that myeloid-specific Fas depletion led to reduced TNFα levels [73], which lends support to our working model of a TNFα-Fas axis in macrophages in MHO. Targeting macrophages may lead to mitigation of the inflammatory conditions that make obesity a long-term health challenge.

Like adipose tissue, liver is a key organ regulating metabolic health, itself possessing a large macrophage component [108]. Several papers document Fas' role in promoting hepatocyte damage [109] [110] [111]. Additionally in NAFLD, which involves ectopic lipid accumulation in the liver, Item *et al.* found that increased Fas dysregulated hepatocyte mitochondrial respiration and fatty acid oxidation. Hepatocyte-specific deletion of Fas led to better outcomes, including improved glucose tolerance, insulin resistance and hepatic steatosis [109].

As previously mentioned, MHO individuals tend to be younger than 40 years old and to regularly exercise. Indeed, Matsuzawa *et al*. reported that sumo wrestlers often transition to MUO from MHO following retirement, hinting that a potential transition mechanism is exercise-related [112]. Another future area of MHO study is the gut microbiome. Intestinal bacterial flora play a vital role in nutrient absorption and cytokine production. In addition to its dysregulation being implicated in atherosclerosis, chronic kidney disease, T2DM, and many more diseases, several studies have reported the connection between obesity and an individual's gut microbiome, specifically an altered microbiome [113] [114] [115] [116]. One possible reason put forth by several papers is activation of immune cells, such as macrophages, in the intestine, leading to inflammation status [117] [118]. To date, gut microbiome has not been studied in MHO [119]. Another avenue for future investigation is understanding how circadian rhythm may affect MUO and MHO [120] [121]. In fact, circadian clocks have been discovered in WAT, where adipokines like leptin and resistin experience scheduled expression and where the transcription factor BMAL1 functions as a master regulator of adipocyte circadian rhythm [122]. Again, research into the circadian rhythm of MHO individuals is non-existent.

The other extremely important arm of studying the MHO phenomenon is understanding why MHO patients are protected from or, at the very least, experience delayed MUO. Determining the molecule(s), or lack thereof, that facilitate this phenomenon is vital to understanding the condition as a whole. Knowing what promotes MHO in the first place and what promotes its transition can inform the clinical treatment of MUO patients. With a more complete understanding, future treatments may involve inducing MUO patients into the MHO phase as well as helping MHO patients maintain their status, thereby preventing their transition in metabolic ill-health.

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