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Supervisor:	Geoff Werstuck	**Defence date:**	2023/09/18

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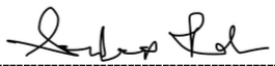

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EFFECTS OF SEX HORMONES ON MACROPHAGE POLARIZATION AND FUNCTION

INVESTIGATING THE EFFECTS OF SEX HORMONE SUPPLEMENTATION ON BONE MARROW-DERIVED MACROPHAGE POLARIZATION AND PRO-ATHEROGENIC FUNCTION

By: SOPHIE ENRIGHT, BMSc

A Thesis Submitted to the School of Graduate Studies in Partial Fulfilment of the Requirements for the Degree Master of Science

McMaster University MASTER OF SCIENCE (2023) Hamilton, Ontario

(Medical Sciences)

TITLE: Investigating the effects of sex hormone supplementation on bone marrow-derived macrophage polarization and pro-atherogenic function

AUTHOR: Sophie Enright, BMSc (University of Western Ontario)

SUPERVISOR: Dr. Geoff H. Werstuck

NUMBER OF PAGES: xiv, 84

LAY ABSTRACT

The risk and presentation of heart disease are different in men and women. Understanding the underlying reasons for these sex differences will allow us to improve clinical strategies to better care for patients with heart disease. Scientists believe that sex hormones, including estrogen and testosterone, affect how heart disease develops in the body over time. Macrophages, which are cells involved in inflammation, guide the progression of heart disease. We believe that sex hormones interact with macrophages and modulate the development of heart disease. This study demonstrates that estrogen and testosterone do alter macrophage functions that relate to heart disease. Sex hormones appeared to have a beneficial effect on these cell functions and may play a protective role in heart disease. This study is significant because it i) provides insight into the complicated role that sex hormones play in heart disease, and ii) provides a baseline for future research on this topic.

ABSTRACT

Atherosclerosis, the main underlying cause of most cardiovascular disease (CVD), is characterized by progressive inflammation of the vascular intima. Macrophages are inflammatory cells centrally involved in atherogenesis. Macrophage polarization into pro-inflammatory or anti-inflammatory phenotypes has a significant impact on disease progression. Sex differences in the development and progression of CVD are well established, but the effects of sex hormones on macrophage polarization and pro-atherogenic functions are not well described. We hypothesized that sex hormones directly modulate macrophage polarization and pro-atherogenic function, and thereby regulate the progression of atherosclerosis.

Bone marrow-derived monocytes from adult male and female C57BL/6 mice were differentiated into macrophages using macrophage colony-stimulating factor (20 ng/mL) and pre-treated with either 17β-estradiol (100 nM), testosterone (100 nM), or a vehicle control for 24 hours. Pre-treated macrophages were polarized into the pro-inflammatory or anti-inflammatory phenotypes and the effects of sex hormone supplementation on gene expression of macrophage phenotypic markers were assessed using RT-qPCR. Protein concentration of inflammatory markers including IL-1β was quantified using an addressable laser bead immunoassay. A Transwell migration assay was employed to determine changes in macrophage migration, and changes in lipid accumulation were determined by cell staining and fluorescence microscopy.

Sex differences were observed in macrophage polarization, inflammatory responses, migration, and lipid accumulation. Pre-treatment with 17β-estradiol significantly impaired gene expression of pro-inflammatory macrophage markers and production of IL-1β in pro-inflammatory macrophages. In anti-inflammatory macrophages, 17β-estradiol significantly upregulated expression of anti-inflammatory markers and enhanced cell migration. Pre-treatment

with testosterone enhanced anti-inflammatory marker mRNA expression in anti-inflammatory macrophages and impaired production of IL-1β by pro-inflammatory macrophages. Our observations suggest a protective role of 17β-estradiol and testosterone in atherogenesis and may contribute to the sexual dimorphisms in CVD observed in human patients.

AKNOWLEDGEMENTS

The completion of this thesis would not have been possible without the support and guidance of several people**.** I would like to thank my supervisor, Dr. Geoff Werstuck for providing me with the opportunity to pursue a research project that aligned exactly with my research interests. His guidance throughout my degree and his confidence in my ability allowed me to develop as a researcher. I would also like to thank my committee members, Dr. Sandeep Raha and Dr. Fox-Robichaud, for their continuous support on my project. Their thought-provoking contributions resulted important research interpretations and helped me develop a broader understanding of my area of study.

Next, I would like to acknowledge and thank all members, past and present, of the Werstuck lab, for their contributions to my research experience. Thank you to Dr. Daniel Venegas Pino, Dr. Monica De Paoli, Dr. Lauren Mastrogiacomo, Dr. Sarvatit Patel, and Rangana Talpe Guruge for their time and patience in passing down laboratory techniques essential to my research, as well as their friendship throughout my degree. To all other lab members and the greater TAARI research group, I am grateful for the friendships that were fostered. I would like to acknowledge and thank Roniya Sebastian for her substantial contribution to the image analysis in this project.

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LIST OF ABBREVIATIONS

ALBIA……..Addressable Laser Bead Immunoassay
ANOVA……....Analysis of Variance
ApoE-/- ……Apolipoprotein E Knockout
AR……...Androgen Receptor
ARE…….Androgen Response Element
Arg1……..Arginase 1
BMDM……..Bone Marrow-Derived Macrophage
CCL19……Chemokine Ligand 19
CD38…….Cluster of Differentiation 38
CD206……Cluster of Differentiation 206
CS-FBS.……...Charcoal-Stripped Fetal Bovine Serum
CVD……..Cardiovascular Disease
DAPI……..4′,6-diamidino-2-phenylindole
DH-T……..Dihydrotestosterone
DMEM……Dulbecco's Modified Eagle Medium
DNA……. Deoxyribonucleic Acid
E1……Estrone
E2 …….17β-Estradiol
E3 ……Estriol
ER ……Endoplasmic Reticulum
ERα ……...Estrogen Receptor Alpha
ERβ ……..Estrogen Receptor Beta
ERK …….Extracellular Signal-Regulated Kinase
Fizz1 …….Found In Inflammatory Zone 1
GPER1 ……...G Protein Coupled Estrogen Receptor 1
GPER30 ……..G Protein Coupled Receptor 30
HA-mac …….Hemorrhage-Associated Macrophage
HSC ……...Hematopoietic Stem Cell
IFNγ …….Interferon Gamma
IHD ……..Ischemic Heart Disease
IL-1 ……...Interleukin-1
IL-1β …….Interleukin-1Beta
IL-4 ……...Interleukin-4
IL-6 ……...Interleukin-6
IL-10 ……...Interleukin 10
IL-13 ……...Interleukin-13
iNOS ……..Inducible Nitric Oxide Synthase
IRF4 …….Interferon Regulatory Factor 4
IRF5 …….Interferon Regulatory Factor 5
LDL ……..Low-Density Lipoprotein
LPS ……Lipopolysaccharide
MAPK ……Mitogen Activated Protein Kinase
M-CSF ……..Macrophage Colony-Stimulating Factor
Mhem ……Hemoglobin-Associated Macrophage

MI ……...Myocardial Infarction
MR ……...Mannose Receptor
mRNA ……Messenger Ribonucleic Acid
NO ……Nitric Oxide
ORO ……..Oil Red O
oxLDL ……...Oxidized Low Density Lipoprotein
PBS ……Phosphate Buffered Saline
PFA ……..Paraformaldehyde
RT-qPCR …….Reverse Transcription Quantitative Polymerase Chain Reaction
SEM ……...Standard Error of the Mean
SHBG …….Sex Hormone Binding Globulin
SMC ……Smooth Muscle Cell
STAT1 ……..Signal Transducer and Activator of Transcription 1
STAT3 ……..Signal Transducer and Activator of Transcription 3
STAT6 ……..Signal Transducer and Activator of Transcription 6
TE ……..Testosterone
TGF-β ……Transforming Growth Factor-Beta
Th-1 ……T-Helper Type 1
Th-2 ……T-Helper Type 2
THP-1……..Tohoku Hospital Pediatrics-1
TLR4 ……...Toll-Like Receptor 4
TNF-α ……..Tumour Necrosis Factor Alpha
Ym1 ……Chitinase-3-Like Protein 3

DECLARATION OF ACADEMIC ACHIEVEMENT

This study has characterized bone marrow-derived macrophage polarization over several time points and has identified sex differences in macrophage polarization and pro-atherogenic functions. It has also revealed that treatment with 17β-estradiol and testosterone enhances macrophage behaviours that could support a role in human atheroprotection.

All methods conducted in this study were completed by Sophie Enright. The bone marrow-derived macrophage differentiation protocol was developed by Dr. Sarvatit Patel and the macrophage polarization scheme was further adjusted by Sophie Enright. The experimental design of determining sex differences in BMDM polarization and pro-atherogenic functions was created by Dr. Geoff Werstuck and Sophie Enright. All sample collection, cell culturing and experimentation was carried out by Sophie Enright. Dr. Daniel Venegas Pino assisted in the processing of Transwell inserts and Roniya Sebastian assisted in the analysis of fluorescence microscopy images.

1.0 INTRODUCTION

1.1 Cardiovascular Disease and Atherosclerosis

Cardiovascular disease (CVD) is the world's leading cause of death[1]. In 2020, the WHO reported that 27% of deaths occurred as a result of complications of CVD[1]. Atherosclerosis, a disease of progressive arterial inflammation at the vascular intima[2], is the underlying cause of most CVD[3]. Atherosclerosis is characterized by arterial hardening, narrowing, and a resultant reduction in blood flow. It primarily occurs in areas where blood flow is turbulent, such as areas with branches or inner curvatures[4]. Atherosclerotic CVD can be subclassified into two major diseases: ischemic heart disease (IHD) and cerebrovascular disease. IHD describes the buildup of plaque in, and the narrowing of, arteries that supply blood to the heart[5]. Reductions in blood flow to the heart result in insufficient oxygen and nutrient delivery to cardiomyocytes and resultant myocardial infarction[4,5]. Cerebrovascular disease involves a buildup of plaque and narrowing of the arteries that supply blood to the brain, resulting in insufficient oxygen and nutrient delivery to the brain, cumulating in a stroke[6].

1.1.1 Atherogenesis

Atherogenesis occurs through a complex lipid-driven mechanism involving an initial endothelial injury and a local inflammatory event, followed by lesion initiation and development, plaque formation, arterial narrowing and reduction of blood flow, and potential plaque rupture[7] (**Figure 1**).

Insults to endothelial cells of the vascular intima, including physical stress, chemical irritants, and elevated circulating low-density lipoprotein (LDL)-cholesterol lead to endothelial dysfunction, injury, and the formation of an initial lesion[7]. Activation of resident cells, including

intimal endothelial cells and medial smooth muscle cells (SMCs) induces a local inflammatory response within the lesion[8]. High circulating levels of LDL cholesterol lead to the sequestration and accumulation of LDL in the arterial intima[8], wherein subendothelial LDL particles are modified into pro-inflammatory molecules (modified LDL) through processes such as oxidation by reactive oxygen species[9]. Modified LDL particles exacerbate the immune response by promoting the expression of adhesion molecules by the endothelium[10]. Adhesion molecule presentation leads to the recruitment of circulating monocytes and lymphocytes to the activated endothelium[8, 10]. Monocytes infiltrate the vessel wall, and micro-environmental stimuli induce monocyte differentiation into macrophages in the sub-endothelium[11,12]. Lesional macrophages scavenge and uptake the accumulating modified LDL and cellular debris, therein leading to foam cell formation[13,14]. In early atherosclerosis, a fatty streak forms in the arterial wall and consists of these foam cells. Macrophage-derived foam cells secrete pro-inflammatory cytokines and growth factors, such as interferon gamma (IFNγ), interleukin-1 (IL-1), and tumour necrosis factor alpha (TNF-α), that lead to continued inflammation[15,16]. In later stages of atherosclerosis, foam cells induce SMC migration from the tunica media to the tunica intima. Migrated SMCs contribute to the production of extracellular matrix proteins that cause fibrosis of the developing plaque. As foam cells continue to uptake modified LDL particles, intracellular accumulation of these particles leads to foam cell apoptosis[16].

A hallmark of atherosclerosis progression is foam cell apoptosis within the lesion[17]. In early atherosclerotic lesions, macrophages efficiently efferocytose apoptotic cells and prevent further lesion progression. However, in advanced atherosclerotic lesions, macrophage efferocytotic capacity becomes impaired, and a buildup of cellular debris in the lipid core of the lesion occurs[18]. This accumulation of cellular debris in the lesion forms the necrotic core.

Necrotic core formation is a key indicator of plaque instability and potential plaque rupture[18]. Further, foam cells release matrix-degrading enzymes, such as collagenases and matrix metalloproteases, that reduce fibrotic cap stability and increase rupture susceptibility[15]. Lesion rupture results in atherothrombosis, which frequently causes vessel occlusion and can result in cardiovascular complications including IHD or stroke.

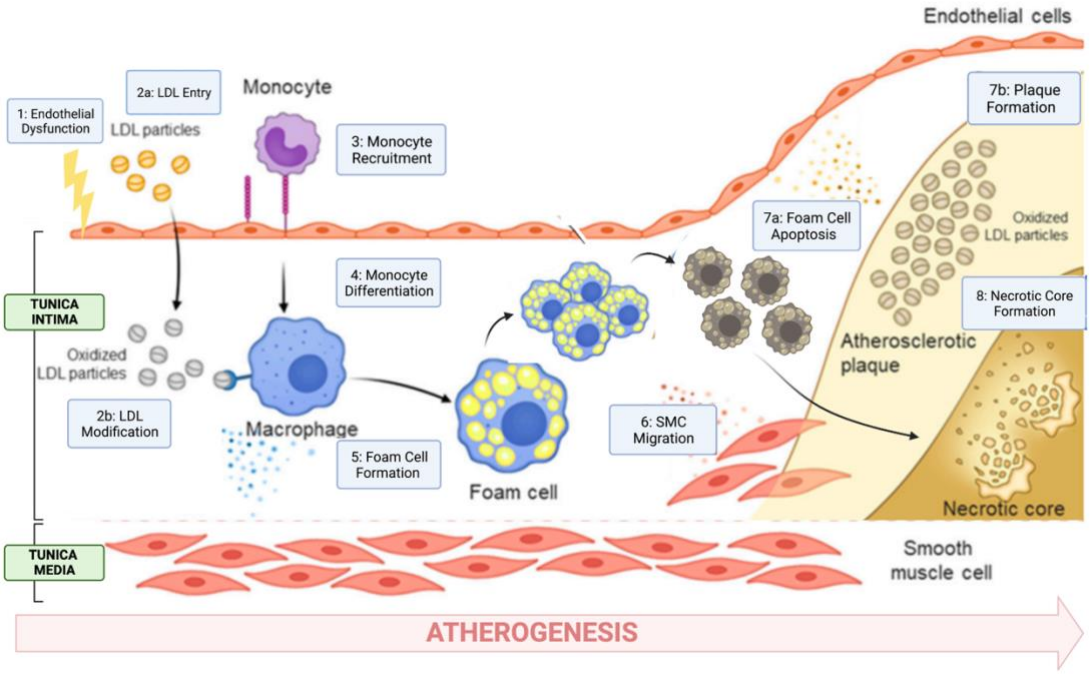


Figure 1: **Key steps in atherogenesis. 1.** Elevated concentrations of circulating LDL-cholesterolresults in initial endothelial injury, activation, and local inflammatory responses. **2a.** Circulating LDL is sequestered and accumulates in the arterial intima. **2b.** LDL particles are modified into oxidized pro-inflammatory particles (modified LDL) in the subendothelium. **3.** Modified LDL particles promote the expression of adhesion molecules by the activated endothelium. Circulating monocytes are attracted to and infiltrate the vessel wall. **4.** Microenvironmental stimuli in the subendothelium induce monocyte differentiation into macrophages. **5.** Macrophages take up accumulating modified LDL particles leading to foam cell formation. Foam cells form the fatty streak of early lesions and produce pro-inflammatory cytokines and growth factors that amplify inflammatory events in the lesion. **6.** Foam cells induce SMC migration from the tunica media into the tunica intima. SMCs produce collagen that contributes to fibrosis of the developing plaque. **7a.** Excess uptake of modified LDL particles by foam cells leads to impaired efferocytosis and eventual foam cell apoptosis. **7b.** Accumulation of cholesterol deposits furthers plaque formation. SMCs continue to produce ECM proteins that stabilize the plaque with a fibrotic cap. **8.** Accumulation of necrotic debris forms a necrotic core. **9. (Not shown)** Loss of plaque stability results in plaque rupture and thrombus. (Figure adapted from "Antibody-Based Therapeutics for Atherosclerosis and Cardiovascular Diseases", 2021[19]).

1.2 Monocyte Differentiation and Macrophage Polarization

Blood cells are produced from hematopoietic stem cells (HSCs) in a process known as hematopoiesis[20]. Briefly, in the bone marrow, HSCs differentiate into common myeloid progenitor cells, which further differentiate into myoblasts[21]. Myoblasts are precursors of

basophils, neutrophils, eosinophils, and monocytes. Emerging evidence suggests an important role for neutrophils in atherosclerosis, including involvement in initial endothelial activation, crosstalk with and recruitment of monocytes, and regulating inflammatory responses of macrophages[22]. Despite this interplay between neutrophils and monocytes, the primary focus of this project is on monocytes alone. Monocytes are released from the bone marrow and circulate in the blood[21]. Monocytes are attracted to regions of tissue damage where they further differentiate into macrophages.

Macrophages are a central part of the innate immune system. They are highly plastic and susceptible to influence by microenvironmental stimuli[22,23], for example, within an atherosclerotic lesion. While many macrophage phenotypes have been identified, two broad classifications exist: pro-inflammatory macrophages (also known as M1 macrophages) and anti-inflammatory macrophages (also known as M2 macrophages)[17] (**Figure 2**).

1.2.1 Pro-Inflammatory Macrophages

Macrophages can be polarized into pro-inflammatory macrophages by exposure to lipopolysaccharide (LPS) alone or in conjunction with T-helper type 1 (Th-1) cytokines, IFNγ or TNF-α[17,24] (**Figure 2**). The use of LPS as an inducer of pro-inflammatory macrophage polarization is often considered a synthetic mechanism. However, increasing evidence of neutrophil involvement in atherogenesis may explain infection-induced endothelial injury in human atherogenesis, which could justify the use of LPS for induction of pro-inflammatory macrophage polarization *in vitro*[22]. Pro-inflammatory macrophages may also be produced through exposure to and uptake of cholesterol crystals or oxidized LDL[25]. In the atherosclerotic lesion, pro-inflammatory macrophages maintain inflammatory responses through the production of pro-inflammatory cytokines including TNF-α, IL-6, and IL-1β[26]. Pro-inflammatory

macrophages are also characterized by their production of nitric oxide (NO) through activation of inducible nitric oxide synthase (iNOS) and reactive oxygen intermediates[12,27,28]. While short-term activation of pro-inflammatory macrophages is beneficial, long-term activity of these macrophages may disrupt tissue repair and may contribute to further tissue damage[29].

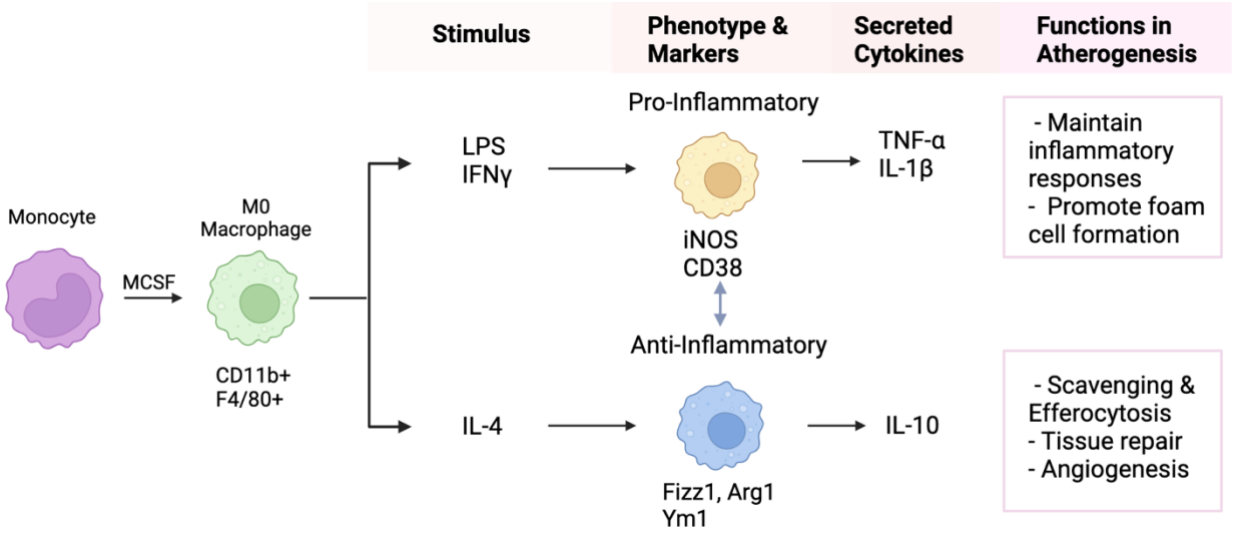


Figure 2: Monocyte differentiation and macrophage polarization. In areas of tissue damage, monocytes are recruited and differentiate into macrophages. M0 macrophages may be identified experimentally through their expression of the surface marker cluster of differentiation molecule 11B (CD11b), and the murine-specific EDF-like module-containing mucin-like hormone receptor-like 1 (F4/80). M0 macrophages may polarize into pro-inflammatory macrophages after exposure to lipopolysaccharide (LPS) and interferon-γ (IFNγ). Pro-inflammatory macrophages may be identified through the upregulated expression of inducible nitric oxide synthase (iNOS) or CD38. Pro-inflammatory macrophages produce high levels of tumor necrosis factor alpha (TNF-α), and interleukin-1β (IL-1β) in order to maintain of inflammatory responses. Pro-inflammatory macrophages also uptake oxidized LDL particles to form foam cells. M0 macrophages polarize to anti-inflammatory macrophages following exposure to IL-4. Anti-inflammatory macrophages may be identified through their expression of found in inflammatory zone 1 (Fizz1), arginase 1 (Arg1), chitinase-3-like protein 3 (Ym1). They also produce high levels of and IL-10. Primary functions of anti-inflammatory macrophages include efferocytosis and debris scavenging, tissue repair, and angiogenesis. *(Created with BioRender).*

1.2.2 Anti-Inflammatory Macrophages

Anti-inflammatory macrophages are produced following exposure of macrophages to T-helper type 2 (Th-2) cytokines including IL-4 and IL-13[30] (**Figure 2**). Their primary functions

include cholesterol efflux and scavenging of debris[31], tissue repair[17], and angiogenesis[32]. Anti-inflammatory macrophages are characterized by their production of anti-inflammatory cytokines including IL-10 and transforming growth factor-β (TGF-β)[12,30]. Principal markers of anti-inflammatory macrophages include found in inflammatory zone 1 (Fizz1), arginase 1 (Arg1), chitinase-3-like protein 3 (Ym1), and mannose receptor (MR, CD206)[17]. These macrophages are believed to counteract the actions of pro-inflammatory macrophages spatially and temporally[29]. Anti-inflammatory macrophages may also be referred to as M2 macrophages, and multiple distinct subtypes of M2 macrophages have been defined[17].

Finally, additional macrophage phenotypes include M4, MOX, hemorrhage-associated macrophage (HA-mac), and hemoglobin-associated macrophage (Mhem)[17]. While the function and significance of these macrophage phenotypes are actively being investigated elsewhere, they are currently poorly understood. Regardless, the precise classification of macrophages is arbitrary, as all phenotypes exist on a spectrum of pro-inflammatory to anti-inflammatory function.

1.2.3 Significance of Phenotypic Distribution in Atherosclerotic Lesions

While disease outcome and plaque stability were once predicted using the absolute number of macrophages present, it is now more evident that the relative phenotypic abundance is a better predictor of plaque stability[29].

A variety of macrophage phenotypes can exist throughout human atherosclerotic lesions (**Figure 3**). Generally, pro-inflammatory macrophages are the dominant phenotype in the lipid core and the plaque shoulder, which is rupture-prone[33,34]. Anti-inflammatory macrophages are more abundant in the adventitia and neo-vascular areas. Interestingly, the distribution of macrophage phenotype differs with plaque severity [34]. An increasing number of clinical studies

demonstrate that pro-inflammatory macrophages are more prevalent in symptomatic patients and severe lesions, whereas anti-inflammatory macrophages are found in greater abundance in asymptomatic patients or early lesions[35,36]. This supports the suggestion that anti-inflammatory macrophages possess anti-atherogenic properties, and conversely, pro-inflammatory macrophages are pro-atherogenic[29].

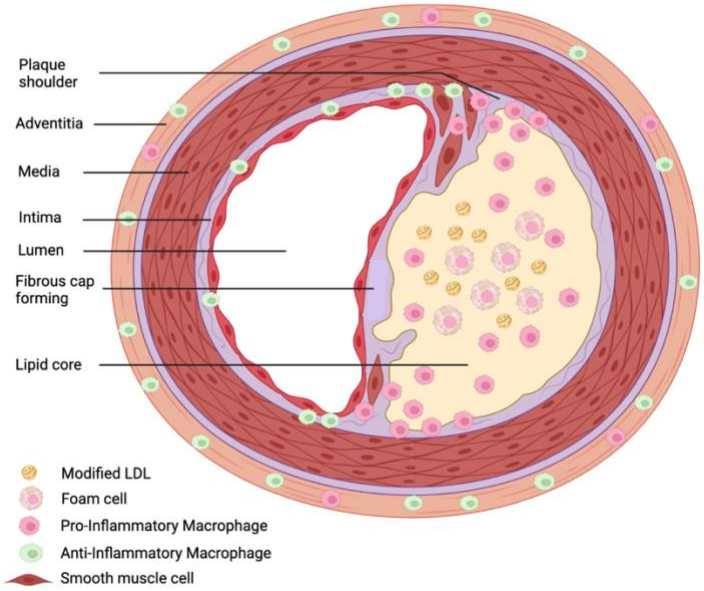


Figure 3: Distribution of macrophage phenotypes in the atherosclerotic lesion. Pro-inflammatory and anti-inflammatory macrophages localize to distinct areas of the atherosclerotic lesion that correspond to their functions in atherogenesis. Pro-inflammatory macrophages are most abundant in the lipid core and the plaque shoulder of moderate to severe atherosclerotic plaques. They transform into foam cells following uptake of modified LDL. Pro-inflammatory macrophages may also be found in other areas of the vessel, including the adventitia. Anti-inflammatory macrophages are present in all stages of plaque development, but are most abundant in early, stable plaques, or in models of plaque regression. These macrophages are localized primarily to the adventitia and areas of neovascularization. *(Created with BioRender).*

Furthermore, these observations extend to experimental models of atherosclerosis. In apolipoprotein E knockout ($ApoE^{-/-}$) mice, anti-inflammatory macrophages were abundant in early atherosclerotic lesions, however, in advanced and unstable plaques, pro-inflammatory macrophages predominated[37,38]. Interestingly, the abundance of pro-inflammatory macrophages was reduced in plaque regression, while the abundance of anti-inflammatory macrophages

increased. Signal transducer and activator of transcription (STAT) 3/6 upregulation in ApoE$^{-/-}$ mice, leading to anti-inflammatory macrophage polarization, resulted in plaque stabilization and regression[39]. Taken together, evidence suggests a role for macrophage polarization in predicting plaque severity and disease outcome. Therefore, targeting macrophage polarization may provide a novel therapeutic strategy to improve plaque stability and slow atherogenesis.

1.2.4 Macrophage Functions in Atherosclerotic Plaque Development

Several macrophage functions, including inflammatory response, migration, and lipid accumulation, affect the production and resolution of atherosclerotic plaques. As mentioned, atherosclerosis is a condition characterized by inflammation in the arterial wall. In particular, the pro-inflammatory cytokine IL-1β has been identified as both a marker and mediator of inflammation in atherosclerosis, imposing several actions upon many cell types in a plaque[40]. Notably, IL-1β inhibition resulted in a significant reduction of CVD events in humans[41]. Therefore, IL-1β is a marker often used to evaluate severity of inflammation in atherosclerotic lesions and can be used as an indicator of inflammatory properties in macrophages[40].

Macrophage migration may be considered both atherogenic and atheroprotective. Increased migratory function in pro-inflammatory macrophages leads to increased cholesterol uptake, foam cell formation, and plaque instability[42]. Alternatively, migration by anti-inflammatory macrophages promotes cholesterol efflux, plaque stability, and plaque resolution[42]. Therefore, it is important to analyze macrophage migration by phenotype. Similarly, lipid accumulation by macrophages can be beneficial or harmful in atherosclerosis. Cholesterol uptake by pro-inflammatory macrophages promotes plaque development and foam cell apoptosis whereas uptake by anti-inflammatory macrophages promotes cholesterol efflux and plaque

resolution[42]. Overall, macrophage functions such inflammatory response, migration, and lipid accumulation may be targets for mediating atherogenesis.

1.3 Sex Differences in Atherosclerosis and their Clinical Significance

Differences in CVD onset, presentation, and severity exist between men and women[43]. Premenopausal women have a much lower prevalence of obstructive IHD and plaque rupture than similarly aged men[44]. Several clinical studies have demonstrated that women with IHD are older and present with more CVD risk factors than men[43–45]. Furthermore, while men had an incidence and related mortality of IHD that is 2-fold greater than women, the disparity narrowed with age[46]. Regardless, CVD risk increases with age in both men and women[47], which coincides with the reduction, or loss, of reproductive hormone production[48]. Observed differences in CVD presentation, risk, and severity with age and between sexes may be attributable, at least in part, to sex hormones[49], including estrogen, progesterone, and testosterone.

1.3.1 Estrogen

Estrogens, a group of cholesterol-derived gonadocorticoids, are the primary female sex hormones[50] (**Figure 4**). However, they are not exclusively female sex hormones and are present in males, generally in lower concentrations. Three forms of estrogens exist: 17β-estradiol (E2), estrone (E1), and estriol (E3). E2 is produced in the ovaries and is the most abundant circulating estrogen[50]. Due to its high affinity for estrogen receptors compared with other estrogens, E2 is also the most potent estrogen[49]. Post-menopausal E2 levels drop drastically due to the cessation of its production by the ovaries[51]. In men, E2 may also be produced through the conversion of testosterone by the enzyme aromatase, present in the bone, brain, adipose tissue, and gonad[52]. E1 is an estradiol metabolite of lower potency, produced in adipose tissue through conversion from androstenedione[49]. Because androstenedione production is maintained by the adrenal cortex post-

menopause, its circulating levels are maintained relative to E2. E3 is a peripheral estradiol metabolite that is produced in abundance by the placenta during pregnancy[49]. However, its production is comparably low in non-pregnant individuals.

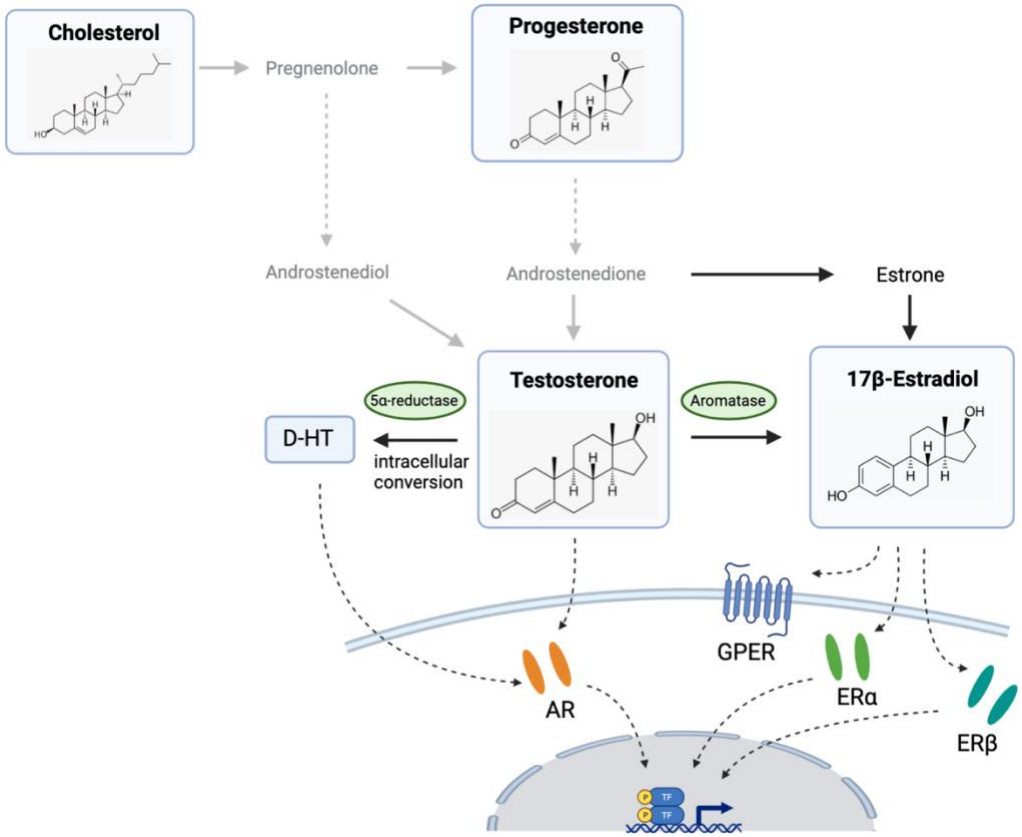


Figure 4: Biosynthesis and receptor targets of estrogen and testosterone. Cholesterol is the common precursor of all sex hormones. Cholesterol is first converted into pregnenolone before enzymatic conversion to progesterone. Progesterone is converted into testosterone through a series of enzymatic reactions involving androstenedione as an intermediate. Testosterone may also be produced from pregnenolone through a separate enzymatic pathway involving androstenediol as an intermediate. Testosterone binds the androgen receptor (AR) and induces receptor dimerization, nuclear translocation, and gene transcription. Intracellularly, testosterone can also be converted to its potent metabolite, dihydrotestosterone (D-HT), by 5α-reductase, which also acts through AR. Alternatively, testosterone can be converted to 17β-estradiol by the enzyme, aromatase. However, 17β-estradiol is generated primarily from estrone. 17β-estradiol acts through three receptors: nuclear receptors, estrogen receptor alpha (ERα) and beta (ERβ), and G protein-coupled estrogen receptor (GPER). Binding to ERα or ERβ in the cytosol induces receptor dimerization, nuclear translocation, and gene transcription. Interaction with GPER at the cell surface induces an intracellular signaling cascade. *(Created with BioRender).*

Estrogens can freely diffuse into the bloodstream[49]. However, circulating estrogens largely bind to sex hormone binding globulins (SHBGs), with a small (2%) proportion remaining free and unbound. Known estrogen-specific receptors include estrogen receptor alpha (ERα) and beta (ERβ)[49], as well as the more recently described G protein-coupled estrogen receptor (GPER)[53]. ERα and ERβ are best known as classical steroidal receptors that exist unbound in the cell cytosol. Unbound estrogens may diffuse through the plasma membranes of cells in target tissues[53]. Within the cell, estrogens bind intracellular estrogen receptors to cause receptor dimerization and nuclear translocation. In the nucleus, ligand-receptor complexes associate with estrogen response elements in DNA and other transcription factors in order to initiate gene transcription and alter physiologic activity[53]. Both receptors are widely expressed and in a variety of cell types. Recent advances suggest that in addition to their known genomic effects, ERs may also exhibit non-genomic effects through association with proteins at the cell membrane resulting in the initiation of intracellular signalling cascades[53]. However, these findings remain subject to controversy.

GPER is a ubiquitously expressed G protein-coupled receptor[54] that, when bound by estrogens, initiates intracellular signaling cascades such as the MAPK/ERK pathway[55]. Controversy exists over GPER localization and signaling cascades. It has been suggested that GPER could be present at the intracellular membrane[56] and within the nucleus[57].

Cardiovascular protection provided by endogenous estrogens is widely accepted. Observed cardiovascular protection in premenopausal women and subsequent increased CVD risk with age coincides with the loss of reproductive hormone production that occurs during menopause[48,58]. In both human and animal studies, estrogens have demonstrated the ability to slow the development of atherosclerosis[58,59]. Furthermore, the post-menopausal loss of estrogens

combined with increases in sex-specific risk factors contribute to increases in CVD incidence in women following menopause[60,61]. However, in men with prostate cancer, E2 treatment is associated with increased cardiovascular morbidity and mortality[62]. Also, in middle-aged male subjects without cardiovascular disease, circulating E2 levels were associated with increases in carotid artery intima-media thickness[63]. Overall, while heavily studied, the actions of E2 in men and women are complex and differential, and the role(s) of E2 in CVD remain debated and poorly understood.

1.3.2 Testosterone

Testosterone (TE) is the primary sex hormone in the male body but is not exclusively a male sex hormone (**Figure 4**). As a steroid hormone, it is produced from a cholesterol precursor, and its production occurs in the greatest quantities in the Leydig cells of the testes, and in smaller proportions in the ovaries of women and the adrenal cortex of both men and women[64]. TE may be metabolized by 5α-reductase into dihydrotestosterone (D-HT), a more potent TE metabolite with a higher binding capacity to target receptors. Conversely, TE may also be converted to E2 by aromatase[64]. In circulation, approximately 60% of TE is bound by SHBG, and to a lesser extent albumin[64]. Only about 1-2% of TE remains unbound and free in circulation.

TE exerts its effects primarily by binding the androgen receptor (AR), a ligand-dependent nuclear receptor that exists in most organs and cell types[64]. Similar to ERs, a conformational change occurs upon ligand binding to the AR, leading to nuclear translocation, association with androgen response promotor elements (AREs), and subsequent modulation of gene transcription. TE may also exhibit non-DNA binding-dependent mechanisms which may activate second messenger signaling cascades involving MAPK, Akt, ERK. However, this mechanism is not well described[64].

Associations between TE and CVD risk are unclear. The involvement of TE in CVD risk protection has been suggested, however, these data are often conflicting[64]. An inverse relationship between endogenous circulating TE levels and cardiovascular mortality in men has been demonstrated in several prospective cohort studies[65,66]. In contrast, other clinical studies have not observed statistically significant associations between these variables[64]. Furthermore, in a cohort study of hormone associations in postmenopausal women, age-adjusted coronary heart disease risks were significantly higher in women with low endogenous bioavailable TE[67]. Overall, evidence suggesting a role for TE in CVD risk is heavily debated, and possible underlying mechanisms by which protection may be conferred are unclear.

Overall, sex hormones, including E2 and TE, may differentially impact CVD risk for both men and women. Further clinical (and pre-clinical) research is required to draw concrete conclusions on this matter.

1.3.3 Preclinical Observations of Sex Differences in Atherosclerosis

Preclinical studies have explored the implications of sex hormones, especially E2 and to a lesser extent TE, on CVD risk. E2 improves vascular function and protects against atherosclerosis in mice[68]. The effects of E2 on the endothelium are relatively well established; in instances of vascular injury, E2 promoted re-endothelialization[69], inhibited smooth muscle cell proliferation[70], and attenuated plaque progression[71]. E2 also attenuated endothelial activation following endothelial injury by inhibiting or interfering with mechanisms that attract circulating inflammatory molecules to the endothelium[72]. Furthermore, in the mesenteric arteries of mice, E2 deprivation resulted in systemic inflammation, which was attenuated by E2 supplementation[73]. Similarly, an anti-inflammatory effect was observed following acute E2 treatment in RAW 264.7 macrophages and primary cultures of microglia from mice *in*

vitro[74]. However, a contrasting effect was observed in macrophages isolated from chronically E2-treated mice. Chronic E2 supplementation of ovariectomized mice resulted in an increase in pro-inflammatory mediator production by peritoneal macrophages[75]. This discrepancy highlights potential differences between effects of short-term and long-term E2 supplementation, as well as a discordance in effects produced in *in vitro* and *in vivo* models[76]. Overall, while the anti-atherogenic effects of E2 supplementation have been described, some discrepancies in the literature suggest that the role of E2 in atheroprotection may be more complex than originally believed.

Experimental studies have also suggested a protective role of TE and AR against cardiovascular disease. TE deficiency through castration increased atherogenesis, an effect which TE supplementation abolished[77]. Further, in male mice, AR deficiency resulted in an increased atherosclerosis burden[77]. While TE has been demonstrated to act through AR, it may also exert its atheroprotective effects after its conversion to E2 by aromatase. In male mice, aromatase inhibition blocked TE's anti-atherogenic effects[78]. This effect might be mediated through modulation of vascular tone because aromatase knockout in mice also resulted in irregular vascular relaxation[79]. Overall, while a relatively new field of study, experimental research suggests a protective role of TE in atherosclerosis.

The Werstuck lab recently developed and characterized a new murine model of hyperglycemia-induced accelerated atherosclerosis: the $ApoE^{-/-}$:$Ins2^{+/Akita}$ mouse[80]. This model is produced through a point mutation in one allele of the insulin 2 gene, which prevents proinsulin processing. Accumulation of proinsulin in the endoplasmic reticulum (ER) and Golgi results in ER stress, eventual β-cell dysfunction, and death. Of note, differences were observed in survival outcomes between male and female $ApoE^{-/-}$:$Ins2^{+/Akita}$ mice fed a Western diet[81]. Male $ApoE^{-/-}$

:Ins2$^{+/Akita}$ mice fed a Western diet did not survive past 25 weeks after suffering from cardiovascular events such as myocardial infarction (**Figure 5 A**). However, female ApoE$^{-/-}$:Ins2$^{+/Akita}$ mice, regardless of diet, did not experience these events and lived a normal lifespan. This finding drew parallels with observed differences in disease progression in men and pre-menopausal women. Furthermore, the results of castration and ovariectomy in this model differed[80]. In male mice, the effects of castration seemed to depend on glycemic status. Castration accelerated atherosclerosis in normoglycemic ApoE$^{-/-}$ mice, but it conversely slowed lesion development in ApoE$^{-/-}$:Ins2$^{+/Akita}$ (hyperglycemic) mice (**Figure 5 B**). In female mice, ovariectomized hyperglycemic females demonstrated the most severe atherosclerotic lesions compared with sham and normoglycemic controls[80] (**Figure 5 C**). Interestingly, our laboratory demonstrated that E2 supplementation in ovariectomized ApoE$^{-/-}$:Ins2$^{+/Akita}$ females reduced plaque severity and restored cardiovascular protection[82] (**Figure 5 D, E**). These observations further suggest differential effects of sex hormones on atherogenesis that extend beyond glucose control.

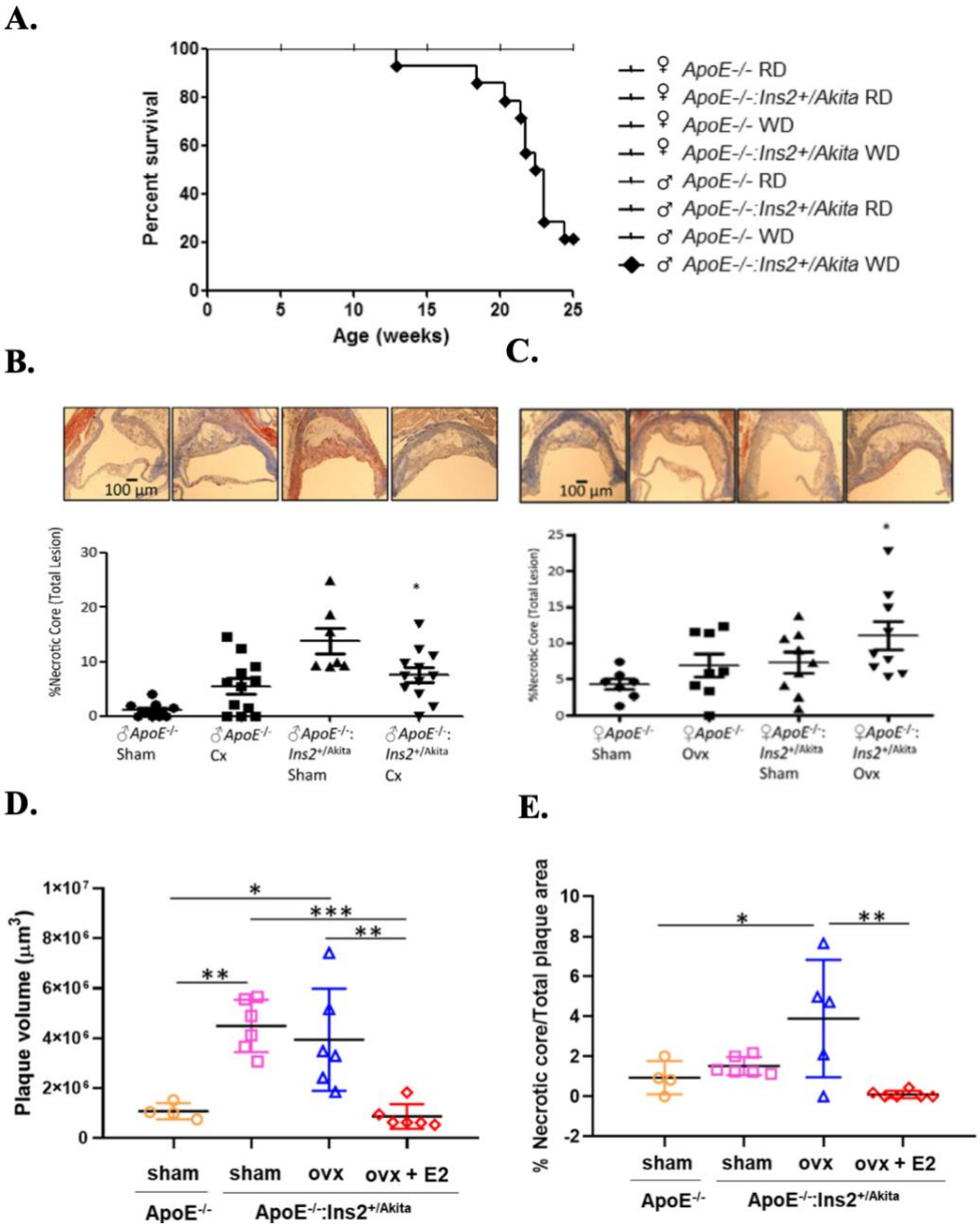


Figure 5: Observations of sex-based differences in the lifespan and progression of atherosclerosis in ApoE$^{-/-}$: Ins2$^{+/Akita}$ mice. A. Survival curves of male and female ApoE$^{-/-}$ and ApoE$^{-/-}$: Ins2$^{+/Akita}$ mice fed a regular chow diet or a western diet from 5 to 25 weeks of age or until endpoint was observed[81]. Necrotic cores in atherosclerotic lesions at the aortic sinus were quantified in 25-week-old **B.** female ovariectomized (Ovx) or sham-operated ApoE$^{-/-}$ and ApoE$^{-/-}$: Ins2$^{+/Akita}$ mice, and **C.** male castrated (Cx) or sham-operated ApoE$^{-/-}$ and ApoE$^{-/-}$: Ins2$^{+/Akita}$ mice[80]. Images represent cross-sections of the aortic sinus. Female ApoE$^{-/-}$ and ApoE$^{-/-}$: Ins2$^{+/Akita}$ mice were ovariectomized and supplemented with 17β-estradiol, and **D.** total plaque volume and **E.** necrotic core area at the aortic sinus were quantified[82].

Owing to increasing evidence for sex hormone involvement in atherosclerosis and accumulating observations that connect macrophage phenotype distribution to atherosclerosis severity, we suggest that sex hormones directly influence macrophage polarization and pro-atherogenic functions. In preclinical *in vitro* studies of macrophage polarization in murine bone marrow-derived macrophages (BMDMs) and THP-1 macrophages, E2 promoted polarization to the anti-inflammatory phenotype and dampened polarization to the pro-inflammatory phenotype[83–86]. Likewise, a smaller body of evidence supports a role for TE in the promotion of the anti-inflammatory phenotype[87,88]. However, differences in experimental protocols, including variability in the type and dosage of polarization agents and sex hormone treatments, as well as the number of sample collection times employed, impede our ability to compare observations from separate studies. Furthermore, very few studies have considered the effects of both E2 and TE on macrophage polarization and functions in the same experimental protocol. Most notably, to our knowledge, no study has connected these observations to the pro-atherogenic functions of macrophages.

2. RESEARCH HYPOTHESIS

We believe sex hormones, including E2 and TE, regulate the progression of atherosclerosis through interactions with macrophages. **We hypothesize that sex hormones directly alter BMDM polarization and pro-atherogenic function.** This project will be the first to directly compare the role(s) of both E2 and TE on BMDM polarization and pro-atherogenic functions in one complete study using one standardized protocol. It will also acknowledge biological sex as an independent factor to be considered in this context. The goal of this research is to further justify and inform efforts to develop new, and more effective, sex-specific strategies to treat patients with CVD.

2.1 Research Objectives

The objectives of this project are:

1) **To characterize macrophage polarization *in vitro* in murine male- and female-derived BMDMs**.

   A comprehensive profile of macrophage polarization that includes expression of phenotypic markers and inflammatory cytokines at more than two time points has not been established in BMDMS. Doing so will provide a reliable baseline useful in further analyses throughout this project and for studies performed elsewhere. This objective also includes an analysis of sex differences in macrophage marker and hormone receptor expression levels.

2) **To establish the effects of sex hormone supplementation on BMDM polarization.**

   The effects of E2 and TE on macrophage polarization have not been investigated together in the context of atherosclerosis. Results from this study will help explain sex

differences in atherogenesis that may reflect observations made in humans or experimental models of atherosclerosis.

3) **To identify sex differences and the effects of sex hormone supplementation on pro-atherogenic functions of BMDMs.**

Our laboratory has previously employed *in vitro* assay systems to identify changes in pro-atherogenic functions of BMDMs, including inflammatory responses, migration, and lipid accumulation[89]. Following this characterization system, identifying changes to pro-atherogenic macrophage function due to sex hormone supplementation will provide greater insights into the roles that sex hormones play in atherogenesis.

3. METHODS

3.1 Bone Marrow-Derived Macrophage Isolation and Polarization

C57BL/6 mice were obtained from Jackson Labs. Mice were provided with unlimited food and water, and were housed in a 12-hour light/dark cycle. All use of mice was pre-approved by the McMaster University Animal Research Ethics Board and followed guidelines regulated by the Canadian Council of Animal Care.

The following bone marrow isolation protocol was carried out as previously described[89]. At ages 8-10 weeks, tibias and femurs were harvested. Bone marrow was collected and passed through a 70 μM nylon filter. Isolated bone marrow was resuspended in phenol red-free Dulbecco's Modified Eagle Medium (DMEM) containing 20% charcoal-stripped fetal bovine serum (CS-FBS), 100 IU/mL penicillin, 100 μg/mL streptomycin, and 1X MEM non-essential amino acids (DMEM plating medium) (**Figure 6)**. Cells were seeded onto 10 cm non-treated Petri dishes at a seeding density of 5×10^6 cells/plate in 10 mL media. To initiate differentiation to M0 macrophages, 20 ng/mL of recombinant macrophage colony-stimulating factor (M-CSF) was applied to each dish[89]. Following 3 days of incubation, 5 mL fresh medium and 20 ng/mL M-CSF were added to each plate. On day 6, cells were washed twice with warm, sterile PBS without calcium or magnesium. Cells were lifted using Accutase®, centrifuged at 200 x g for 5 minutes, and replated in 12-well tissue culture dishes at a density of 4×10^5 cells/well in 1 mL DMEM plating medium. After 24 hours rest, subsets of cells were treated with 100 ng/mL LPS and 20 ng/mL IFNγ to polarize to the pro-inflammatory phenotype[89,90], 10 ng/mL IL-4 to polarize to the anti-inflammatory phenotype[39,89] or left unstimulated in fresh DMEM plating medium with 1X PBS. Sample collection occurred after 6, 24, and 48 hours of incubation.

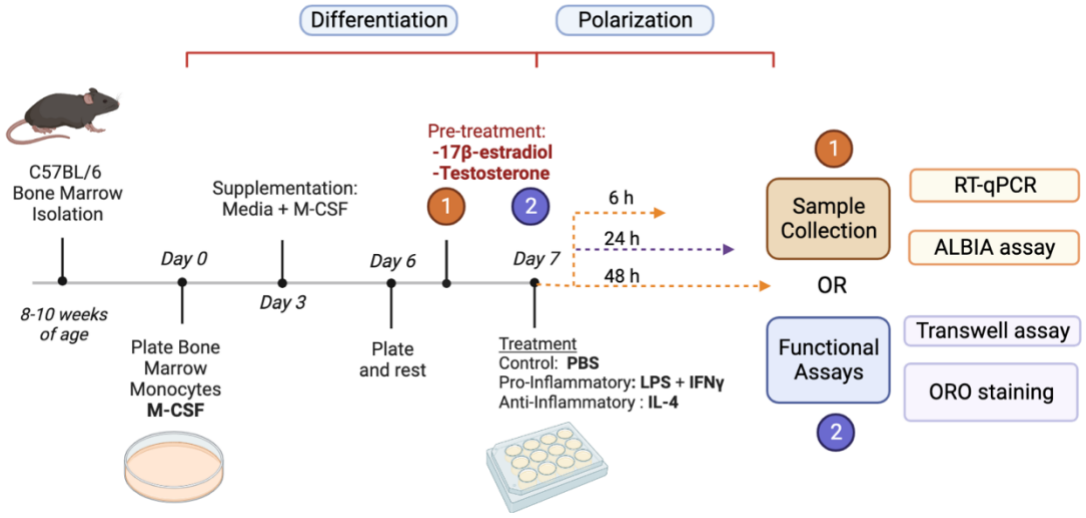


Figure 6: Experimental design for BMDM differentiation, polarization, experimentation, and analysis. Bone marrow monocytes were isolated from male and female C57BL/6 mice at 8 to 10 weeks of age. Cells were treated with macrophage-colony stimulating factor (M-CSF) to induce differentiation to M0 macrophages. On day three, cells were supplemented with additional media and MCSF. 1) On day 6, cells were re-plated and cultured for two hours prior to treatment with testosterone, 17β-estradiol, or vehicle overnight. On day 7, cells were treated with PBS vehicle control, lipopolysaccharide (LPS) and interferon-gamma (IFNγ) to induce polarization to the pro-inflammatory phenotype, or interleukin-4 (IL-4) to induce polarization to the anti-inflammatory phenotype. Cells and culture media were collected 6, 24, or 48 hours following treatment. Cytokines and chemokines in cell culture media were analyzed using an addressable laser bead immunoassay (ALBIA) assay. Gene expression of macrophage markers was quantified by RT-qPCR. 2) On day 7, cells were supplemented with hormone or vehicle treatments and polarized using the described agents for 24 hours. Cells were collected and functional assays, including an ALBIA, a Transwell assay and lipid accumulation assay, were performed. *(Created with BioRender).*

3.2 Hormone Pre-Treatment

Bone marrow-derived monocytes were differentiated into macrophages using M-CSF as described. Two hours following cell replating onto 12-well culture dishes, cells were supplemented with 100 nM E2[83], TE[91,92] or PBS as vehicle control, and incubated for 24 hours. Cells were polarized into their respective phenotypes using the polarization protocol as described, with or without the presence of hormone treatments depending on the assay performed. Concentrations of hormone pre-treatments were selected primarily by reviewing

existing studies that applied hormone treatments to macrophages *in vitro*. Due to a wide range of E2 treatment concentrations employed in the literature, a dose-response experiment was carried out using E2 at concentrations of 100 nM, 200 nM, and 400 nM, and macrophage marker gene expression as the measured response. Changes in macrophage marker gene expression appeared to plateau following 100 nM E2 (data not shown) thus this treatment concentration was selected.

3.3 Analysis of Gene Expression

Bone marrow-derived macrophages were differentiated, pre-treated with E2 or TE, and polarized in 12-well tissue culture dishes at 4 x 10^5 cells/well in 1 mL DMEM plating medium. Following polarization, TRIzol® Reagent was applied to adhered macrophages and total RNA was collected using the TRIzol® Reagent manufacturer's protocol. Isolated RNA was resuspended in RNase-free water and quantified using a Nanodrop. The purity and quantity of RNA were determined using A260/A280 and A260/A230 ratios produced by the Nanodrop. cDNA was produced from 1 μg of total RNA that was reverse transcribed using the High-Capacity cDNA Reverse Transcription kit[89]. Real-time quantitative polymerase chain reaction (qPCR) reaction mixture was created using 12.5 μL SensiFAST SYBR No-ROX Kit, 1.25 μL of forward and reverse primers (500 nM), 8 μL RNase-free water, and 2 μL of resultant cDNA. qPCR was performed and relative fold change ($2^{-\Delta\Delta Ct}$) for each target gene was calculated by normalizing data to the reference gene, β-actin. Common markers of pro-inflammatory macrophages include iNOS, TNF-α, IL-1β, and CD38[83,84,93,94] and anti-inflammatory macrophages have been characterized by the expression of Fizz1, Arg1, Ym1 and CD206[83,84,93,94]. To maintain consistency with the literature, these markers were employed in our study to characterize pro-inflammatory and anti-inflammatory macrophage phenotypes. Primers used for this reaction were specific for murine iNOS, TNF-α, IL-1β, CD38, Fizz1, Arg1, Ym1

and CD206 **(Table 1).** Sex hormone receptor expression was quantified using primers specific for ERα, ERβ, GPER1, and AR (**Table 1**).

Table 1. Primer sequences for RT-qPCR analyses.

qPCR Primers		
Gene	**Forward Primer (5' → 3')**	**Reverse Primer (5' → 3')**
β-Actin	GGC ACC ACA CCT TCT ACA ATG	GGG GTG TTG AAG GTC TCA AAC
iNOS	CAG CTG GGC TGT ACA AAC CTT	CAT TGG AAG TGA AGC GGT TCG
TNF-α	ACC ACA GTC CAT GCC ATC AC	CAC CAC CCT GTT GCT GTA GCC
Fizz1	TCC AGC TGA TGG TCC CAG TGA ATA	ACA AGC ACA CCC AGT AGC AGT CAT
Arg1	ACC TGG CCT TTG TTG ATC TCC CTA	AGA GAT GCT TCC AAC TGC CAG ACT
IL-1β	CTG CTT CCA AAC CTT TGA CC	AGC TTC TCC AGA GCC ACA AT
CD38	TTG CAA GGG TTC TTG GAA AC	CGC TGC CTC ATC TAC ACT CA
Ym1	AGA AGG GAG TTT CAA ACC	GTC TTG CTC ATG TGT AGT GA
CD206	CAG GTG TGG GCT CAG GTA GT	TGT GGT GAG CTG AAA GGT GA
AR	TTG CAA GAG AGC TGC ATC AGT T	ACT GTG TGT GGA AAT AGA TGG GC
ERα	ACC ATT GAC AAG AAC CGG AG	CCT GAA GCA CCC ATT TCA TT
ERβ	TGT GTG TGA AGG CCA TGA TT	TCT TCG AAA TCA CCC AGA CC
GPER1	TCA TTT CTG CCA TGC ACC CA	GTG GAC AGG GTG TCT GAT GT
IRF4	GAA CGA GGA GAA GAG CGT CTT C	GTA GGA GGA TCT GGC TTG TCG A
IRF5	CCT ACA GAA CCA CTC TTG CCT G	CCT TGT GGG TTG CTG ATG GTG A
STAT1	CTG AAT ATT TCC CTC CTG GG	TCC CGT ACA GAT GTC CAT GAT
STAT6	TGA GGT GGG GAC CAG CCG G	GTG ACC AGG ACA CAC AGC GG

3.4 Cytokine/Chemokine Detection

To assess pro-inflammatory responses of BMDMs, the production of pro-inflammatory cytokines and chemokines was analyzed using an addressable laser bead immunoassay (ALBIA), also referred to as a multiplexing LASER bead assay as previously described[89]. Briefly, following BMDM polarization and hormone pre-treatment, cell culture media was collected, centrifuged at 3000 x g for 5 minutes, and the pellet was discarded. The concentration of cytokines and chemokines in supernatant was detected using an ALBIA assay ([Mouse Cytokine Array / Chemokine Array 31- Plex (MD31), Eve Technologies, Calgary, AB]).

3.5 Macrophage Migration Assay

Following differentiation with M-CSF, BMDMs were seeded into 12-well non-treated tissue culture dishes and were treated with 100 nM E2, TE, or PBS vehicle control for 24 hours. Cells were then polarized using LPS and IFNγ or IL-4 in conjunction with 100 nM E2, TE, or PBS treatment.

The following cell migration protocol was carried out as previously described[89]. After 24-hour incubation, cells were re-suspended in serum-free DMEM plating medium and incubated for 1 hour. Transwell inserts (3 μm pore size, 6.5 mm diameter) were coated with rat tail collagen I (4 mg/mL) for 1 hour. Cells were detached using Accutase® and seeded into the upper chamber of the Transwell insert at a density of 0.4 x 10^5 cells/200 μL/insert in serum-free DMEM plating medium and incubated for 30 minutes at 37°C. Chemokine ligand 19 (0.5 μg/mL, CCL19) or DMEM only were dispensed into the lower chambers of the Transwell support system. Following incubation, loaded Transwell inserts were placed into the lower chambers and incubated at 37°C. After 4 hours, cells were rinsed with 1x PBS and fixed with 4% paraformaldehyde (PFA) for 15 minutes. Cells were rinsed with 1x PBS and stained with 4′,6-diamidino-2-phenylindole (DAPI, 1:5000) for 5 minutes. Following 1x PBS rinse, membranes were washed twice with 1x PBS. Non-migrated cells located on the upper surface of the membrane were carefully scraped off using a cotton swab. Migrated cells on the lower surface of the membrane were imaged and quantified using fluorescent microscope (Olympus BX41 microscope connected to a DP71 Olympus camera, with 10x objective).

For quantification, 4 images were taken of each insert representing one biological replicate, and DAPI-stained nuclei were counted using ImageJ 1.52q software. The total number of cells migrated per replicate was determined by combining the cell count from each image in a

biological replicate (ie. 4 images combined). Passive migration was accounted for by subtracting the number of cells that migrated in CCL19-free control wells from each biological replicate of the corresponding macrophage phenotype. Data were normalized to either male-derived controls or vehicle-treated controls, depending on the analysis. Data presented are the mean cell migration of three to four biological replicates represented as a percentage of the control.

3.6 Lipid Accumulation Assay

In conjunction with polarization treatment, cells seeded into 12-well non-treated tissue culture dishes were supplemented with 100 nM E2, TE, or PBS vehicle control. The following lipid accumulation assay was conducted as previously described[89]. Briefly, after a 24-hour incubation, cells were re-suspended in serum-free DMEM plating medium and incubated for 4 hours. Cells were detached using Accutase® and seeded at a density of 0.8 x 10^5 cells/ 200 μL/ well into 8-well chamber slides (ThermoFisher Scientific) in normal DMEM plating medium containing FBS. Following 24-hour incubation at 37°C, cells were washed with 1x PBS and fixed with 4% PFA for 15 minutes and washed again with 1x PBS. To prepare Oil Red O (ORO) stock solution, 0.25 g ORO powder (Sigma) was dissolved in 50 mL 100% isopropanol in a 70°C water bath for 10 minutes. While warm, stock solution was filtered. Working ORO solution was prepared by creating a 3:2 mixture of ORO stock and ddH_2O. Fixed and rinsed cells were permeabilized with 0.5% Triton X-100 for 5 minutes and stained with ORO working solution for 15 – 20 minutes. Cells were washed twice with 60% isopropanol for 15-30 seconds each and rinsed with 1x PBS twice. Cells were then stained with DAPI (1:5000) for 2-5 minutes and mounted using Fluoromount Aqueous Mounting Medium (Sigma). Mounted cells were stored in the dark at 4°C and lipid content was later visualized using a fluorescent microscope (Olympus BX41 microscope connected to a DP71 Olympus camera, 20x objective). Four images were

captured for each biological replicate and background-corrected fluorescence intensity and cell number were quantified using ImageJ 1.52q software. For each image, a fluorescence intensity per cell ratio was created. The intensity per cell ratio of four images was combined to create a mean intensity per cell value for one biological replicate. Data were normalized to the vehicle control or male BMDMs depending on the study conducted. Normalized lipid accumulation values were represented as a percentage of the control.

3.7 Statistical Analysis

Statistical analyses were performed using GraphPad Prism 7 software. Data were analyzed using two-tailed unpaired t-test or a one or two-way analysis of variance (ANOVA) test followed by Tukey's post hoc test to detect significant differences in sample means between groups. Statistical significance was determined by a p-value less than 0.05.

4. RESULTS

4.1 Sex differences and characterization of BMDM polarization

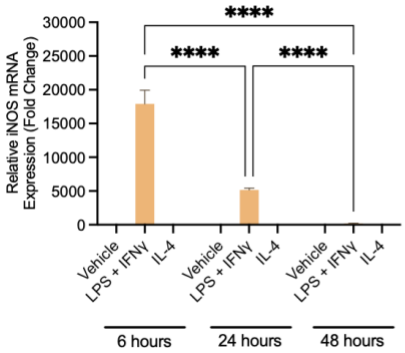
Our laboratory has previously established protocols to polarize murine BMDMs into pro-inflammatory or anti-inflammatory macrophages[89]. To create a baseline phenotypic profile over time for pro-inflammatory and anti-inflammatory BMDMs, male- and female-derived bone marrow monocytes were differentiated into BMDMs in the presence of M-CSF. Differentiated macrophages were polarized into pro-inflammatory or anti-inflammatory phenotypes by exposure to LPS (100 ng/mL) and IFNγ (20 ng/mL) or IL-4 (10 ng/mL), respectively, for 6, 24 or 48 hours. Controls (unpolarized BMDMs) were treated with vehicle alone. Gene expression of pro-inflammatory (iNOS, TNF-α, IL-1β, CD38) and anti-inflammatory (Arg1, Fizz1, Ym1, CD206) markers was quantified using RT-qPCR (**Figure 7, 8**).

When BMDMs derived from either male or female mice were treated with LPS and IFNγ, mRNA expression of iNOS, TNF-α, IL-1β, and CD38 was significantly upregulated (**Figure** 7). Expression of all pro-inflammatory markers, except for CD38 (in males only, (**Figure 7 E)**), was the greatest following 6 hours of polarization. Conversely, when BMDMs were treated with IL-4, mRNA expression of Fizz1, Arg1, Ym1, and CD206 was significantly upregulated (**Figure 8**). Expression of Fizz1 and Ym1 was the lowest following 6 hours of treatment (**Figure 8 A, B, G, H)**. However, Arg1 and CD206 expression was the greatest following 6 hours of treatment (**Figure 8 C-F**). While Arg1 and CD206 expression was also significantly upregulated after 24 and 48 hours of polarization, no significant differences in expression levels were observed between these time points in female-derived BMDMs. The data at each time point are consistent with existing data in the literature and signify that treatment with LPS+IFNγ and IL-4 was sufficient to polarize BMDMs to the pro- and anti-inflammatory phenotypes, respectively.

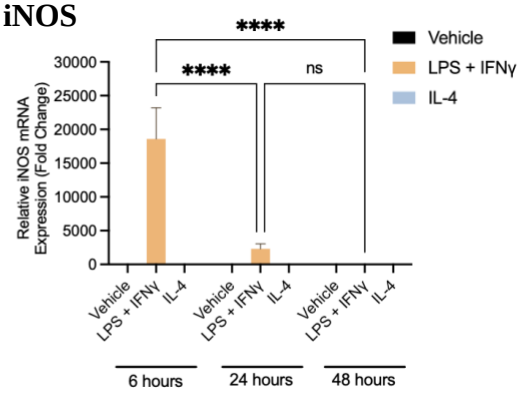
Notably, after 24 hours, pro-inflammatory markers were significantly upregulated in pro-inflammatory macrophages and anti-inflammatory markers were significantly upregulated in anti-inflammatory macrophages. Thus, polarization for 24 hours was selected as an ideal time frame to carry out further analysis.

MALES **FEMALES**

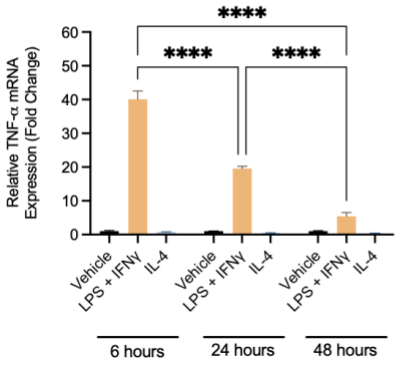
A. iNOS



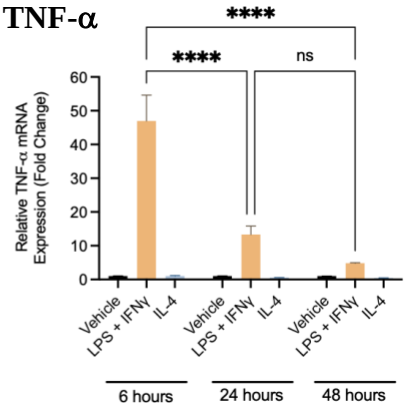
B. iNOS



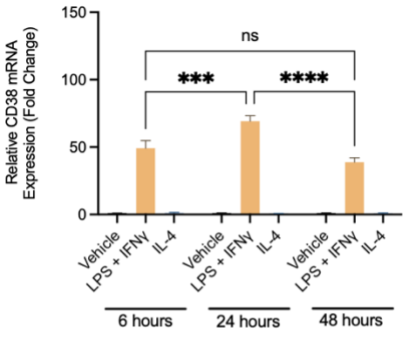
C. TNF-α



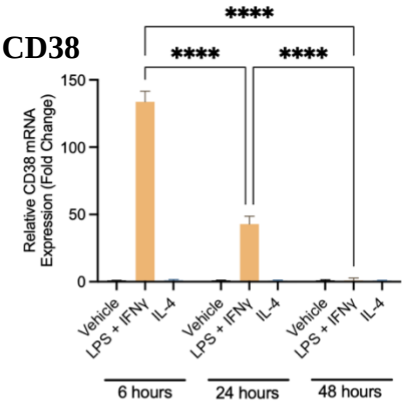
D. TNF-α



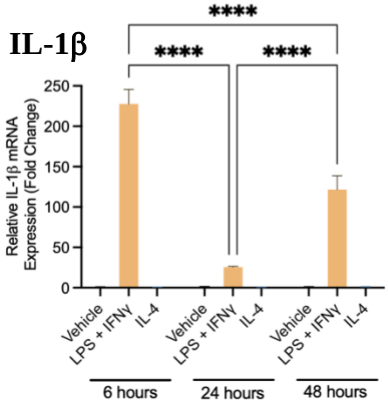
E. CD38



F. CD38



G. IL-1β



H. IL-1β

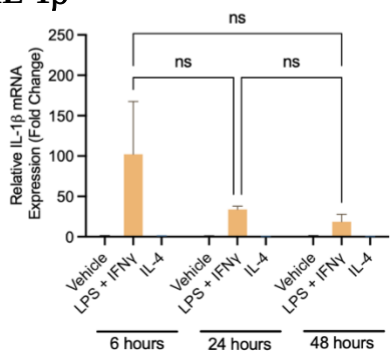
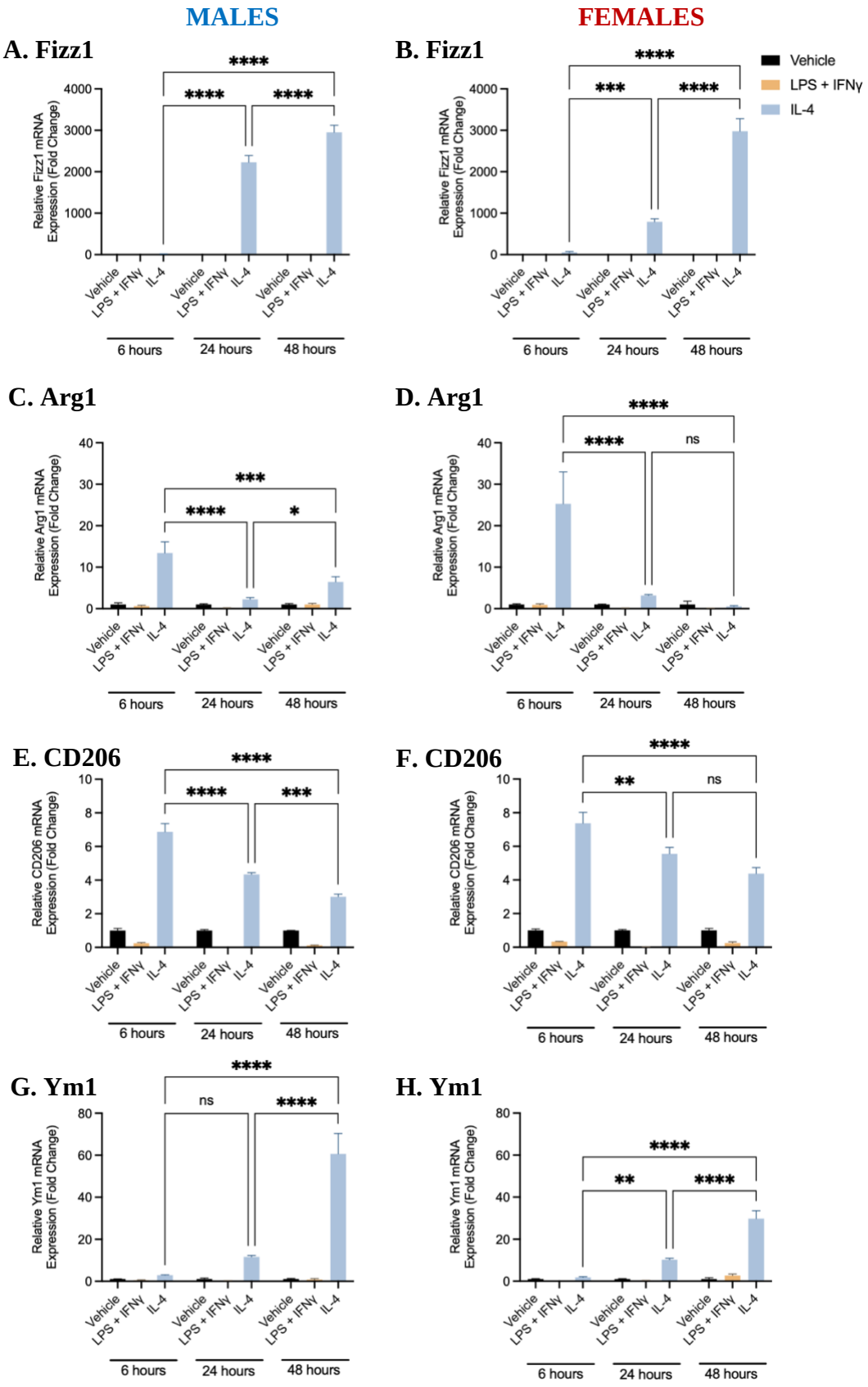


Figure 7: Gene expression of pro-inflammatory markers in male- and female-derived BMDMs. Bone marrow monocytes from male and female C57BL/6 mice were differentiated into BMDMs and treated with LPS and IFNγ, IL-4, or a vehicle control for 6, 24, or 48 hours to polarize cells to the pro- and anti-inflammatory phenotypes, respectively, or to maintain an unpolarized phenotype. Gene expression of pro-inflammatory markers was quantified in male- (**A, C, E, G)** and female- (**B, D, F, H)** derived BMDMs using RT-qPCR. Data shown represent the mean ± SEM fold change ($2^{-\Delta\Delta Ct}$) in marker expression relative to the reference gene β-actin, normalized to the vehicle control. Two-way ANOVA and Tukey's multiple comparison test were performed. (n=4, *p<0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001).


MALES
FEMALES
A. Fizz1
B. Fizz1
C. Arg1
D. Arg1
E. CD206
F. CD206
G. Ym1
H. Ym1
Vehicle
LPS + IFNγ
IL-4
Relative Fizz1 mRNA Expression (Fold Change)
Relative Arg1 mRNA Expression (Fold Change)
Relative CD206 mRNA Expression (Fold Change)
Relative Ym1 mRNA Expression (Fold Change)
6 hours
24 hours
48 hours
****
***
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*
ns

Figure 8: Gene expression of anti-inflammatory markers in male- and female-derived BMDMs. Bone marrow monocytes from male and female C57BL/6 mice were differentiated into BMDMs and treated with LPS and IFNγ, IL-4, or a vehicle control for 6, 24, or 48 hours to polarize cells to the pro- and anti-inflammatory phenotypes, respectively, or to maintain an unpolarized phenotype. Gene expression of anti-inflammatory markers was quantified in male- (**A, C, E, G)** and female- (**B, D, F, H)** derived BMDMs using RT-qPCR. Data shown represent the mean ± SEM fold change ($2^{-\Delta\Delta Ct}$) in marker expression relative to the reference gene β-actin, normalized to the vehicle control. Two-way ANOVA and Tukey's multiple comparison test were performed. (n=4, *p<0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001).

In the above experiment, differences in BMDM polarization between male- and female-derived BMDMs could not be directly assessed because the sexes were not examined concurrently. To directly investigate potential sex differences, the same protocol was carried out, using male and female mice at the same time. Differentiated macrophages derived from male or female mice were polarized to pro- or anti-inflammatory phenotypes, or left unpolarized for 24 hours, and gene expression of pro- or anti-inflammatory markers was quantified. Gene expression of TNF-α **(Figure 9 B)** and IL-1β (**Fig 9 C)** in female-derived pro-inflammatory BMDMs was significantly elevated compared to male-derived BMDMs. In addition, female-derived anti-inflammatory BMDMs demonstrated significantly upregulated gene expression of Fizz1 **(Fig 9 E)** and Ym1 **(Fig 9 H)**. These data suggest that female-derived BMDMs may respond more readily to polarization treatments or that these cells may be more susceptible to environmental stimuli. To investigate whether biological sex affects gene expression of estrogen or androgen receptors, unpolarized BMDMs were collected and gene expression of AR, ERα, ERβ, and GPER1 was assessed. Female-derived BMDMs demonstrated significantly greater expression of AR (**Figure 10 A)** compared to male BMDMs. No sex differences were observed in the expression of estrogen receptors **(Fig 10 B-D).**

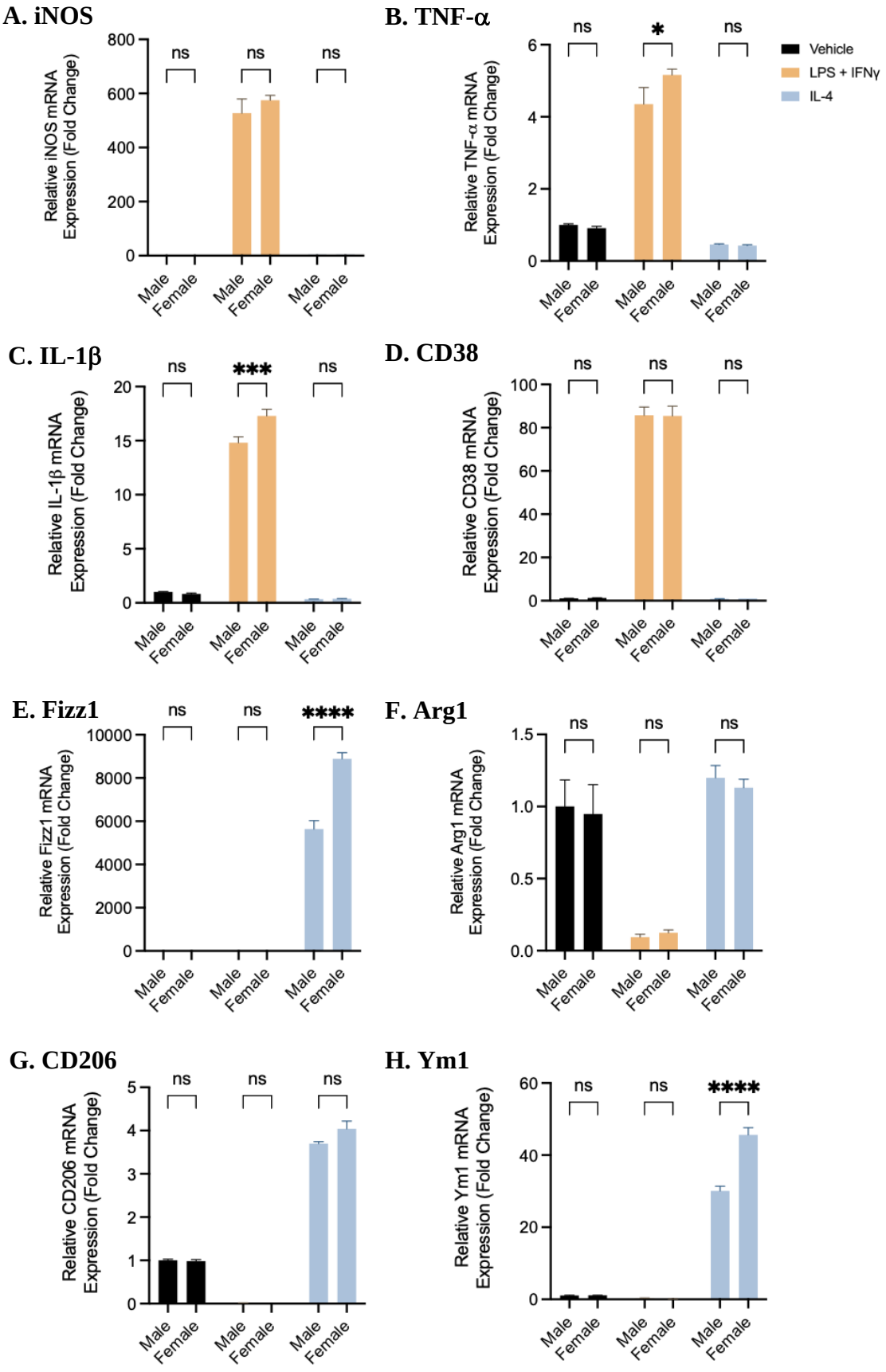

A. iNOS
Relative iNOS mRNA Expression (Fold Change)
800
600
400
200
0
ns
ns
ns
Male
Female
Male
Female
Male
Female
B. TNF-α
Relative TNF-α mRNA Expression (Fold Change)
6
4
2
0
ns
*
ns
Male
Female
Male
Female
Male
Female
Vehicle
LPS + IFNγ
IL-4
C. IL-1β
Relative IL-1β mRNA Expression (Fold Change)
20
15
10
5
0
ns
***
ns
Male
Female
Male
Female
Male
Female
D. CD38
Relative CD38 mRNA Expression (Fold Change)
100
80
60
40
20
0
ns
ns
ns
Male
Female
Male
Female
Male
Female
E. Fizz1
Relative Fizz1 mRNA Expression (Fold Change)
10000
8000
6000
4000
2000
0
ns
ns
****
Male
Female
Male
Female
Male
Female
F. Arg1
Relative Arg1 mRNA Expression (Fold Change)
1.5
1.0
0.5
0.0
ns
ns
ns
Male
Female
Male
Female
Male
Female
G. CD206
Relative CD206 mRNA Expression (Fold Change)
5
4
3
2
1
0
ns
ns
ns
Male
Female
Male
Female
Male
Female
H. Ym1
Relative Ym1 mRNA Expression (Fold Change)
60
40
20
0
ns
ns
****
Male
Female
Male
Female
Male
Female

Figure 9: Sex differences in gene expression of BMDM polarization markers. Bone marrow monocytes from male and female C57BL/6 mice were differentiated into BMDMs and treated with LPS and IFNγ, IL-4, or a vehicle control for 24 hours to polarize cells to the pro- or anti-inflammatory phenotypes, respectively, or to maintain an unpolarized phenotype. Gene expression of pro-inflammatory (**A-D)** and anti-inflammatory (**E-H)** macrophage markers was quantified in female- and male-derived BMDMs using RT-qPCR. Data shown represent the mean ± SEM fold change ($2^{-\Delta\Delta Ct}$) in marker expression relative to the reference gene β-actin, normalized to male BMDMs. Two-way ANOVA and Tukey's multiple comparison test were performed. (n=4, *p<0.05, ***p < 0.001, ****p < 0.0001).

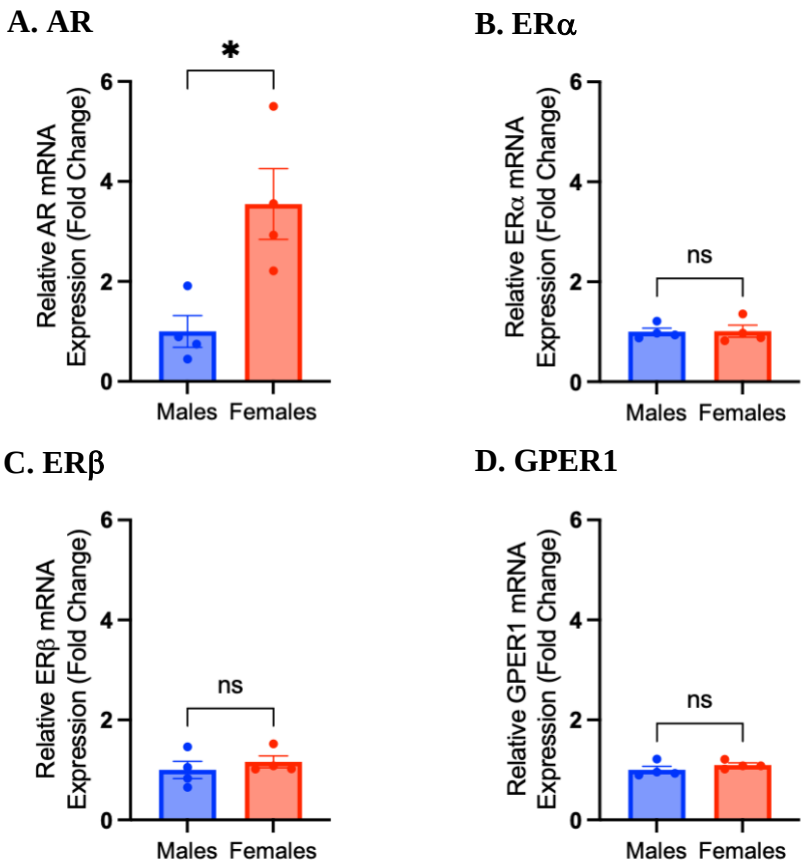


Figure 10: Sex differences in gene expression of sex hormone receptors in BMDMs. Bone marrow monocytes from male and female C57BL/6 mice were differentiated into BMDMs. Gene expression of **A.** AR, **B.** ERα, **C.** ERβ, and **D.** GPER1 was quantified by RT-qPCR. Data shown represent the mean ± SEM fold change ($2^{-\Delta\Delta Ct}$) in receptor expression relative to the reference gene β-actin, normalized to male BMDMs. Two-tailed unpaired t-test was used to evaluate significant differences. (n=4, *p<0.05).

4.2 BMDM polarization is affected by 17β-estradiol and testosterone supplementation

To determine if BMDM polarization is affected by supplementation with E2 or TE, differentiated BMDMs derived from male or female mice were treated with E2 (100 nM) or TE (100 nM) for 24 hours, and then polarized to the pro- or anti-inflammatory phenotypes, as described above. mRNA expression of pro- and anti-inflammatory markers was quantified using RT-qPCR.

Figure 11 illustrates the effects of E2 on BMDM polarization after 24 hours. Treatment with E2 resulted in a significant reduction in iNOS and TNF-α mRNA expression compared to vehicle controls in pro-inflammatory macrophages derived from female (**Fig 11 B, D**), but not male (**Fig 11 A, C**) BMDMs. A (non-significant) a trend towards a reduction in pro-inflammatory marker expression in response to E2 treatment can be observed in male-derived BMDMs (**Fig 11 A, C**). In anti-inflammatory macrophages derived from female mice, treatment with E2 significantly increased mRNA expression of Fizz1 (**Fig 11 F**) and Arg1 (**Fig 11 H**) compared to the vehicle control. No significant differences were observed in male derived BMDMs, but a trend towards an increase in anti-inflammatory marker expression was observed following E2 treatment compared with the vehicle control. These data suggest that E2 may alter polarization to both the pro- and anti-inflammatory phenotype and may affect male- and female-derived BMDMs differently, or to a different extent.

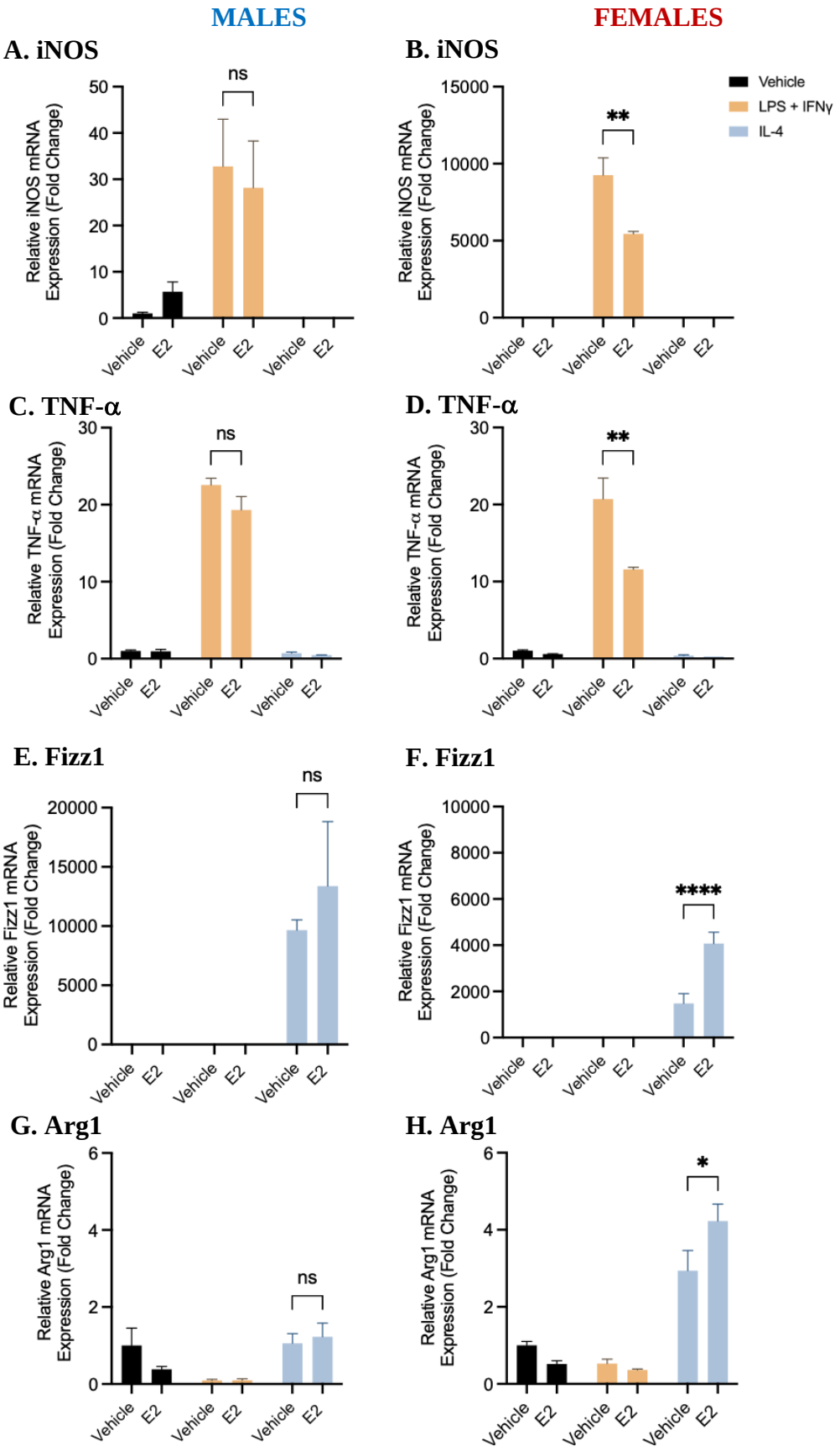

MALES
FEMALES
A. iNOS
B. iNOS
C. TNF-α
D. TNF-α
E. Fizz1
F. Fizz1
G. Arg1
H. Arg1
Vehicle
LPS + IFNγ
IL-4
Relative iNOS mRNA Expression (Fold Change)
Relative TNF-α mRNA Expression (Fold Change)
Relative Fizz1 mRNA Expression (Fold Change)
Relative Arg1 mRNA Expression (Fold Change)
ns
**
****
*
Vehicle
E2

Figure 11: Gene expression of pro- and anti-inflammatory markers in male- and female-derived BMDMs pre-treated with 100 nM 17β-estradiol (E2). BMDMs were pre-treated with 100 nM E2 for 24 hours and polarized to pro- and anti-inflammatory macrophages using LPS and IFNγ or IL-4, respectively, or the vehicle control, for 24 hours. Gene expression of **A.** iNOS, **C.** TNF-α, **E.** Fizz1, and **G.** Arg1 from male-derived BMDMs and **B.** iNOS, **D.** TNF-α, **F.** Fizz1, and **H.** Arg1 from female-derived BMDMs 24 hours following polarization was quantified by RT-qPCR. Data shown represent the mean ± SEM fold change ($2^{-\Delta\Delta Ct}$) in marker expression relative to reference gene β-actin, normalized to the vehicle control. Two-way ANOVA and Tukey's multiple comparison test were performed to evaluate significant differences (n=3-5, *p<0.05, **p < 0.01, ****p < 0.0001).

The effects of TE on BMDM polarization after 24 hours are presented in **Figure 12**. Treatment with TE did not significantly impact iNOS or TNF-α expression in female- (**Figure 12 B, D**) or male-(**Figure 12 A, C**) derived pro-inflammatory BMDMs compared to vehicle-treated controls. A trend of increasing pro-inflammatory marker expression with TE supplementation can be observed in these cells. TE treatment significantly downregulated Fizz1 mRNA expression in anti-inflammatory BMDMs (**Figure 12 E, F**) compared to vehicle controls. Conversely, TE treatment significantly upregulated Arg1 mRNA expression in anti-inflammatory BMDMs derived from male (**Figure G**) but not female (**Figure H**) mice, compared to vehicle controls. These data suggest that TE treatment significantly affects polarization to the anti-inflammatory phenotype and may enhance pro-inflammatory macrophage polarization.

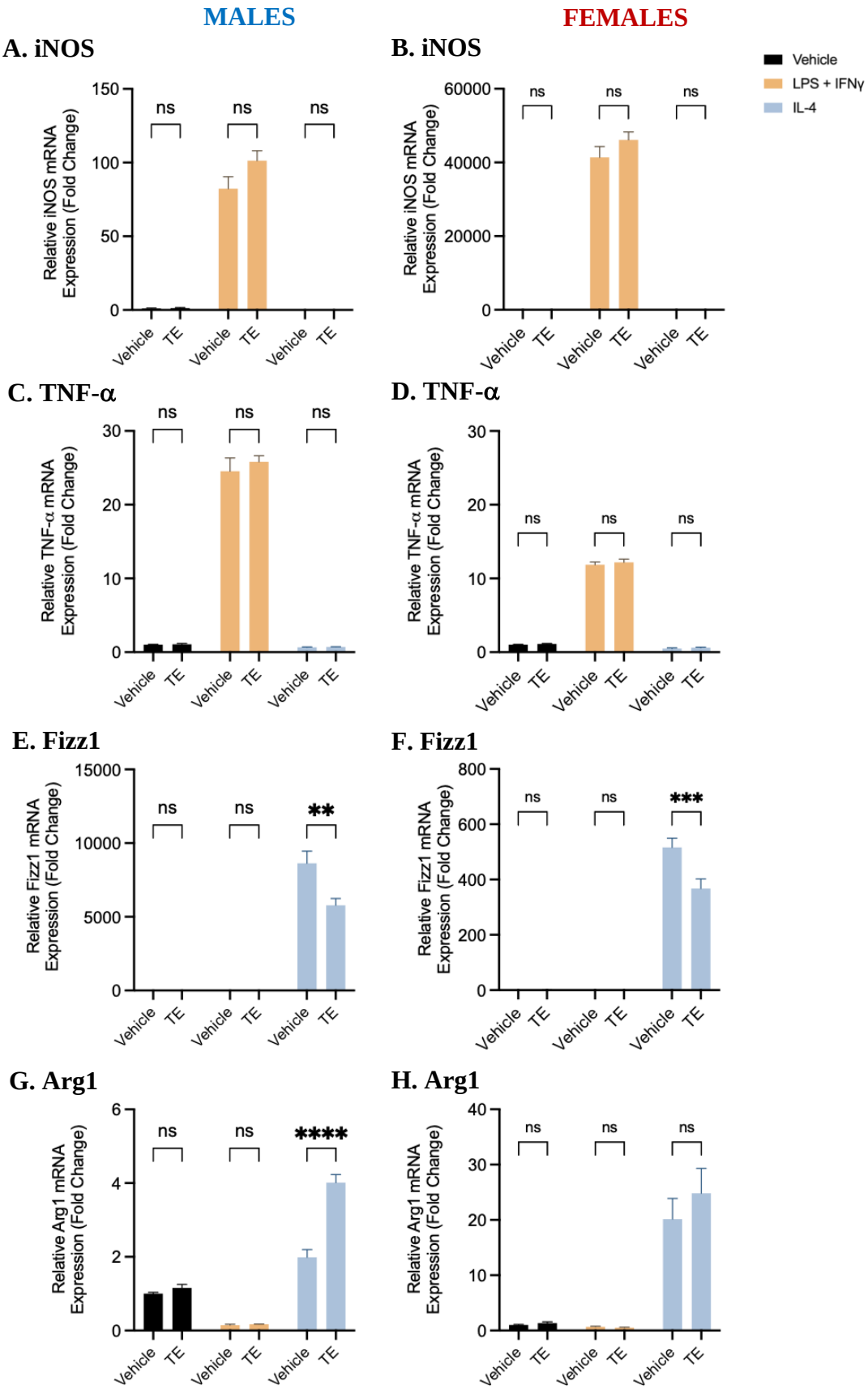

MALES
FEMALES
A. iNOS
B. iNOS
C. TNF-α
D. TNF-α
E. Fizz1
F. Fizz1
G. Arg1
H. Arg1
Vehicle
LPS + IFNγ
IL-4
Relative iNOS mRNA Expression (Fold Change)
Relative TNF-α mRNA Expression (Fold Change)
Relative Fizz1 mRNA Expression (Fold Change)
Relative Arg1 mRNA Expression (Fold Change)
ns
**
***
****
Vehicle
TE

Figure 12: Gene expression of pro- and anti-inflammatory markers in male- and female-derived BMDMs pre-treated with 100 nM testosterone (TE). BMDMs were pre-treated with 100 nM TE for 24 hours and polarized to pro- and anti-inflammatory macrophages using LPS and IFNγ or IL-4, respectively, or the vehicle control, for 24 hours. Gene expression of **A.** iNOS, **C.** TNF-α, **E.** Fizz1, and **G.** Arg1 from male-derived BMDMs and **B.** iNOS, **D.** TNF-α, **F.** Fizz1 **H.** Arg1 from female-derived BMDMs 24 hours following polarization was quantified by RT-qPCR. Data shown represent the mean ± SEM fold change ($2^{-\Delta\Delta Ct}$) in marker expression relative to reference gene β-actin, normalized to the vehicle control. Two-way ANOVA and Tukey's multiple comparison test were performed to evaluate significant differences (n=3-5, *p<0.05, **p < 0.01, ***p<0.001, ****p < 0.0001).

Different subsets of female-derived BMDMs were supplemented with TE or E2 for 24 hours and polarized for 6 hours. Contrary to 24-hour data, E2 supplementation significantly increased iNOS (**Supplementary Figure 1 A**) and TNF-α (**Supplementary Figure 1 C**) mRNA expression after 6 hours of polarization with LPS+IFNγ compared to the vehicle control. In anti-inflammatory macrophages polarized for 6 hours, E2 supplementation did not affect Fizz1 (**Supplementary Figure 1 E)** mRNA expression but resulted in a significant increase in Arg1 (**Supplementary Figure 1 G)** mRNA expression compared to the vehicle. Equivalent treatment with TE did not alter iNOS (**Supplementary Figure 1 B**) mRNA expression but significantly reduced TNF-α (**Supplementary Figure 1 D**) mRNA expression in pro-inflammatory macrophages polarized for 6 hours. Further, TE treatment did not alter Fizz1 (**Supplementary Figure 1 F)** mRNA expression but significantly increased Arg1 (**Supplementary Figure 1 H)** expression in anti-inflammatory macrophages compared to the vehicle control.

The pro-inflammatory macrophage polarization pathway involves several mechanistic steps. IFNγ binding to the IFNγ receptor results in downstream STAT1 phosphorylation and activation, and eventual gene transcription of iNOS[95,96]. Also, activation of the TLR4 by LPS results in binding to interferon regulatory factor 5 (IRF5), causing nuclear translocation and gene transcription of pro-inflammatory mediators, as well as inhibition of interferon regulatory factor

4 (IRF4), a key transcription factor mediating anti-inflammatory macrophage pathways[96]. Conversely, anti-inflammatory macrophage polarization is mediated by the IL-4 receptor[97]. Receptor activation results in STAT6 phosphorylation and IRF4 activation, resulting in nuclear translocation and gene transcription of Arg1, Ym1, and Fizz1[97,98]. Given that E2 and TE supplementation altered pro-inflammatory and anti-inflammatory macrophage polarization, we hypothesized that E2 and TE supplementation may affect STAT1/6 and IRF4/5 activity.

While analysis at the protein level was preferred, experimental constraints interfered with our ability to carry out these analyses. Alternatively, we investigated whether E2 or TE supplementation altered STAT1, STAT6, IRF4, or IRF5 at the level of gene expression. Analysis at the level of expression is relevant in this context because STAT6 upregulation in RAW264.7 macrophages promoted anti-inflammatory macrophage polarization[39]. **Figure 13** demonstrates the effects of E2 supplementation on gene expression of STAT1, STAT6, IRF4, and IRF5 in pro- and anti-inflammatory macrophages derived from male or female mice. E2 supplementation significantly reduced STAT1 gene expression in female-derived BMDMs **(Figure 13 A)**. No other significant differences in transcription factor gene expression were observed in E2-treated BMDMs (**Figure 13 B-H)**. TE supplementation did not significantly alter gene expression of STAT1, STAT6, IRF4, or IRF5 in pro- or anti-inflammatory BMDMs (**Figure 14)**. These data indicate that E2 and TE may not play a meaningful role in regulating macrophage polarization at the level of transcription, however, mediation at the protein level is possible.

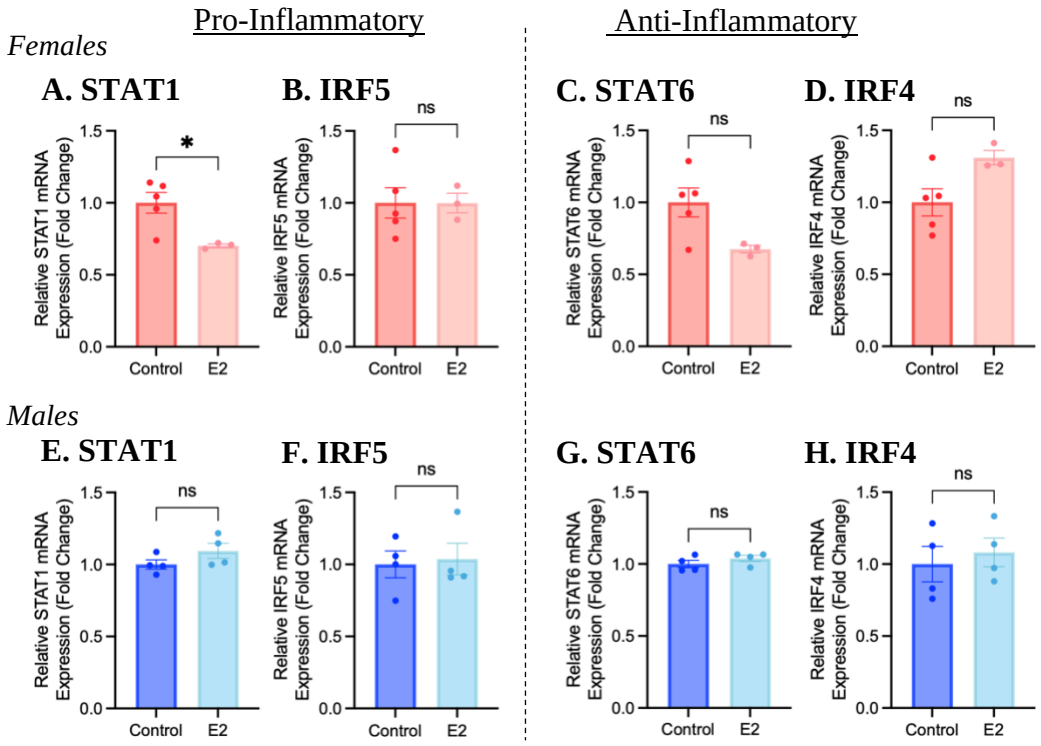


Figure 13: Gene expression of transcription factors in pro- or anti-inflammatory BMDMs treated with 17β-estradiol (E2). BMDMs were pre-treated with 100 nM E2 for 24 hours and polarized into pro- or anti-inflammatory macrophages using LPS and IFNγ, IL-4, or the vehicle control, for 24 hours. Gene expression of transcription factors from **A-D** female- and **E**-H male-derived BMDMs was quantified by RT-qPCR. Data shown represent the mean ± SEM fold change ($2^{-\Delta\Delta Ct}$) in target gene expression relative to reference gene β-actin, normalized to the vehicle control. Two-tailed unpaired t-test was used to evaluate significant differences. (n=4, *p<0.05).

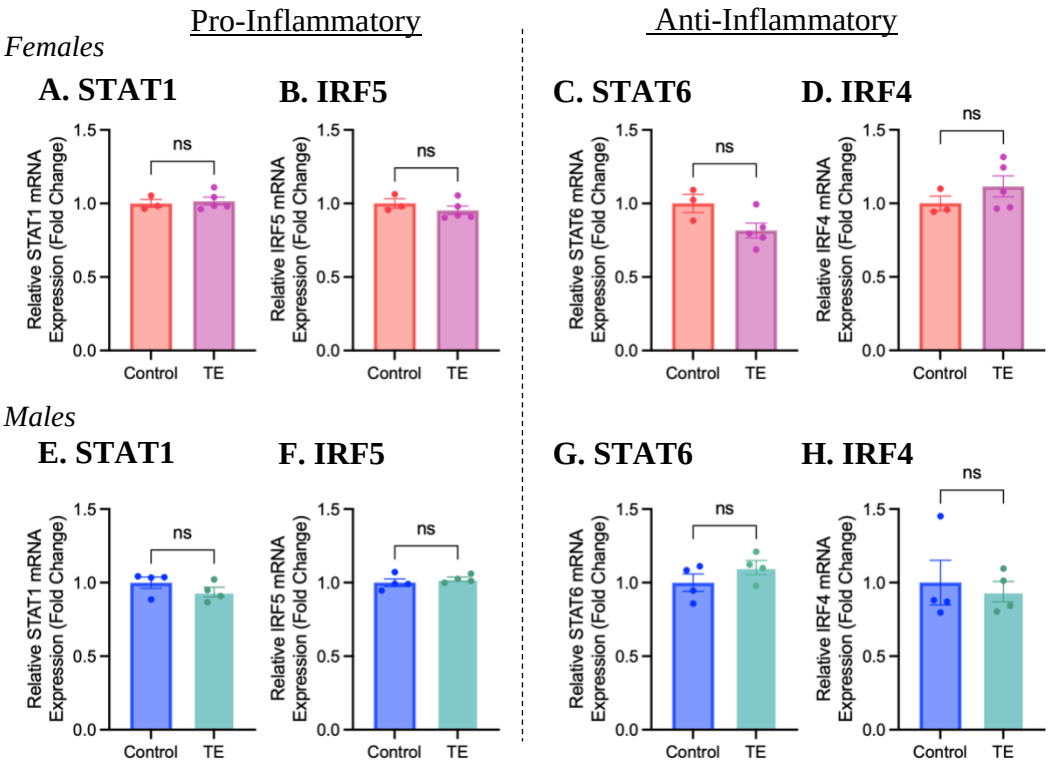


Figure 14: Gene expression of transcription factors in pro- and anti-inflammatory BMDMs treated with 100 nM testosterone (TE). BMDMs were pre-treated with 100 nM TE for 24 hours and polarized into pro- or anti-inflammatory macrophages using LPS and IFNγ, IL-4, or the vehicle control for 24 hours. Gene expression of transcription factors from **A-D** female- and **E-H** male-derived BMDMs was quantified by RT-qPCR. Data shown represent the mean ± SEM fold change ($2^{-\Delta\Delta Ct}$) in target gene expression relative to reference gene β-actin, normalized to the vehicle control. Two-tailed unpaired t-test was used to evaluate significant differences. (n=4, p = 0.05).

Taken together, treatment with TE or E2 resulted in similar expression patterns in macrophage markers, with some deviances. E2 significantly dampened pro-inflammatory macrophage polarization and both E2 and TE promoted anti-inflammatory macrophage polarization. Thus, macrophage polarization may be similarly impacted by the sex hormones they encounter. Also, biological sex may be an independent factor that determines how BMDM polarization is altered by sex hormone supplementation.

4.3 Sex differences and the effects of sex hormone supplementation on BMDM inflammatory response

Inflammatory response is a critical function of macrophages in an atherosclerotic plaque. To assess whether sex is an independent factor affecting inflammatory response of BMDMs, differentiated BMDMs derived from male or female mice were polarized into pro- or anti-inflammatory macrophages for 24 hours, and protein concentration of secreted cytokines and chemokines in cell culture media was analyzed using an ALBIA assay. A comprehensive analysis of all analytes measured in M0 macrophages is presented in **Figure 15**. BMDMs derived from female mice displayed a significantly greater production of IL-1β, IL-3, IL-6, and LIF, and significantly lower production of MIP-1β compared to male-derived BMDMs. Pro-inflammatory macrophages derived from female BMDMs displayed significantly upregulated levels of IL-7, IL-9 and KC, and significantly downregulated production of MIG compared to male-derived BMDMs (**Supplementary Figure 2**). Lastly, anti-inflammatory macrophages derived from female mice exhibited significantly higher levels of IL-7 and MIP-2 compared to male-derived BMDMs (**Supplementary Figure 3**).

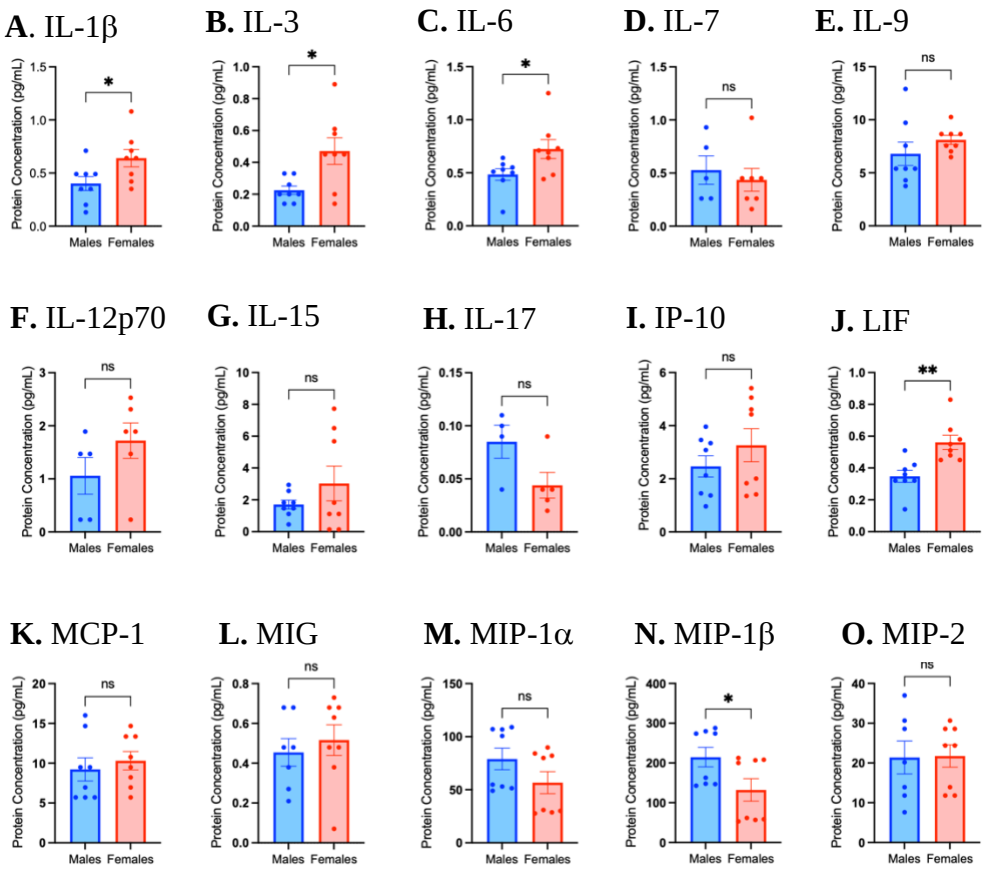
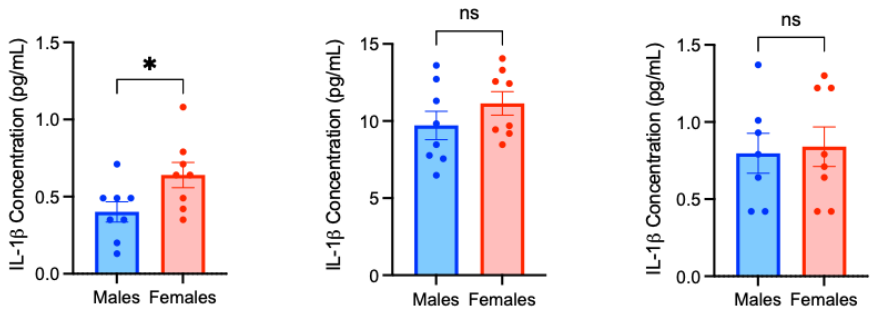


Figure 15: Protein concentration of pro-inflammatory cytokines and chemokines secreted by unpolarized BMDMs derived from male and female mice. Bone marrow monocytes were differentiated into BMDMs, and cells were treated with the vehicle control for 24 hours. Data shown represent the mean ± SEM protein concentration of secreted cytokines and chemokines from cell culture media of BMDMs derived from male and female mice, quantified by an addressable laser bead immunoassay (ALBIA). Two-tailed unpaired t-test was used to evaluate significant differences. (n=5-8, *p<0.05, **p<0.01).

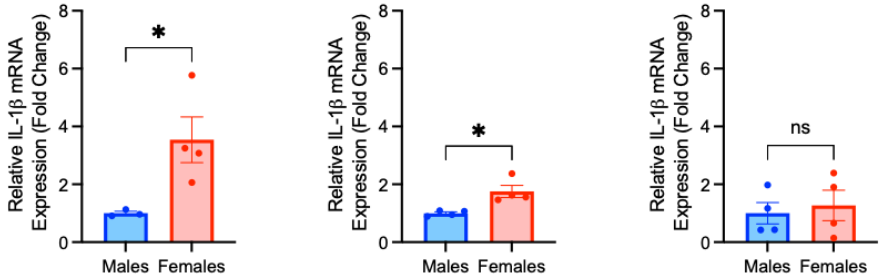
To further investigate these observed sex differences, differentiated BMDMs were pre-treated with E2 (100 nM) or TE (100 nM) for 24 hours prior to polarization to the pro-inflammatory or anti-inflammatory phenotype. Protein concentration of cytokines and chemokines present culture media was analyzed using an ALBIA assay and mRNA expression of IL-1β in vehicle controls was compared. Because IL-1β is a key inflammatory mediator in atherosclerosis[40], we focused our analysis on this cytokine. As previously mentioned, IL-1β

protein concentration was significantly greater in M0 macrophages derived from female mice (**Figure 16 A**) but no significant sex differences in IL-1β protein concentration were observed in pro- or anti-inflammatory macrophages (**Figure 16 B, C**). IL-1β mRNA expression in female-derived M0 and pro-inflammatory BMDMs was significantly greater compared to male-derived BMDMs (**Figure 16 D, E**) thereby corroborating ALBIA data. E2 and TE treatment resulted in a significant reduction in IL-1β production in female- but not male-derived pro-inflammatory macrophages (**Figure 16 G, I**). Overall, biological sex may represent an independent factor affecting pro-inflammatory response, and both E2 and TE may alter the production of IL-1β in pro-inflammatory, female-derived BMDMs.

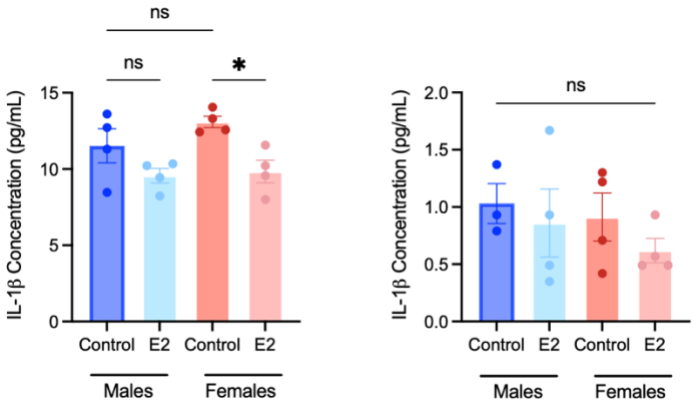
A. Unpolarized **B**. Pro-Inflammatory **C**. Anti-Inflammatory



D. Unpolarized **E**. Pro-Inflammatory **F**. Anti-Inflammatory



G. Pro-Inflammatory **H**. Anti-Inflammatory



I. Pro-Inflammatory **J**. Anti-Inflammatory

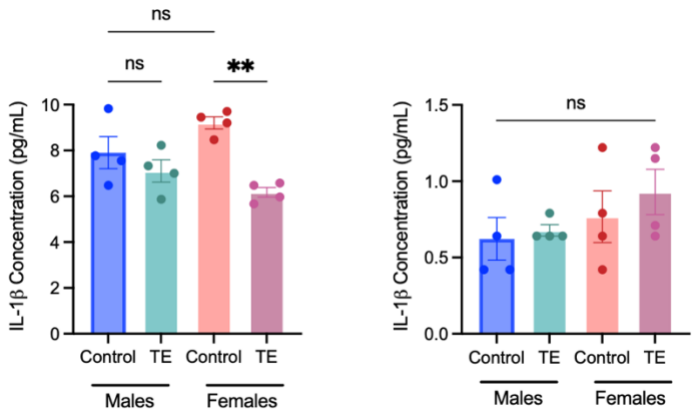


Figure 16: Sex differences and the effects of 17β-estradiol (E2) and testosterone (TE) on IL-1β protein concentration and mRNA expression in BMDMs. A-F: Differentiated BMDMs from female and male mice were polarized to pro- or anti-inflammatory phenotypes or left untreated for 24 hours. Protein concentration of IL-1β from **A.** unpolarized **B.** pro-inflammatory, and **C.** anti-inflammatory BMDM culture medium was quantified using an addressable laser bead immunoassay (ALBIA). Gene expression of IL-1β from **D.** unpolarized **E.** pro-inflammatory, and **F.** anti-inflammatory BMDMs was quantified by RT-qPCR. Data shown are the mean ± SEM fold change ($2^{-\Delta\Delta Ct}$) in IL-1β expression relative to reference gene β-actin, normalized to the vehicle control (**A-C)**, or mean ± SEM IL-1β protein concentration (**D-F)**. Significant differences were detected using two-tailed unpaired t-tests. **G-J:** Differentiated BMDMs were pre-treated with 100 nM E2, TE, or vehicle control for 24 hours prior to polarization. Data shown are the mean ± SEM IL-1β protein concentration from cell culture medium. One-way ANOVA and Tukey's post-hoc test were used to evaluate significant differences. (n=4-8, *p<0.05, **p<0.01).

Due to the use of E2-free cell culture conditions, it was unclear if the observed increases in expression and abundance of pro-inflammatory cytokines by female-derived BMDMs was a result of true sex differences in this model, or rather due to the deprivation of endogenous E2 that female-derived cells experienced in culture. To address this question, bone marrow was collected from male and female mice, and the same bone marrow monocyte differentiation protocol was carried out with the addition of daily treatments of 100 nM E2 or vehicle control. The addition of daily E2 treatment represented exposure to endogenous estrogen. Differentiated macrophages were collected, and gene expression of IL-1β and was measured using RT-qPCR. E2 supplementation for 6 days resulted in a significant reduction in IL-1β expression in female-derived BMDMs **(Figure 17)**. Control (untreated) female BMDMs demonstrated significantly greater expression of IL-1β compared to male control-treated BMDMs (p-value < 0.0001). However, while IL-1β expression in female E2-treated BMDMs was significantly greater than male control-treated BMDMs, the significance level of this comparison was lower (p value = 0.0395). These data suggest that E2-free experimental conditions did not precipitate the sex differences previously observed in this study.

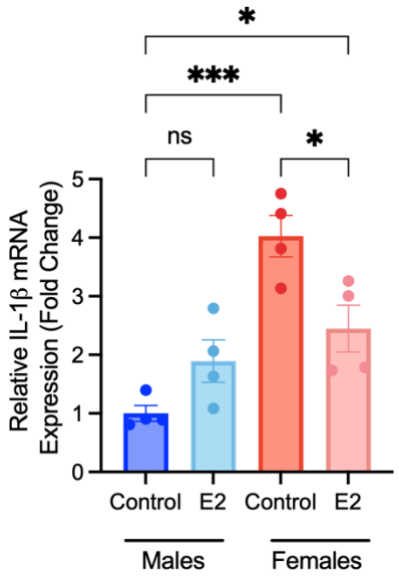


Figure 17: Effects of long-term 17β-estradiol (E2) exposure on IL-1β expression in BMDMs. Isolated bone marrow monocytes were differentiated into BMDMs with or without daily treatments of E2 (100 nM). Gene expression of IL-1β was quantified by RT-qPCR. Data shown are the mean ± SEM fold change ($2^{-\Delta\Delta Ct}$) in IL-1β expression relative to reference gene β-actin, normalized to the vehicle-treated male control. One-way ANOVA and Tukey's post-hoc test were used to evaluate significant differences. (n=4, *p<0.05, ***p<0.001).

4.4 Sex differences and the effects of sex hormone supplementation on BMDM migratory response

Migration is an important function of macrophages in an atherosclerotic plaque[42]. To assess whether biological sex is a factor affecting macrophage migration, BMDMs derived from male and female mice were polarized and a Transwell migration assay was performed. Cell migration calculations accounted for passive migration and were presented as percentages normalized to male-derived BMDMs or untreated controls. No sex difference was observed in the normalized number of pro-inflammatory macrophages migrated (**Fig 18 A**). However, significantly fewer anti-inflammatory macrophages derived from female mice migrated compared to male-derived BMDMs (**Figure 18 B)**.

To address this difference observed, BMDMs were treated with E2 (100 nM) or vehicle for 24 hours prior to macrophage polarization and Transwell migration assay. E2 treatment did not significantly affect pro-inflammatory macrophage migration regardless of sex (**Figure 18 C, D**). Interestingly, E2 significantly enhanced female-derived anti-inflammatory BMDM migration (**Figure 18 E)**. Treatment with TE (100 nM) did not significantly alter BMDM migration **(Supplementary Figure 4).** These data indicate that E2 but not TE may promote migration of anti-inflammatory BMDMs.

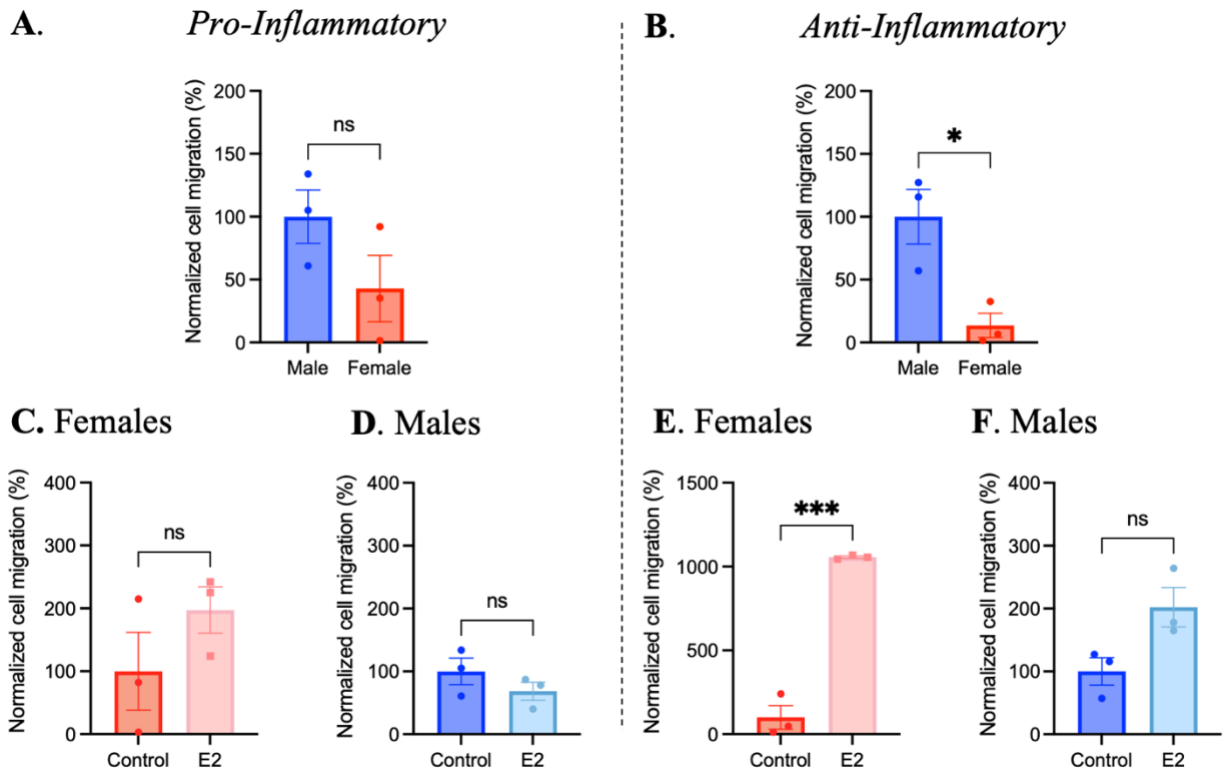
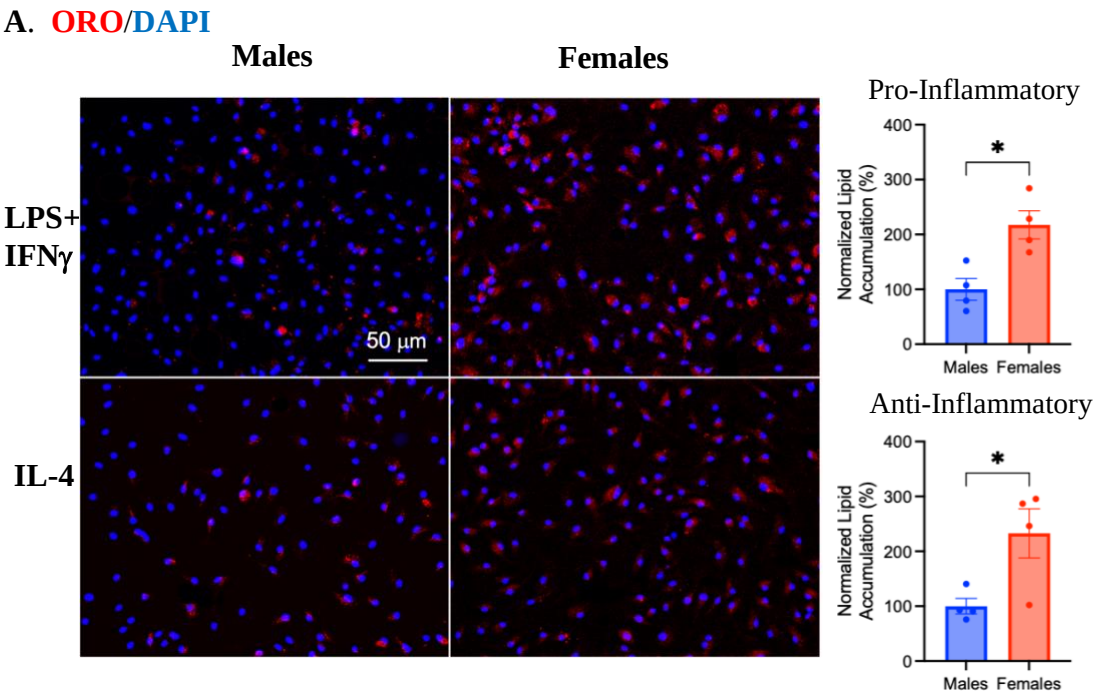


Figure 18: Sex differences and the effects of 100 nM 17β-estradiol (E2) on BMDM migration. Differentiated BMDMs from female and male mice were polarized to pro- or anti-inflammatory phenotypes. A Transwell assay was performed with or without chemoattractant CCL19 in culture medium of the lower assay chamber. Data represent the mean ± SEM cell migration (%) of female **A.** pro-inflammatory or **B.** anti-inflammatory BMDMs, normalized to male BMDMs. **C-F:** Differentiated BMDMs were pre-treated with 100 nM E2 for 24 hours prior to polarization. Following the same Transwell protocol, mean ± SEM cell migration (%) of **C., D.** pro-inflammatory or **E., F.** anti-inflammatory BMDMs, normalized vehicle-treated controls, was calculated. Two-tailed, unpaired t-tests were performed to evaluate significant differences (n=3-4, *p<0.05, ***0.001).

4.5 Sex differences and the effects of 17β-estradiol on BMDM lipid accumulation

Within the atherosclerotic lesion, pro-inflammatory macrophages accumulate lipids and become lipid-laden foam cells whereas anti-inflammatory macrophages transport deposited cholesterol out of the developing plaque[31,99]. To determine sex differences and the effects of E2 and TE on BMDM lipid accumulation, male- and female-derived BMDMs were pre-treated with E2 or TE (100 nM), or vehicle and polarized as described above. Polarized BMDMs were

incubated in serum-free DMEM plating medium before incubation in regular DMEM plating medium with or without FBS. Cells were stained with ORO to visualize lipid molecules and DAPI for nuclei. Images were captured and normalized lipid accumulation was calculated as a percentage of the control (male or untreated BMDMs). Pro- and anti-inflammatory BMDMs derived from female mice demonstrated greater lipid accumulation compared to male-derived BMDMS (**Figure 19 A**). Treatments with E2 or TE did not significantly alter lipid accumulation of pro- or anti-inflammatory BMDMs (**Figure 19 B-I**). However, a non-significant reduction in lipid accumulation was observed in male-derived pro-inflammatory BMDMs (**Figure 19 G**). These data indicate that female-derived BMDMs may have a greater capacity to accumulate lipids, and that E2 and TE may not affect this cell function.


A. ORO/DAPI
Males
Females
LPS+ IFNγ
IL-4
50 µm
Pro-Inflammatory
Anti-Inflammatory
Normalized Lipid Accumulation (%)
Males
Females
*

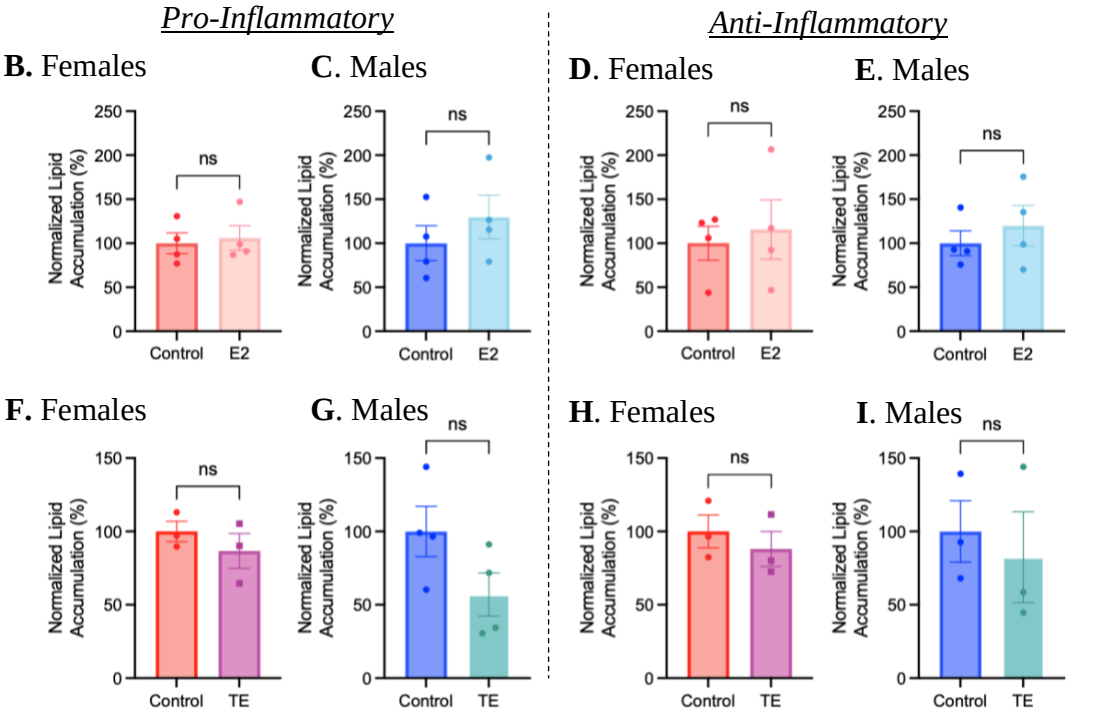

Pro-Inflammatory
Anti-Inflammatory
B. Females
C. Males
D. Females
E. Males
F. Females
G. Males
H. Females
I. Males
Normalized Lipid Accumulation (%)
ns
Control
E2
TE

Figure 19: Sex differences and the effects of 100 nM 17β-estradiol (E2) and testosterone (TE) on BMDM lipid accumulation. Differentiated BMDMs from female and male mice were polarized to pro- or anti-inflammatory phenotypes. Cells were incubated in serum-free medium and then transferred into chamber slides in medium containing fetal bovine serum for 4 hours. Cells were stained with Oil Red O and DAPI. Fluorescence intensity of stained lipid droplets was quantified, and nuclei were counted. Mean intensity per cell ratios for each biological replicate were calculated and data were normalized to male BMDMs and represented as a percentage. Data represent the mean ± SEM lipid accumulation (%) of female **A.** pro-inflammatory or anti-inflammatory BMDMs, relative to male BMDMs. Representative images show male- and female-derived BMDMs stained with ORO and DAPI. **B-I:** Differentiated BMDMs were pre-treated with 100 nM E2 (**B-E)** or TE (**F-I**) for 24 hours and were polarized to the pro- or anti-inflammatory phenotypes. Mean ± SEM lipid accumulation (%) of pro-inflammatory or anti-inflammatory BMDMs, normalized vehicle-treated controls, was calculated. Two-tailed, unpaired t-tests were performed to evaluate significant differences (n=3-4, *$p<0.05$).

5. DISCUSSION

Biological sex and concentrations of circulating sex hormones play a role in the progression of CVD[43,48,49]. Macrophages are a primary cell type abundant in atherosclerotic lesions and their phenotypic distribution in a lesion has been correlated to plaque severity[33]. In this study, we hypothesized that sex hormones alter macrophage polarization and pro-atherogenic function and thereby regulate the progression of atherosclerosis. To address our hypothesis, bone marrow was isolated from male and female mice, and bone marrow-derived monocytes were differentiated into BMDMs. BMDMs were polarized into pro- or anti-inflammatory phenotypes. In order to investigate the effects of sex hormones, BMDMs were pre-treated with E2 or TE (100 nM) and gene expression of pro- and anti-inflammatory macrophage markers, protein concentration of inflammatory mediators, and migratory and lipid accumulation properties were examined.

5.1 Characterizing BMDM polarization in male- and female-derived BMDMs

The first objective of our study was to characterize BMDM polarization. This objective involved establishing a BMDM polarization profile over three time points stratified by biological sex, followed by a direct comparison of male- and female-derived BMDMs, and an analysis of sex hormone receptor expression. An important aspect of this investigation is the extensive use of macrophage polarization markers. Due to the complex and often overlapping nature of macrophage phenotypes and their phenotypic markers, quantification of several phenotypic markers allows for distinct characterization.

Our lab previously demonstrated that 95% of bone marrow-derived cells subjected to differentiation with M-CSF were F4/80+ and CD11b+ by flow cytometry, which verifies the efficiency of our macrophage differentiation protocol[89]. In the present study, we expanded upon

this protocol by analyzing the gene expression of pro-inflammatory (iNOS, TNF-α, Il-1β, and CD38) and anti-inflammatory (Arg1, Fizz1, Ym1, and CD206) macrophage markers following polarization. Macrophages treated with LPS and IFNγ demonstrated significantly upregulated expression of pro-inflammatory markers (**Figure 7**) and significantly downregulated expression of anti-inflammatory markers (**Figure 8**). This indicates successful polarization to the pro-inflammatory phenotype and is corroborated extensively in the literature [83,84,93,94]. Macrophages treated with IL-4 demonstrated significantly upregulated expression of anti-inflammatory markers (**Figure 8**) and significant downregulation of pro-inflammatory markers (**Figure 7**). These findings align with previous descriptions of anti-inflammatory macrophage polarization [83,84,93]. Therefore, we can conclude that macrophages were polarized successfully into pro- and anti-inflammatory BMDM phenotypes.

A characterization study of both male-and female-derived BMDMs over three time points, to our knowledge, has never been performed. To address this knowledge gap and to create a profile of macrophage polarization over time, we assessed the gene expression of macrophage markers following 6, 24, and 48 hours of polarization. Pro-inflammatory macrophage markers were most abundantly expressed following 6 hours of LPS and IFNγ treatment in both male and female derived cells, except for CD38 expression in female-derived cells (**Figure 7)**. The early expression of pro-inflammatory cytokines IL-1β and TNF-α may be attributable to the acute phase response[100]. However, our findings reflect previous observations in which these markers were labelled as early pro-inflammatory macrophage markers[83]. In anti-inflammatory macrophages, gene expression of Arg1 and CD206 was the greatest following 6 hours of polarization and are in line with observations that Arg1 is an early marker for anti-inflammatory macrophages[83]. Our observations that Ym1 and Fizz1 are most abundantly

expressed by anti-inflammatory macrophages following 48 hours of polarization reflect findings that that implicate these markers as late anti-inflammatory macrophage markers[101]. Overall, we have successfully created a comprehensive profile of pro- and anti-inflammatory macrophage marker expression over time.

In addition to performing a side-by-side analysis of male- and female-derived BMDMs, we also directly compared gene expression of macrophage markers and sex hormone receptors in male- and female-derived BMDMs. Pro-inflammatory markers TNF-α and Il-1β, and anti-inflammatory markers Fizz1 and Ym1, were significantly elevated in female-derived cells compared to male-derived cells, at the same time point (**Figure 9**). These data suggest that female-derived BMDMs may be more sensitive to environmental stimuli. These observations may also be attributable to the hormone deprivation that occurred as a result of our study protocol. This possible explanation will be further evaluated at a later point in this discussion.

In addition to direct effects of sex hormones, the influence of epigenetics and the effect of X and Y chromosomes in pathology are emerging areas of study that might contribute to the sex differences observed in this study (and elsewhere). Recently, it has been suggested that unresolved inflammation in atherosclerotic lesions could be caused, at least in part, by an impaired phenotypic switch of atherosclerosis-associated macrophages into anti-inflammatory macrophages[102]. These findings relate to the hypothesized epigenetic mechanism termed macrophage repolarization, in which M1-like macrophages are skewed towards an M2-like phenotype by turning off M1 machinery[103]. Also, evidence of E2-and estrogen receptor-induced epigenetic mechanisms including DNA methylation, histone modification, and chromatin remodelling has been reviewed extensively elsewhere[104]. These findings suggest that macrophage polarization in atherosclerosis could be regulated by E2 and estrogen receptors

through epigenetic mechanisms, thereby relating to sex differences observed in disease progression.

The number of X chromosomes has been demonstrated to affect pro-inflammatory cytokine production following toll-like receptor stimulation in human purified monocytes[105]. Elevated levels of cytokines were reported in stimulated whole blood derived from men compared to women, and compared to individuals with Klinefelter syndrome (phenotypic males who carry two X chromosomes; XXY). These data suggest a potential therapeutic benefit related to the X chromosome. While these observations conflict with our findings that pro-inflammatory markers were upregulated in female-derived BMDMs, they may relate to the net effect of the X chromosome in whole blood analyses rather than on one cell type alone. Overall, epigenetics and chromosomal influence are emerging fields of research that may lead to connections between sex differences in atherosclerosis and macrophage polarization and functions.

We evaluated gene expression of AR, ERα, ERβ, or GPER1 in male- and female-derived BMDMs (**Figure 10**). Female-derived BMDMs demonstrated a significantly greater expression of AR while no significant differences were observed in the gene expression of ERα, ERβ, or GPER1. These data conflict with previous findings indicating that BMDMs from male rats exhibit significantly greater expression of AR than female-derived BMDMs[106]. However, critical flaws in the experimental protocol including the use of reagents that may contain estrogenic activity, such as RPMI (containing phenol red) and standard FBS may have contributed to this result. It is possible that the increased expression of macrophage markers seen in female-derived BMDMs relates to the observed increase in AR expression in these cells. Further research is needed to determine a mechanistic basis for these sex differences observed in BMDM polarization.

Overall, within our first objective, we have created a comprehensive profile of BMDM polarization over 6, 24, and 48 hours stratified by biological sex. Further, we observed sex differences in BMDM polarization and in the expression of AR. Therefore, our first objective has been completed.

5.2 Effects of sex hormones on BMDM polarization

Once polarization was confirmed, we then investigated whether sex hormones impact macrophage polarization. Differentiated BMDMs were pre-treated with E2 or TE prior to polarization. We observed that pre-treatment with E2 impaired polarization to the pro-inflammatory phenotype by diminishing mRNA expression of pro-inflammatory markers and promoted polarization to the anti-inflammatory phenotype by enhancing expression of anti-inflammatory markers (**Figure 11**). These findings are supported by evidence that E2 supplementation reduced pro-inflammatory marker expression including TNF-α, IL-1β, and IL-6 in murine peritoneal macrophages and human peripheral blood monocytes[107]. Pro-inflammatory macrophage polarization is regulated through pathways involving STAT1 phosphorylation and IRF5[95], whereas STAT6 phosphorylation and IRF4 activity are important for anti-inflammatory macrophage polarization[96]. E2 may impact iNOS and TNF-α expression through its actions on AP1, NF-κB, and STAT1[86]. Also, E2 may regulate Arg1 expression through STAT6[86]. In contrast, E2 promoted STAT1 DNA binding activity but did not increase expression of total or phosphorylated STAT1 protein in murine splenocytes[108]. To investigate this mechanism further, we assessed the effects of E2 on gene expression of STAT1, IRF4, STAT6 and IRF5 (**Figure 13)**. Due to experimental limitations, we were unable to assess these molecules at the protein level. E2 supplementation significantly reduced STAT1 transcript levels which is consistent with the dampened pro-inflammatory phenotype previously observed in **Figure 11**. This means that

STAT1 and downstream signaling, including STAT1 phosphorylation and nuclear translocation, may be involved in mechanisms by which E2 provides protection in atherosclerosis.

Significant differences in gene expression of iNOS and TNF-α in pro-inflammatory macrophages and Fizz1 and Arg1 in anti-inflammatory macrophages in response to E2 supplementation were only observed in cells derived from female mice (**Figure 11**)**.** This may further support our interpretation that female-derived cells may be more sensitive to environmental stimuli, especially given that gene expression of estrogen receptors was not significantly different when comparing male- and female-derived BMDMs. This may also indicate a sex difference in the effects of E2 on BMDM polarization, or that a greater sample size in future studies is needed to overcome variability in the sample. Overall, we demonstrated that E2 may impair pro-inflammatory macrophage polarization and enhance anti-inflammatory macrophage polarization (**Figure 20**).

Pro-inflammatory marker expression was not affected by pre-treatment with TE, indicating that TE does not alter polarization to the pro-inflammatory phenotype (**Figure 12**). However, pre-treatment with TE resulted in an increase in Arg1 expression and a decrease in Fizz1 expression in anti-inflammatory macrophages, suggesting at least two opposing mechanistic actions of TE on anti-inflammatory macrophage polarization. To further delineate this mechanism, we analyzed the effects of TE on STAT6 and IRF4, which represent two separate pathways through which anti-inflammatory macrophage polarization is mediated[96]. Because anti-inflammatory macrophage polarization was promoted in response to STAT6 upregulation[39], we hypothesized that TE supplementation may alter STAT6 expression level. However, no significant differences in expression of STAT6 or IRF4 were observed in response to TE supplementation (**Figure 14**), suggesting that TE may not affect these transcription factors

at the transcript level. These data could be strengthened by an analysis at the protein level, including assessing STAT6 and IRF4 phosphorylation[109]. Regardless, TE has been reported as an anti-inflammatory agent[110] and our results partially support these claims. While our results indicate opposing actions of TE, it is possible that the net effect of TE results in a dampened anti-inflammatory phenotype. Overall, the effects of TE on macrophage polarization to the pro-inflammatory phenotype are unclear, but TE may promote polarization to the anti-inflammatory phenotype (**Figure 20**).

Taken together, our second objective of determining the effects of sex hormone supplementation on BMDM polarization was addressed. Our evidence supports a possible anti-atherogenic role for E2 or TE in the context of macrophage polarization (**Figure 20**).

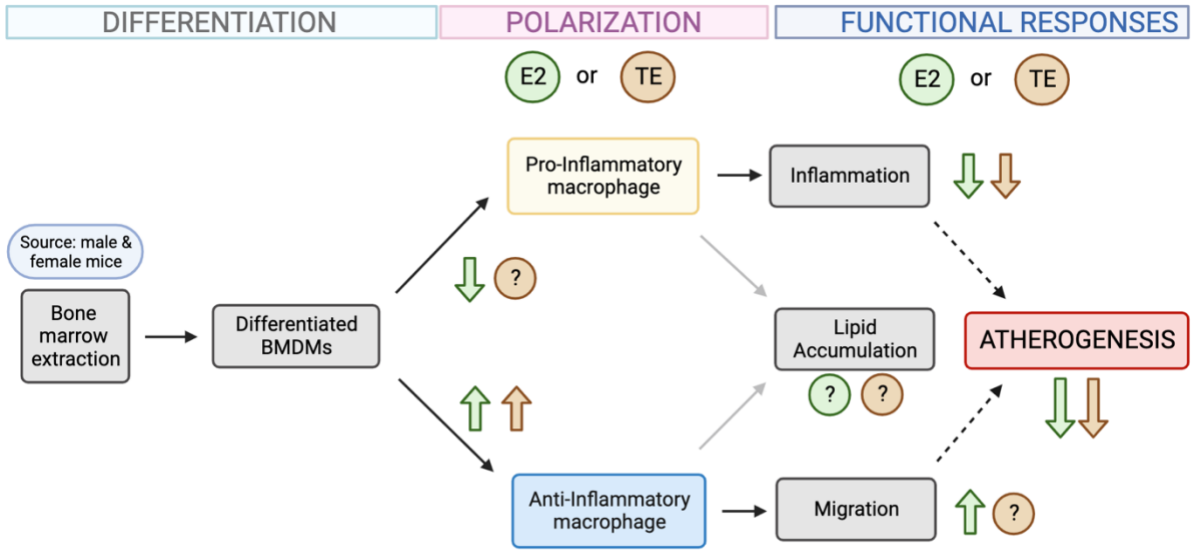


Figure 20: Proposed role of 17β-estradiol (E2) and testosterone (TE) on BMDM polarization and pro-atherogenic function. Bone marrow monocytes derived from male and female mice were differentiated into macrophages in E2-free medium. Differentiated BMDMs derived from female mice produced lower levels of pro-inflammatory cytokines, including IL-1β. E2 pre-treatment impaired pro-inflammatory macrophage polarization and promoted anti-inflammatory macrophage polarization, reduced the inflammatory response of pro-inflammatory macrophages, and promoted migration of anti-inflammatory macrophages. TE pre-treatment promoted anti-inflammatory macrophage polarization and reduced inflammatory response of pro-inflammatory macrophages. The effects of ET and TE on lipid accumulation are unclear. Taken together, these actions suggest a protective role of E2 and TE in atherosclerosis. *(Created with BioRender).*

5.3 Sex differences and the effects of sex hormones on BMDM pro-atherogenic functions.

5.3.1 Sex differences and the effects of sex hormones on BMDM inflammatory responses

Next, our third objective was to investigate sex differences in pro-atherogenic functions of BMDMs, including inflammatory response, migration, and lipid accumulation. To determine sex differences in inflammatory responses of BMDMs, we measured protein concentration of pro-inflammatory cytokines produced by unpolarized BMDMs derived from male or female mice (**Figure 15**). We observed that female-derived BMDMs produced significantly greater quantities of pro-inflammatory cytokines IL-1β, IL-3, and IL-6 than male-derived BMDMs, suggesting a sex difference in inflammatory profiles at in M0 macrophages. Further research is

needed to determine the specific mechanisms by which biological sex affects the regulation of these cytokines Alternatively, these observed sex differences may have been produced due to the loss of E2 exposure once cells were removed from their host. As E2 has been suggested to act as an anti-inflammatory agent[74] the loss of E2 exposure that BMDMs derived from female mice experienced during the requisite 6 days of culturing might relate to increased pro-inflammatory cytokine expression compared to male-derived BMDMs.

To address these questions, we conducted a brief study to determine if E2-free experimental conditions produced a confounding effect on inflammatory responses of female-derived macrophages (**Figure 17**). Interleukin-1β (IL-1β) is a pro-inflammatory cytokine that has been identified as both a marker and mediator of inflammation in atherosclerosis[40]. Notably, inhibition of IL-1β resulted in a significant reduction of CVD events in humans[41]. Therefore, IL-1β often used as a marker to evaluate severity of inflammation in atherosclerotic lesions and may be employed as an indicator of inflammatory properties of macrophages[40]. For this reason, IL-1β was a primary marker of inflammation in our study. Isolated bone marrow-derived monocytes were supplemented with 100 nM E2 daily for 6 days while differentiation into BMDMs using M-CSF was carried out. We observed that daily E2 treatment dampened IL-1β expression in female-derived M0 macrophages, however, IL-1β expression in these E2-treated BMDMs remained significantly greater than in male-derived BMDMs. These data demonstrate that experimental conditions only partially precipitated inflammatory responses in female-derived BMDMs, and that sex differences in BMDM inflammatory response may still exist.

To further investigate the effects of E2 or TE on BMDM pro-inflammatory responses, we pre-treated BMDMs with E2 or TE prior to polarization and assessed the concentration of IL-1β in cell culture media (**Figure 16**). We observed that E2 and TE treatment diminished IL-1β

production by pro-inflammatory macrophages from female mice with a closely aligned trend observed in BMDMs from male mice. Interestingly, these findings are in line with the anti-atherogenic effect of E2 on macrophage polarization previously observed in this study and elsewhere[68,84]. Also, the anti-inflammatory role of TE previously suggested[110] is further supported by these findings. Cleaved IL-1β protein levels are largely regulated by the NLRP3 inflammasome through caspase-1[111,112]. The NLRP3 inflammasome also regulates IL-18 protein levels in a similar manner [111,112]. Therefore, determining whether E2 and TE affect NLRP3 mRNA expression and IL-18 production could provide further insights into the mechanism by which E2 and TE regulate IL-1β production. Taken together, E2 and TE may dampen inflammatory responses in pro-inflammatory macrophages, thereby supporting a protective role of these hormones in atherosclerosis (**Figure 20**).

5.3.2 Sex Differences and the effects of sex hormones on BMDM migration

Migration is a key function of macrophages with a dual effect. Migration of pro-inflammatory macrophages allows for the localization to areas of cholesterol content, which leads to foam cell formation[42]. However, migratory properties seen in anti-inflammatory macrophages may relate to increased cholesterol efflux and plaque stabilization[42]. To continue to address our third objective, we investigated whether any differences exist between male- and female-derived BMDMs in their ability to migrate towards the chemoattractant CCL19 (**Figure 18**). A Transwell migration assay was performed with CCL19 supplemented in the lower chamber, and data suggest a significantly lower migratory ability of female-derived anti-inflammatory BMDMs compared to male-derived BMDMs. These data contribute to the trend observed in this study that female-derived BMDMs may be more sensitive to environmental stimuli than male-derived BMDMs. Furthermore, we supplemented BMDMs with E2 or TE prior

to performing the Transwell assay. In anti-inflammatory macrophages from both male- and female-derived mice, E2 supplementation increased migration towards CCL19. These data suggest a protective role for E2 in atherosclerosis by enhancing migration of anti-inflammatory macrophages (**Figure 20**).

One line of research suggests that rate of migration may be related to the expression of integrins $\alpha_M\beta_2$ and $\alpha_D\beta_2$[113]. While integrins $\alpha_M\beta_2$ and $\alpha_D\beta_2$ affected pro-inflammatory macrophage migration in an opposing manner, both molecules promoted anti-inflammatory macrophage motility. Therefore, it is possible that E2 actions may regulate integrin molecule expression, which could explain the increase in migration of anti-inflammatory macrophages observed in our study. More research is needed in this area to better interpret these data. Additionally, TE supplementation did not appear to alter BMDM migration, despite evidence that 100 nM TE supplementation significantly increased T-cell regulator migration[114]. Overall, E2 may play a protective role in atherosclerosis by promoting migration of anti-inflammatory macrophages (**Figure 20**).

5.3.3 Sex Differences and the effects of sex hormones on BMDM lipid accumulation

Lipid accumulation by pro-inflammatory macrophages results in foam cell formation and eventual apoptosis and deposition of necrotic debris within the atherosclerotic plaque[17,18]. However, lipid uptake by anti-inflammatory macrophages may promote cholesterol efflux and plaque regression[31,99]. We investigated whether biological sex or sex hormones alter lipid accumulation of BMDMs. Differentiated BMDMs were treated with either E2 or TE, and polarized to the pro- or anti-inflammatory phenotypes. Polarized cells were stained with ORO to detect intracellular lipid droplets. We observed a greater percent lipid accumulation in female-derived pro- and anti-inflammatory BMDMs compared to male-derived BMDMs (**Figure 19**).

This indicates female biological sex as both an atherogenic and atheroprotective factor through actions on pro- and anti-inflammatory BMDMs, respectively[29]. This result also reflects the trends in this study that female-derived BMDMs were more inflammatory and polarized to a different extent than male-derived BMDMs. Taken together, this information could indicate that female-derived BMDMs in this model are more sensitive to environmental stimuli, potentially due to the loss of E2 during cell culturing. Further investigation into the mechanisms by which this potential effect occurs would provide useful insight for future research using this model.

E2 and TE supplementation did not affect lipid accumulation by pro- or anti-inflammatory BMDMs (male- or female-derived) in this study (**Figure 19**). This result may contrast findings that E2, but not TE, lowered human monocyte-derived macrophage cholesterol content[115]. It has been suggested that E2 may mediate its effects through ERα signaling involving sterol regulatory element binding protein-1 in a study of lipid accumulation and LDL uptake in HepG2 cells and THP-1 macrophages[116]. These data provide an example of how E2-stimulated pathways may be manipulated to enhance cellular sensitivity to statins[116]. Overall, our data demonstrate that female-derived BMDMs accumulate lipids more readily, but the effects of E2 and TE on BMDM lipid accumulation remain inconclusive.

5.3.4 Summary of sex differences and the effects of sex hormones on BMDM pro-atherogenic functions.

The third objective of our investigation was to determine sex differences and the effects of sex hormones on BMDM pro-atherogenic functions. Thus far, have elucidated a possible sex difference in BMDM inflammatory response at baseline, in anti-inflammatory macrophage migration, as well as in lipid accumulation by pro-and anti-inflammatory macrophages. We did not observe significant differences in lipid accumulation after treatment with E2 or TE. However, the data indicated that both E2 and TE reduce inflammatory properties of pro-

inflammatory BMDMs. Furthermore, we have suggested that E2 may promote migration of anti-inflammatory macrophages. These observations suggest a protective role of E2 and TE in atherosclerosis (**Figure 20**).

5.6 Limitations

The primary limitation of our study is that the use of BMDMs *in vitro* as a proxy for macrophages in atherogenesis, and the application of E2 and TE to these cells, are both oversimplifications of dynamic and complicated pathological and endocrinological systems. Also, our study does not evaluate the effects of progesterone, or the combination of sex hormones, both of which are relevant and necessary to examine. The goal of this study is to create a snapshot of the interactions between sex hormones and macrophages. Insights gained from this study can be expanded upon using *in vivo* models of atherosclerosis, but not to draw conclusions about human physiology or pathology.

Another limitation of our study is that we were unable to investigate mechanistic effects of E2 and TE at the protein level despite successful quantification of total protein. Detection of total and phosphorylated STAT6 and STAT1 was unsuccessful due to extremely low abundance of total and phosphorylated target protein relative to loading controls such as β-actin. More work is needed to better optimize this system.

Additionally, executing a study with a large scope came the expense of a small sample size. However, the sources of BMDMs (C57BL/6 mice) are genetically identical and were not subject to any interventions that may introduce variability, such as drug injections or diet control. Also, care was taken to make direct and reliable comparisons. This includes accounting for time as a variable that may affect the quality of comparisons. We are confident that the results

presented in this study are accurate because of the internal consistency demonstrated throughout our findings.

5.4 Future Directions

5.4.1 Investigating the effects of E2 and TE on macrophage functions: apoptosis and efferocytosis.

Atherosclerotic plaque severity can be signified by the presence and development of a necrotic core[17,18]. Necrotic cores are formed by foam cell apoptosis and deposition of necrotic debris[18]. Macrophages within the plaque can efferocytose this necrotic debris and slow plaque progression[31,99]. Thus, apoptosis and efferocytosis are prominent cell functions that could be affected by E2 and TE. Future research should investigate the effects of E2 and TE on these functions to gain further insight into the role of sex hormones in atherosclerosis.

5.4.2 Investigating the effects of progesterone on macrophage polarization and functions.

Progesterone is a secondary sex hormone that plays an important role in promoting and maintaining pregnancy[117]. Circulating levels of progesterone oscillate throughout the menstrual cycle and are reduced in post-menopausal individuals[117]. As such, it has been suggested that pre-menopausal CVD protection may also be attributable to circulating progesterone[118–121]. However, the role of progesterone in CVD is infrequently studied and unclear. Future research should examine the role that progesterone might play in macrophage polarization and functions, and atherosclerosis in general.

5.4.3. Investigating the effects of E2 and TE on macrophage phenotypes and functions *in vivo*.

Plaque severity has been related to the predominance of pro- or anti-inflammatory macrophages in mice and humans[29]. While our study elucidates effects of *in vitro* E2 and TE

supplementation on macrophage polarization and function, it is unclear how this may translate to *in vivo* models. Administering E2 or TE treatments to ovariectomized and sham-operated control ApoE$^{-/-}$mice and observing the phenotypic distribution of macrophages in an atherosclerotic lesion is an example of a realistic extension of our experimental model.

5.4.4 Identifying the specific mechanisms by which sex hormones act on macrophage polarization and functional pathways

Our study demonstrated that sex hormones may regulate macrophage polarization and pro-atherogenic functions, which could ultimately provide protection from atherosclerosis. However, the global effect that sex hormones have throughout the body creates a limitation in our ability to treat cardiovascular diseases using exogenous hormones without harmful or off-target adverse effects[122]. Therefore, identifying specific mechanisms by which sex hormones act on pathways that regulate macrophage polarization and pro-atherogenic functions is a crucial long-term goal of this research. Identifying specific mechanistic actions could lead to the discovery of key targets that could be manipulated by more specific, and safer, drug therapies.

6.0 CONCLUSION

The purpose of our investigation was to identify sex differences and the effects of sex hormone supplementation on macrophage polarization and pro-atherogenic function. Our findings suggest that sex differences may exist in macrophage polarization, pro-inflammatory responses, migration, and lipid accumulation. We propose an atheroprotective role for E2 by dampening polarization to the pro-inflammatory phenotype and promoting polarization to the anti-inflammatory phenotype, and by dampening inflammatory responses of pro-inflammatory macrophages. E2 may also promote migration of anti-inflammatory macrophages. Furthermore, our data supports an atheroprotective role for TE by promoting anti-inflammatory macrophage polarization and by impairing inflammatory responses of pro-inflammatory macrophages. Altogether, these actions suggest a protective role for both E2 and TE in atherosclerosis.

While sex hormones appeared to have a positive effect on macrophage pro-atherogenic functions, sex differences can also arise from differences in chromosome number[105] and actions of sex hormones on epigenetics[104]. Also, while we proposed that TE and E2 may have a net positive effect atherogenesis, we demonstrated that these hormones affect individual functions of macrophages differently. This illustrates that E2 and TE act through differing, and sometimes opposing mechanisms in macrophages, and may further explain sex differences observed in this model. E2 and TE both impact several diverse signaling networks throughout the body, many of which have yet to be discovered. Identifying and delineating these mechanisms will contribute to significant advances in understanding their effects in males and females, separately.

Our study provides a unique opportunity to explain the effects of sex hormones on macrophage polarization and function in atherogenesis. Because of the important role that

macrophages play in atherosclerosis, observations made from this work will provide insights into the interactions of biological sex and cardiovascular disease. Ultimately, this research supports the development of more effective sex-specific strategies to better treat individuals with CVD.

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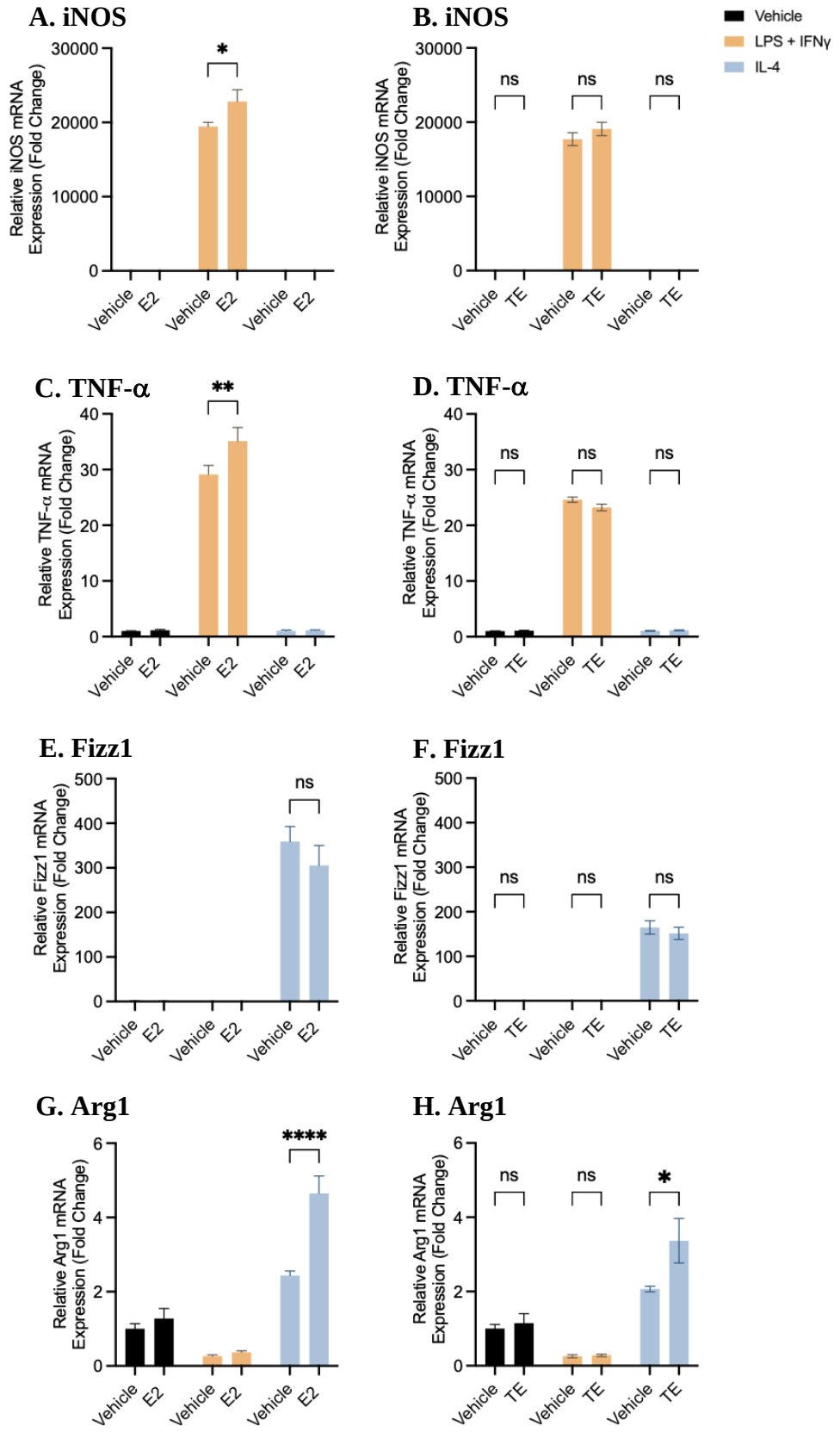
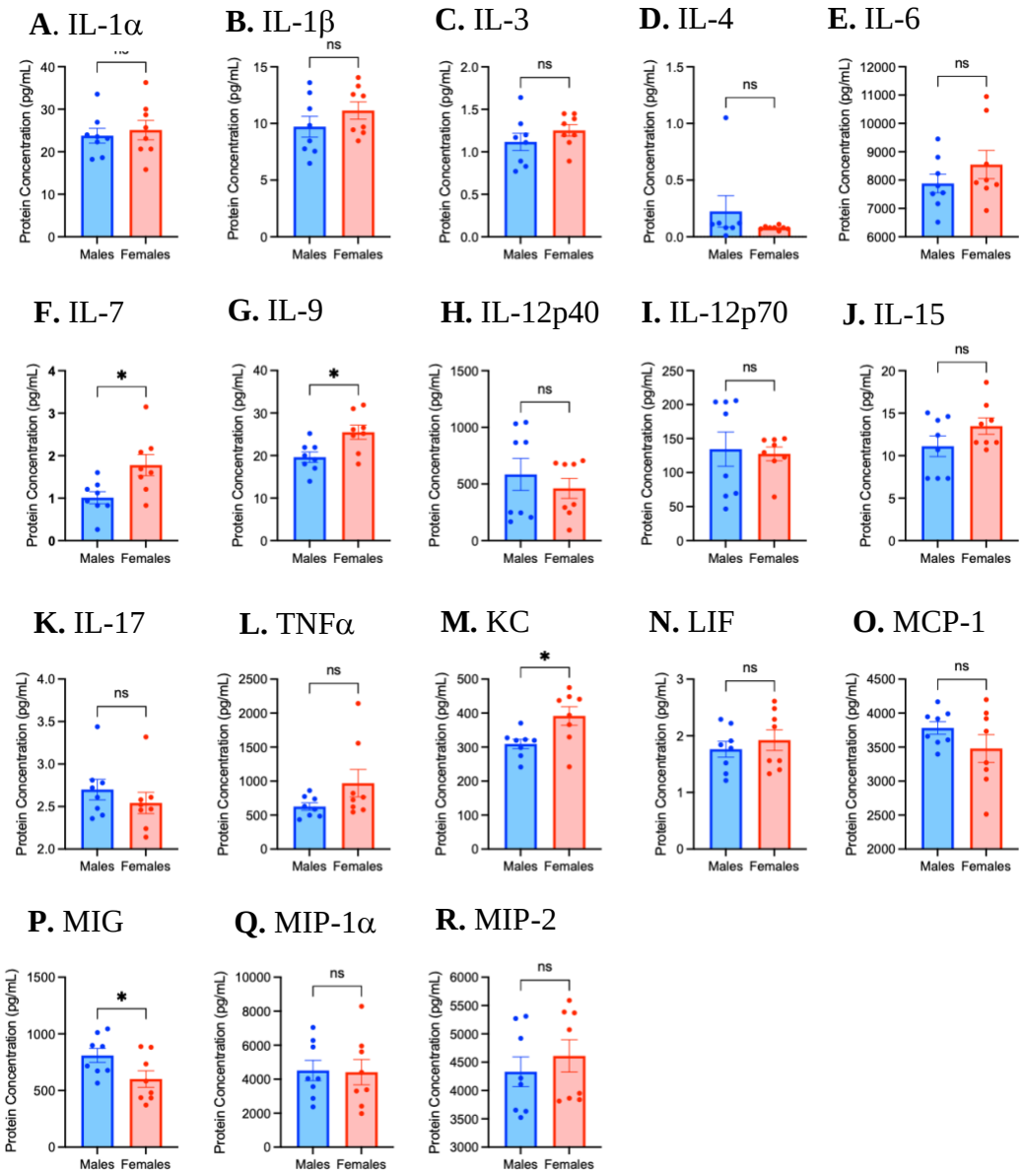
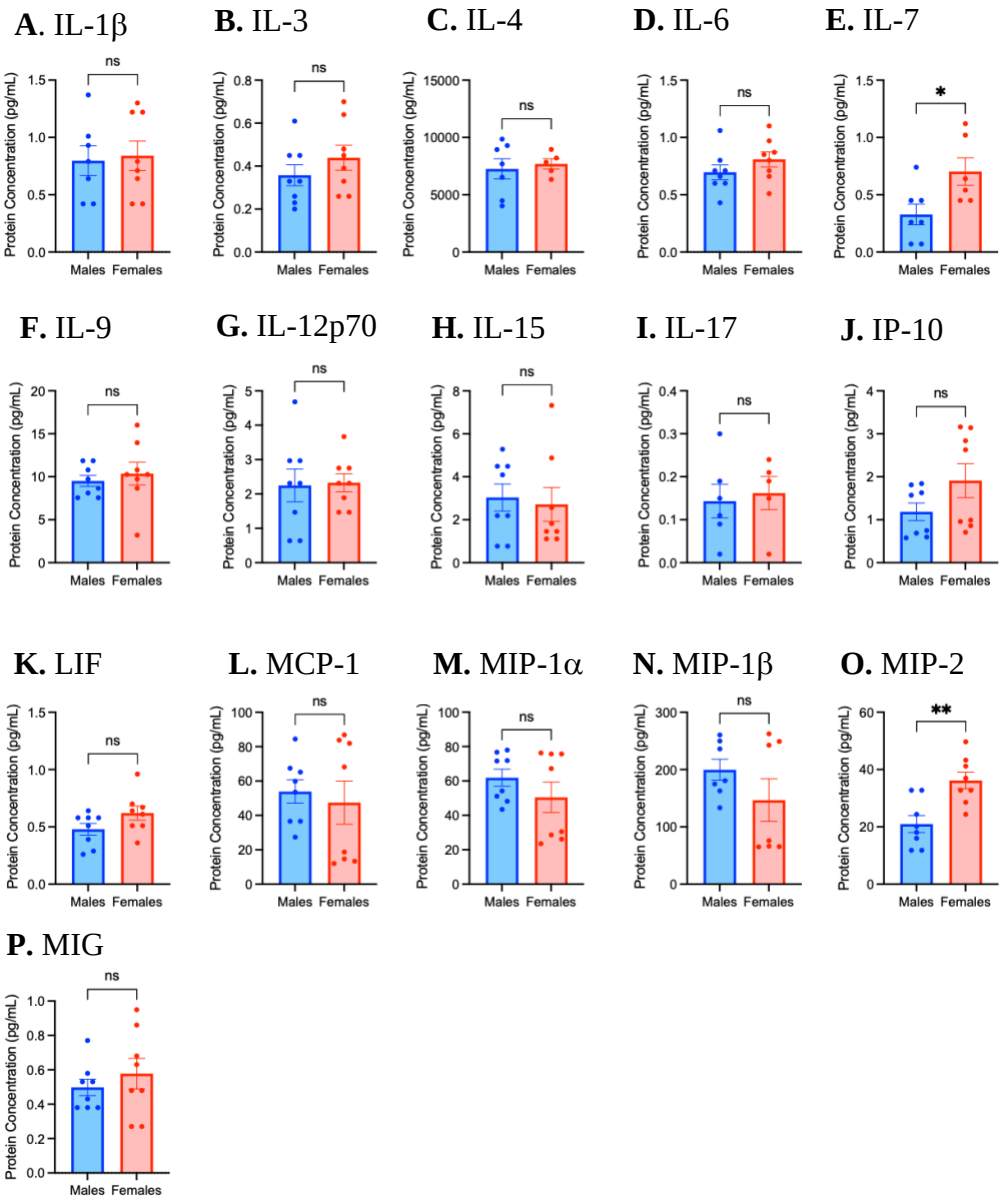
8.0 SUPPLEMENTARY FIGURES



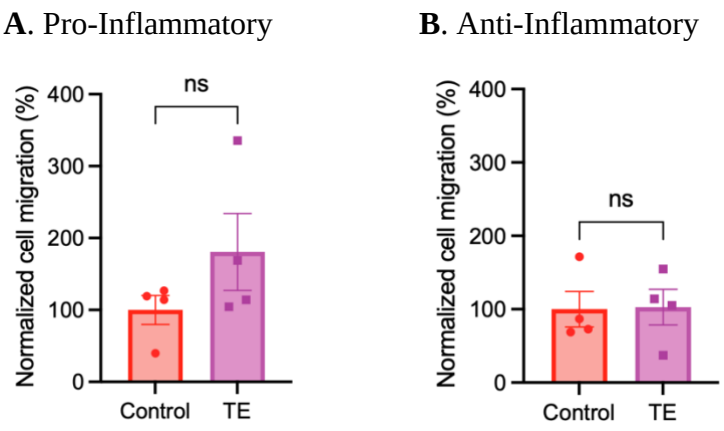
Supplementary Figure 1: Gene expression of pro- and anti-inflammatory markers in female-derived BMDMs pre-treated with 100 nM 17β-estradiol (E2) or testosterone (TE). Female-derived BMDMs were pre-treated with 100 nM E2 or TE for 24 hours and polarized to pro- and anti-inflammatory BMDMs using LPS and IFNγ and IL-4, respectively, or the vehicle control, for 6 hours. Gene expression of **A., B.** iNOS, **C., D.** TNF- α, **E., F.** Fizz1, and **G., H.** Arg1 from E2- or TE-treated was quantified by RT-qPCR. Data shown represent the mean ± SEM fold change ($2^{-\Delta\Delta Ct}$) in marker expression relative to reference gene β-actin, normalized to the vehicle control. Two-way ANOVA and Tukey's multiple comparison test were performed to evaluate significant differences (n=4, *p<0.05, **p < 0.01, ****p < 0.0001).



Supplementary Figure 2: Protein concentration of pro-inflammatory cytokines and chemokines secreted by pro-inflammatory BMDMs derived from male and female mice. Bone marrow monocytes were differentiated into BMDMs, and cells were treated with LPS and IFNγ for 24 hours. Data shown represent the mean ± SEM protein concentration of secreted cytokines and chemokines from cell culture media of BMDMs, quantified by an addressable laser bead immunoassay (ALBIA). Two-tailed unpaired t-test was used to evaluate significant differences. (n=5-8, *p<0.05).



Supplementary Figure 3: Protein concentration of pro-inflammatory cytokines and chemokines secreted by anti-inflammatory BMDMs derived from male and female mice. Bone marrow monocytes were differentiated into BMDMs, and cells were treated with IL-4 for 24 hours. Data shown represent the mean ± SEM protein concentration of secreted cytokines and chemokines from cell culture media of BMDMs, quantified by an addressable laser bead immunoassay (ALBIA). Two-tailed unpaired t-test was used to evaluate significant differences. (n=5-8, *p<0.05).



Supplementary Figure 4. The effects of 100 nM testosterone (TE) on BMDM migration. Differentiated BMDMs from female mice were pre-treated with 100 nM TE and polarized to pro- or anti-inflammatory phenotypes. A Transwell migration assay was performed with or without chemoattractant CCL19 in culture medium of the lower assay chamber. Data represent the mean ± SEM cell migration (%) of **A.** pro-inflammatory or **B.** anti-inflammatory BMDMs, normalized to the vehicle-treated control. Two-tailed, unpaired t-tests were performed to evaluate significant differences (n=4, $p<0.05$).