Epigenetics and Sexual Dimorphism of Macrophages in repair of Myocardial Infarction

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Abstract

Cardiovascular disease (CVD) continues to be the predominant cause of death and injury across America (Centers for Disease Control and Prevention). For the majority of these cases, women have been known to show better outcomes and different symptoms compared to men (Roth et al., 2018). Historically, discrepancies in health and biological responses in women have been attributed to estrogen. However, studies have shown mixed results on the role of estrogen and its protective effects (Hodis et al., 2022). Here we show a larger, more convincing contributing factor: epigenetic regulation of the genes responsible for controlling and initiating inflammatory and healing responses. In the cell model macrophages, methyltransferase Euchromatic histone lysine methyltransferase 2/Gene 9a (Ehmt2/G9a) di/tri-methylates the histone 3 lysine 9 mark (H3K9me2/3). This action controls the expression of two key genes of interest: CC motif ligand 3 (CCL3), a proinflammatory gene, and Vascular endothelial growth factor (VEGF). The research demonstrates sex-dependent differences in pro-angiogenic signaling, and potentially different relationships of methyltransferase activity with CCL3 and VEGF in macrophages compared to other cell types previously used in research (such as endothelial progenitor cells (EPC)). Looking at the differences in gene expression and regulation of CCL3 and VEGF, we can begin to understand how this drives macrophage response to myocardial infarction and how this may vary in females versus males; potentially contributing to higher inflammation and worse outcomes in males compared to females. Understanding the

epigenetics of the sexual dimorphism seen post-MI can open doors to new potential therapies to rescue the male response to mimic that of the female.

Sexual dimorphism in post-myocardial infarction

Cardiovascular disease (CVD) remains the leading cause of morbidity and mortality for both men and women; however, differences in onset, progression, and outcomes persist. Sex differences in CVD outcomes have been largely attributed to effects of estrogen, despite trials showing mixed results (Manson *et al.,* 2003; Prentice *et al.,* 2005; Regitz-Zagrosek & Gebhard, 2023). However, the lower incidence of CVD in women persists decades beyond menopause, and prepubescent females exhibit reduced susceptibility to ischemic injury; hormone replacement trials have shown that neither estrogen nor estrogen with medroxyprogesterone acetate (a progestin hormone treatment) significantly halted any CVD risk (Charan Thej and Raj Kishore, 2024; Hodis & Mack, 2022). Increasing evidence indicates that epigenetic regulation of immune and progenitor cells, rather than sex hormones, contributes to these differences.

Following myocardial infarction (MI), circulating monocytes infiltrate the injured myocardium and differentiate into macrophages that trigger inflammation and tissue repair (Yang et. al., 2024). These signals initiate epigenetic regulatory mechanisms in genes responsible for controlling and initiating inflammatory and healing responses; CC motif ligand 3 (CCL3) and Vascular Endothelial Growth Factor (VEGF). Preliminary data reveals that male monocytes secrete higher levels of cytokines such as CCL3, whereas female monocytes exhibit reduced inflammatory activity. These differences correlate with distinct histone H3 lysine-9-di and trimethylation (H3K9me2/3) marks, governed by the histone methyltransferase EHMT2 (G9a). This represses inflammatory genes. In this review, I propose that epigenetic mechanisms, rather than sex hormones, drive the reparative mechanisms of female monocytes to macrophages

following MI, which are superior to that of the male phenotype. By defining EHMT2–H3K9me2/3–CCL3 and modifying male monocytes toward a low-inflammatory and proangiogenic state that resembles that of the females, we aim to enhance post-infarction cardiac repair. This demonstrates sex-differential cardiac protection by establishing epigenetic control of monocytes and reparative function as a key determinant of myocardial recovery, offering new therapeutic avenues to mimic the reparative advantage of the female immune system.

Epigenetics

Post translational modifications alter the epigenome of genes in response to environment and external factors. Though these modifications were described in the 1940s, first introduced by Waddington, only decades later were they suggested to modulate gene expression (Dupont et al., 2009). Modifications to histones, organizational proteins in which the DNA wraps around, are implicated in physical regulation of expression by organizing chromatin structure. Consisting of histones H1, H2A, H2B, H3, and H4, DNA coiled around it forms a nucleosome and connected by linker DNA throughout forms the chromatin structure (Figure 1). Histone modifying enzymes are responsible for modifications to specific residues on the histone "tails" to aid in the condensing or opening of the chromatin structure; namely to specific residues of lysine, serine, or arginine residues (Dupont *et al.*, 2009). Due to DNA's negative charge, factors such as acetylation (addition of a neutrally charged polar methyl group bound to a carbonyl) or methylation (addition of a neutral nonpolar methyl group) control the tightness or looseness of the DNA coiled around the histone and thus its transcription. Acetylation on lysine residues for a number of histone marks remains associated with transcriptional activation (e.g., H3K9, H3K14, H3K18, H3K23, H4K5, H4K8, H4K12, and H4K16) compared to methylation on lysine residues, which depending on the location either yields transcriptionally activating or repressing

effects (Dupont et al., 2009). Due to the nucleophilic nature of its side chain, lysine is easily susceptible to methylation. Because of this, mono-, di-, or even tri-methylation events at the lysine residue of a histone tail can lead to gene suppression or expression.

Heterochromatic histone marks histone 3 lysine 9 trimethylation (H3K9me3) and histone 3 lysine 27 tri-methylation (H3K27me3), are constitutive gene repression marks that exhibit different alterations between male and female mice (Charan Thej & Raj Kishore. 2024; Thej *et al.,* 2024). H3K9me3, another histone mark, mediates the condensing of heterochromatin and thus gene silencing. It is also a reciprocal alteration of histone H3K27 and H3K4 methylation that regulates M2-Macrophages (Wang *et al.*, 2024). The significance of H3K9me2/3 is that its methylation leads to gene repression. The methyltransferase responsible for this, euchromatic histone lysine methyltransferase 2 (Ehmt2, or Gene 9a), controls the methylation of this histone mark in CCl3 gene leading to its repression and thus less inflammatory activity.

The sex differences in the gene expression of vascular endothelial growth factor and macrophage inflammatory protein-1 alpha (MIP-1α; CCL3) in macrophages can be attributed to the regulation by the histone methyltransferase Ehmt2/G9a. Euchromatic histone modifications such as H3K4me3, H3K27ac, and H3K36me3 differed between males and females, regulating X-chromosome inactivation –DNA methylation that balances the gene dosage in females with males–among other functions (Thej *et al*., 2024). This was also observed in the heterochromatic histone marks H3K9me3 and H3K27me3, which are constitutive gene-repression marks that exhibit distinct alterations between male and female mice (Thej *et al.,* 2024). Histone 3 Lysine 9 di/trimethylation is particularly notable as this mediates the condensing of heterochromatin and thus gene silencing. It is also a reciprocal alteration of histone H3 lysine 27 and histone 3 lysine 4 methylation that regulates M2-Macrophages (Levinsky *et al.*, 2022). Gene 9a (Ehmt2) acts as a

methyltransferase to catalyze the trimethylation of the histone H3K9, downregulating CCL3 expression in macrophages which differs from endothelial progenitor cells (EPCs); where Ehmt2 methylates and silences CCL3 and VEGF due to the vessel forming nature of these cells (Thej *et al.*, 2024). Upon inhibiting Ehmt2 methylation activity on CCL3 in mouse EPCs in male, female, and ovariectomized, it was shown that the cell secretion was enhanced, demonstrating Ehmt2 regulating activity of CCL3 and that there is a relationship with the methyltransferase that can reduce the inflammatory mark and suppress the gene (Thej *et al.*, 2024).

However, the question then remains of *why, in females, there is improved angiogenic activity compared to males.* The relationship of this methyltransferase extends to VEGF, as epigenetic sequencing revealed similar occupancies of the gene repressive mark H3K9me3 at transcription start sites of key angiogenic and inflammatory genes in Male EPCs, that were not shown in F-EPCs or OVX-EPCs , despite this not yet being shown in macrophages (Thej *et al.*, 2024). Additionally, female mesenchymal stromal cells (MSC's), were shown to secrete higher amounts of VEGF and less MIP-1α compared to male matched cells (Thej *et al.*, 2024). When examining this in monocytes, it was found that pro-inflammatory cytokines and chemokines including CCL3 were highly secreted in male EPCs, and promoted greater migration of monocytes, and did not promote polarization into M2-like macrophages. Female cells, on the other hand, promoted polarization of monocytes into M2-like phenotypes. These results, compared to the similar bone-marrow derived nature of macrophages, are an interesting basis to examine their secretion patterns and if they bare resemblances.

Vascular Endothelial Growth Factor (VEGF)

The primary mechanism in which macrophages regulate angiogenesis is by modulating the amount of expression of VEGF. VEGF responds to the hypoxia resulting from cardiac injury

to promote this angiogenic response, and according to the study done by Zhao *et al.*, VEGF mRNA expression in ischemic zones post-MI increased along the border of these zones, and diminished in later stages (after day 2) (Zhao *et al.*, 2010).

This indicates that VEGF expression is crucial for angiogenesis in early stages of myocardial infarction. Three major isoforms of VEGF arise from alternative splicing and take on various cell surface receptors to mediate responses to VEGF (Zhao *et al*., 2010). When looking at *in situ* hybridization, it was found that VEGF in the normal heart pre-ischemia was evenly expressed throughout the myocardium. Two hours post-MI, an increase in expression in VEGF at the border zones of the ischemic region were detected, however the mRNA levels decreased over the course of 4 weeks. However, post MI VEGF binding increased at the border zones in day 1 and by day 7, the VEGF expression levels *returned back to control level* (Figure 3). Furthermore, this indicates that VEGF activity is at its peak right in the beginning of an infarction event. In connection with what was shown in endothelial progenitor cells, female and ovariectomized EPCs showed a lower inflammatory profile and higher promotion of cardiac reparative activity.

In situ hybridization involves labeling single stranded DNA probes that detect localization of various proteins being expressed, and allow for spatial mapping of the expression levels of the specified protein. Here, single stranded DNA (ssDNA) get tagged with chemical or fluorescent dye, and a labeled probe binds to matching sequences within the ssDNA which can be visualized with fluorescent microscopy (Solomon, 2020). In terms of the results from this study, localization and detection of density of expression of VEGF was done by looking specifically at the mRNA, due to RNA's ability to better reflect the changes in DNA in 'real time' in response to physiological changes in the body and gene activity (Glasscock, 2020).

In the context of macrophage activity, this tight regulation of VEGF expression translates to M2-like macrophage activity to better heal and repair cardiac tissue (Zhao *et al.*, 2010).

CCL3, Signaling, and Communication with Monocytes post-MI

CC motif ligand 3 (CCL3), also known as macrophage inflammatory protein 1-alpha (MIP-1α), is a member of the CC-subfamily of chemokines characterized by the position of their N-terminal cysteine residues. This ~8kD chemokine complexes with glycosylated proteins, promoting direction for cell movement (Bhavsar et al., 2015). This gene is present in macrophages and expresses the chemokines responsible for the M1-like macrophage ability to initiate an inflammatory response; specifically, Il-L (α and **β**), TNF-α, and IL-6 signaling results in macrophage infiltration into the infarct post-MI (Yang et. al., 2024). The events that occur begin with the necrotic tissue resulting from myocardial infarction, which release CC-chemokines. The hypoxia that results from the damaged cardiac tissue triggers cardiomyocytes to release damaged molecular patterns (DAMPs), and stimulates factors that induce expression of VEGF in the myocardium (Zhao et al., 2010). These signals are detected by residential and circulating monocytes and promote phosphatidylserine "eat me signal" recognition and enhance pinocytosis (phagocytic activity of macrophages) (Yang et. al., 2024). Originating from bone marrow, the circulating monocytes report to the infarct area, and are stimulated by macrophage-colony stimulating factors (MCSFs) to differentiate into macrophages that either express interleukin-1β (IL-β) and tumor necrosis factor-α (TNF-α), or interleukin-10 (IL-10) and interleukin-4 (IL-4) (figure 2). The secretion of either of these signals will lead to polarization of M1-like macrophage or M2-like macrophages that are responsible for initiating angiogenesis and cardiac remodeling (Yang et. al., 2024). Macrophages exhibit a high expression of receptors to respond to a multitude of signals, however in the context of angiogenesis post-MI,

the expression of VEGF within the myocardium along border zones of the infarct in conjunction with signaling of circulating monocytes show the complexity of the post-MI response. This demonstrates that early VEGF expression in myocardium, in anticipation of a cardiac injury event, could result in a more angiogenic-driven response post-MI (Zhao et al., 2010). In the context of macrophage activity, this almost 'pre-determining' step could contribute to myocardium already geared for signaling events that promote circulating monocytes to more rapidly polarize into M2-like macrophage phenotype; thus resulting in a pro-healing response. Further analysis in gene and protein expression will be beneficial supplemental material for this argument.

Techniques to measure VEGF and CCL3 in macrophage activity

When looking at gene expression of vascular endothelial growth factor and macrophage inflammatory protein-1 alpha, MIP-1α (CCL3) in female and male macrophages (of each activation level), evidence suggests a sexual dimorphism that echoes what was previously determined in EPC data (Thej, C., *et al* 2024). This continues to be due to the apparent regulation of the histone methyl transferase Ehmt2/G9a. Techniques such as quantitative polymerase chain reaction (qPCR) and Enzyme-linked immunosorbent assays (ELISA) that were used in epigenetic analysis of EPC activity were used to analyze gene expression and protein concentrations in activated, M1-like, and M2-like macrophages in male and female cells (figure 5).

Quantitative PCR, also referred to as real time PCR, is a method used to evaluate gene expression, in which primers, deoxynucleotide triphosphates, a DNA polymerase reagent (Taqman or SYBR Green), buffers, and a fluorescent detection system is incorporated with cDNA to quantify the genes expressed. Rather than performing a polymerase chain reaction, the

reaction occurs in a thermocycler in ‘real-time’, and a plot of cycle threshold (CT) values show the number of cycles required for a fluorescent signal to reach a threshold value (often compared to a known set of values from housekeeping genes such as GAPDH or 18S), and thus quantifying DNA (*Introduction to Gene Expression*). In the instance of measuring gene expression of CCL3, Ehmt2/G9a, and VEGF (figure 4b), Expression levels of pro-inflammatory genes were significantly higher in M-EPCs compared with F-EPCs and OVX-EPCs and there are no differences in the inflammatory panel between female and ovariectomized EPCs. This showed gene expression of a multitude of factors that are associated with inflammation, which included IFNγ, and TNFα that we previously observed to determine M1-like phenotype in macrophages (Thej *et al.,* 2024). In terms of angiogenic factors and their results, however, observing VEGF factor expression showed that there was no significance between M-EPC and OVX-EPC expression– something that is counterintuitive to what we thought previously and could suggest potential effects of estrogen when in the context of angiogenesis. However, further investigation and continuation of this into cell model macrophages could provide more context for any areas where there could be a role of estrogen. Additional assays however, showed greater contributions to sex differences in post- MI response from epigenetic regulation mechanisms.

Along with gene quantification, protein quantification techniques such as ELISA can be used to measure proteins and cytokine secretion. ELISA operates by antigen-antibody specificity, by first letting the sample interact with primary antibody (already coated onto the wells of the ELISA plate) to isolate the antigen of interest, and then by using a detection antibody that binds a different epitope on the antigen forming a sandwich ELISA. The sample is measured against a specific wavelength of light in a measurable enzyme-substrate reaction that directly correlates to antigen concentration (*Sandwich ELISA Protocol*). When using ELISA to measure the secretion

of CCL3 and VEGF in male and female macrophages of each activation level, in conjunction with gene quantification, it is shown that there is higher activation of CCL3 genes (Figure 5a) as well as H3 marks and secretion of CCL3 (Figure 5b) in activated male macrophages compared to female macrophages. When comparing secretion of VEGF, there appears to be no significant difference between M1-like or M2-like macrophages, or differences between sexes. Taken together, these results show sex-dependent differences in pro-angiogenic signaling. In addition, this raises potential different relationships of methyltransferase activity with CCL3 and VEGF in macrophages compared to EPC's. Due to endothelial progenitor cell function to form blood vessels and promote angiogenic activity, more mechanisms to control the activity of pro-angiogenic factors such as VEGF would be more likely to be present in EPCs that is not yet apparent in macrophages.

In addition, interesting similarities were apparent when measuring tube forming capabilities in female and male macrophages from each activation level. In the previous assay with EPCs, results showed higher tube formation and number of branches in female and OVX EPCs than in males. In macrophages, however, this resulted in M2-like macrophages showing a higher number of branches and total length in both male and female; thus reflecting M2-like macrophages' role in pro-angiogenic activity (figures 4 and 6). Further investigation into the gene expression and regulation of macrophages may be beneficial to understanding this discrepancy of secretion of these cytokines.

Euchromatic histone modifications such as H3K4me3, H3K27ac, and H3K36me3 have been shown to differ between male and females, regulating gene expression, X-chromosome inactivation, and a number of other functions (Thej et al., 2024).While research has shown that the action of Ehmt2 is replicated also in gene repression of VEGF in endothelial cells, there is

still no strong evidence to support the role of Ehmt2 as a methyltransferase and thus a mechanism of regulation of VEGF in macrophages (Thej et al., 2024). In a disease model such as myocardial infarction, these findings pose important questions of the mechanism that controls macrophage response to cardiac injury. When observing the results from the experimental model with EPCs replicated in macrophages, both female and male M2-like macrophages showed increased angiogenic activity. While EPCs showed more apparent differences in gene expression and correlation with sex, results with macrophages are less acute. This does raise the question if the epigenetic mechanisms seen in EPCs are slightly different in macrophages, and if there could be less Ehmt2 control for VEGF activity in macrophages compared to EPCs (figure 5b).

Clinical applications and relevance

While it is known that males exhibit worse outcomes than females in response to myocardial infarction, estrogen hormones have been believed to be the cause. However, this does not explain why post-menopausal women continue to exhibit better outcomes post-myocardial infarction compared to males, without the role of estrogen to be the alleged cause. While it is still unclear as to the differences between the H3K9 methylation patterns of CCL3 between males and females, there is increasing evidence to support it altering macrophage activity in the post MI response. In the context of MI, understanding how signals from infarction communicate with circulating monocytes to activate them into macrophages and their polarization to carry out inflammation and healing provides physiological information on what cytokines could better promote M2-like activity. It is shown prior that male response to cardiac injury stalls in the inflammatory phase, while females show improvements in continuing to a healing phase post-MI, further investigation into the relationship and effectiveness of Ehmt2 inhibition of

VEGF and methods to promote M2-like macrophage activity could be beneficial and pose potential therapies (Thej *et al.,* 2024).

Understanding the mechanics of the sex differences in macrophage behavior can aid in our understanding the role of epigenetic regulation of macrophages and thus the cardioprotective differences observed in males compared to females; ultimately bringing us closer to learning more about hormone role in health, rescuing the male phenotype to mimic that of the female, and more inclusivity in medicine.

Figures

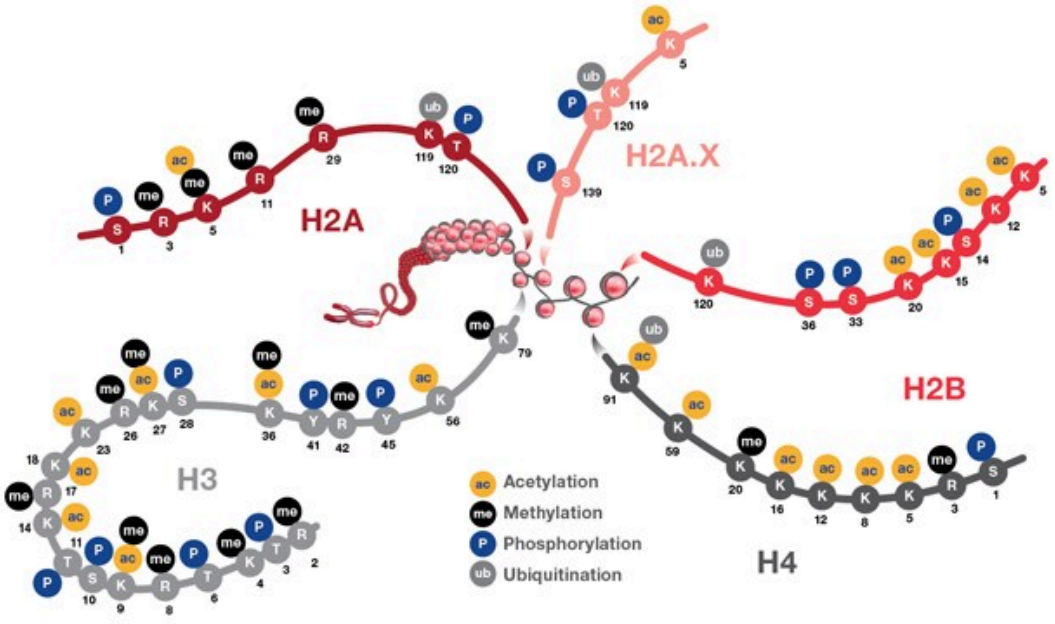


Figure 1: Post-translational marks of histone tails, adapted from ThermoFisher scientific. Nucleosomes are represented by red spheres wrapped by DNA shown in gray. Modifications can be seen in acetylation (in yellow), methylation (in black), and ubiquitination (in light gray). Two modification patterns to the histone 3 lysine 9 tail are shown; acetylation and methylation, which the effects of these marks are discussed in this review.

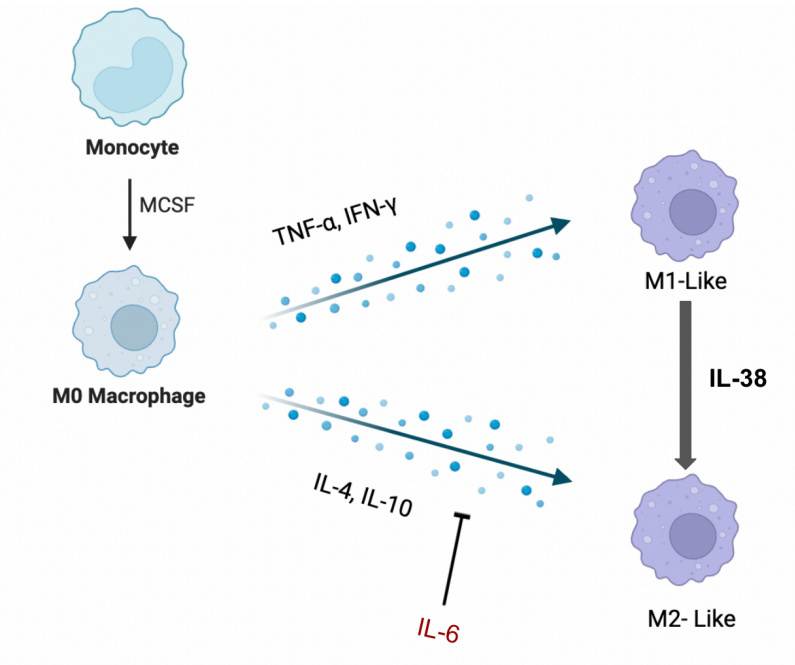


Figure 2: Activation of circulating monocytes into macrophages, and their activation based on varying cytokines released, created using https://BioRender.com. Cytokines responsible for promoting polarization of the M0 macrophage into M1-like or M2-like macrophages are shown, and the transformation of M1-like to M2-like macrophage. In the context of MI, chemokines released from necrotic tissue are the key piece that leads to activation of circulating monocytes, which results in their assigned roles of what macrophage to polarize into and thus function to carry out.

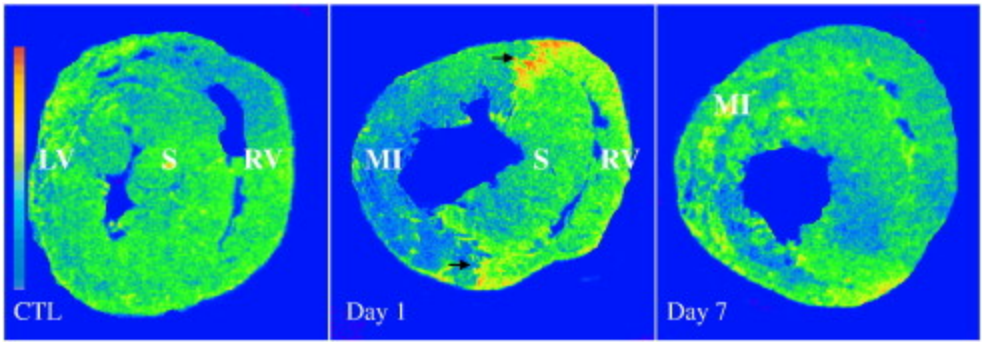


Figure 3: *In situ* hybridization of ischemic heart showing increase of expression of VEGF mRNA post-MI, and then a decline in the weeks after, adapted by Zhao et al., 2010. Here they showed that VEGF was normally expressed in both the left and right ventricles, however post MI VEGF binding increased at the border zones in day 1 and not in later stages. VEGF showed to be largely decreased in the infarct zone at day 1 post-mI and *returned to control level at day 7*. This is a significant finding, as it shows that VEGF expression fluctuates in response to cardiac injury and is at its most active right in the beginning of the formation of the infarct.

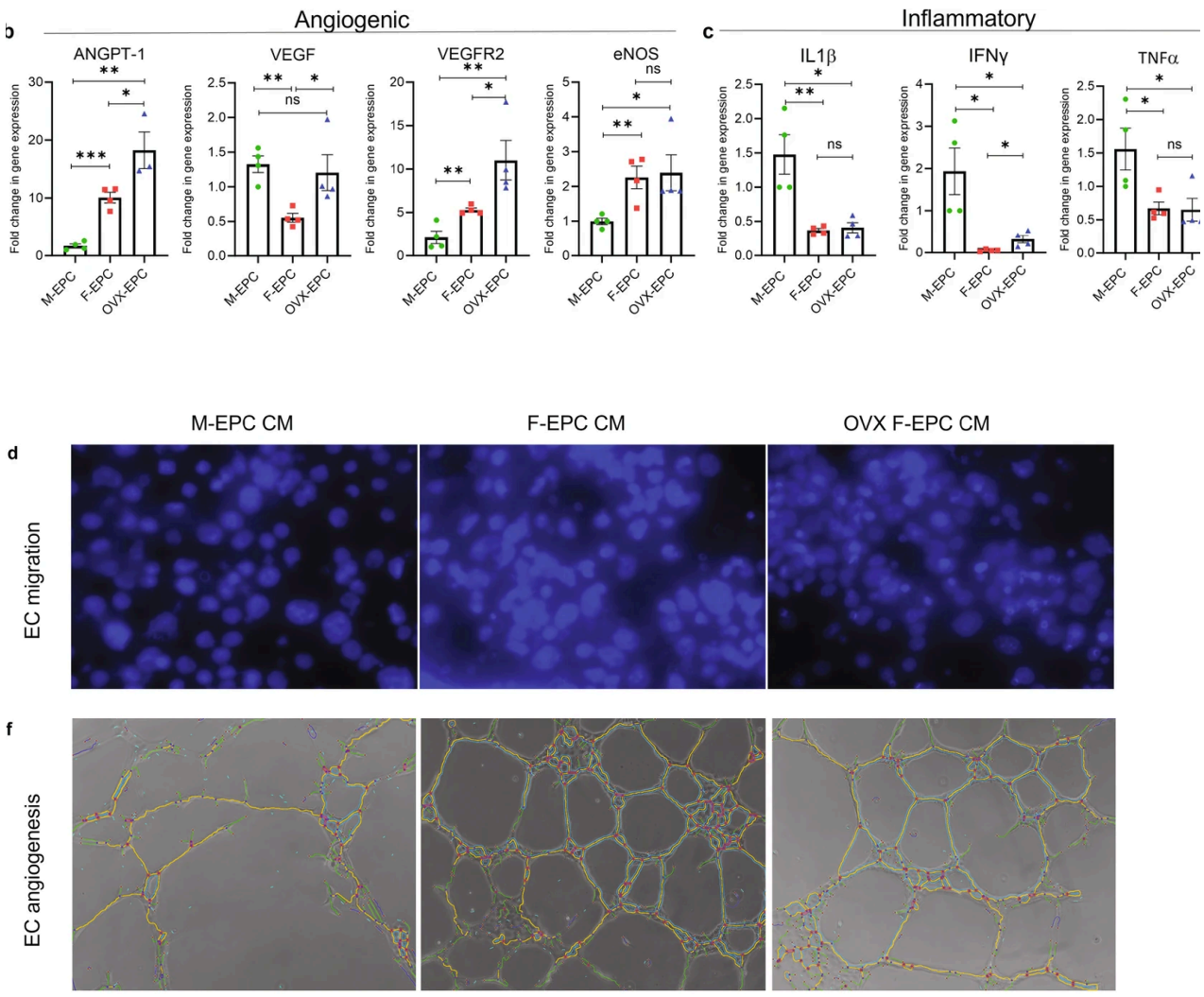
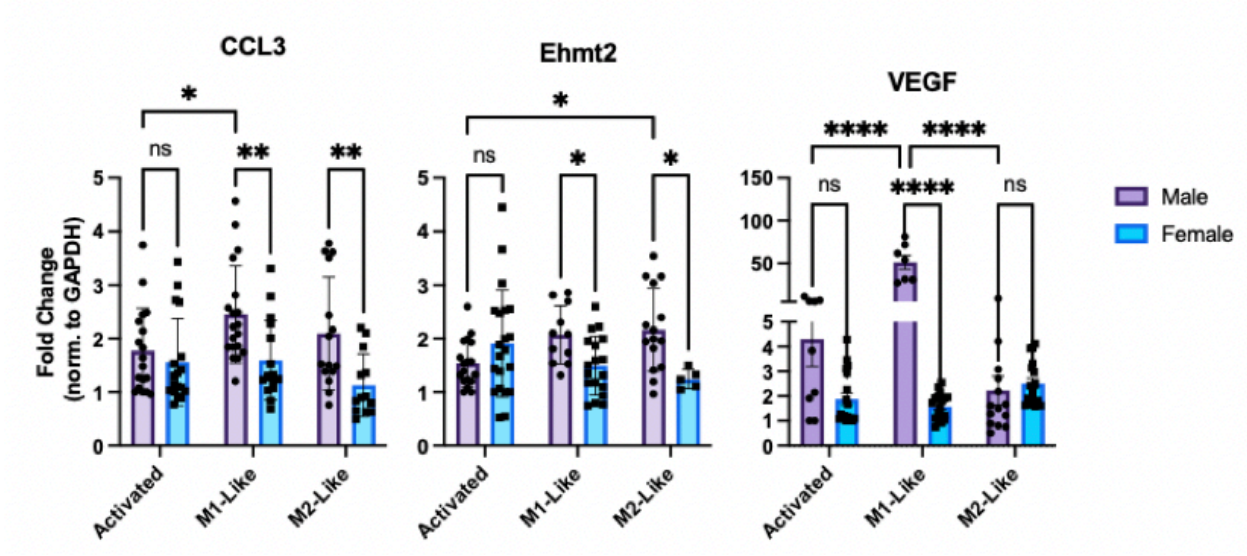


Figure 4: Angiogenic and Inflammatory qPCR results adapted from figure 2 Thej et al., 2024. Panel above (4b) shows qPCR gene expression of angiogenic factors (left) and inflammatory factors (right). Expression levels of pro-inflammatory genes were significantly higher in M-EPCs compared with F-EPCs and OVX-EPCs and there are no differences in the inflammatory panel between female and ovariectomized EPCs. In the migration assay (d), high localization of CCL3 in the male cells demonstrates higher inflammatory activity in males, Tube formation (f) in the female and OVX cells demonstrates higher angiogenic activity than in the male cells.

a



b

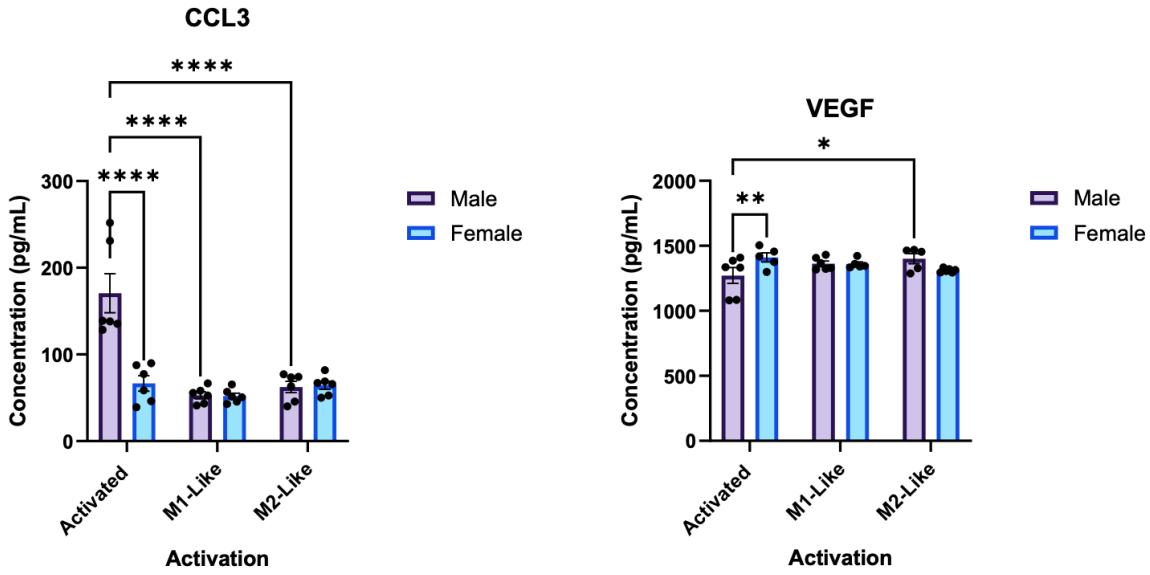


Figure 5: Data generated by the author in the Kishore lab. Gene expression of CCL3, Ehmt2, and VEGF in WT BMDMs (normalized to GAPDH) (5a). Data are presented as mean ± SEM; two-way ANOVA analysis highlights differences in expression across sex and macrophage activation states. VEGF expression is highest in male M1-like macrophages, indicating

sex-dependent differences in pro-angiogenic signaling. Cytokine expression done by ELISA, generated by the author in the Kishore lab shows the corresponding protein expression (5b).

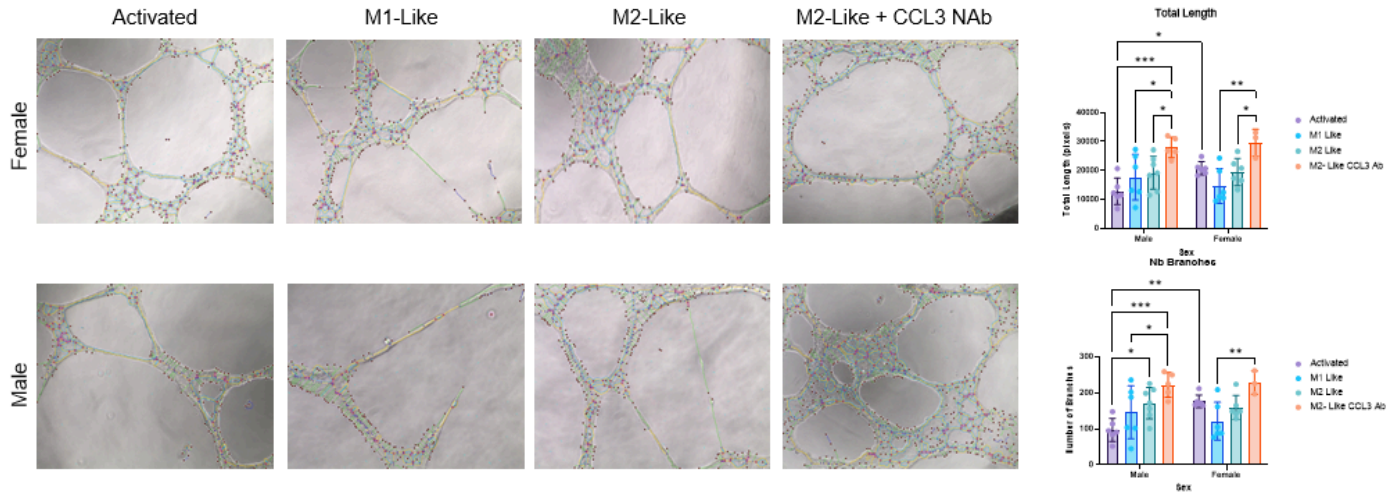


Figure 6: Data generated by the author in the Kishore lab. Macrophages showed that M2-like macrophages in both male and female showed higher total length and nb branches. Importantly, there is no OVX-mice included for comparison as the EPC results showed.

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