Sex-specific and Hormonal Effects on Macrophage MMP-2 Activity in Response to Vitamin D

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Abstract

Macrophages regulate inflammation and tissue remodeling partly through matrix metalloproteinase-2 (MMP-2), an enzyme involved in extracellular matrix (ECM) degradation and tissue remodeling. Vitamin D and 17β-estradiol (E2) are important immunomodulatory hormones, but their specific interactions with MMP-2 remain poorly understood. The following study investigates how polarization state, hormonal environment, and cell sex derivation affect macrophage MMP-2 activity. Female-derived J774A.1 (XX) and male-derived RAW264.7 (XY) murine macrophages were polarized to pro-inflammatory (M1) or anti-inflammatory (M2) states or remained unpolarized (M0) and were cultured in basal or hormone-free media. They were then treated with low- (5 nM) or high- (100 nM) dose vitamin D and/or E2, separately and in combination. MMP-2 activity was subsequently quantified by gelatin zymography. In J774A.1 cells, M1 polarization increased MMP-2 activity in basal media, whereas M2 polarization reduced it. RAW264.7 cells showed the highest basal MMP-2 activity in the M0 state, with reduced activity after M1 polarization. High-dose vitamin D generally suppressed MMP-2 activity across both cell lines and polarization states, whereas low-dose responses were more variable, including a notable spike in RAW264.7 M1 cells. E2 often exhibited a “rescue effect” on vitamin D-induced MMP-2 suppression in a dose- and phenotype-dependent manner. Overall, these findings show that macrophage MMP-2 activity is shaped by cellular sex-derivation, macrophage polarization, endocrine context, and hormone dosage, highlighting the need for greater consideration of cellular sex-derivation and hormone microenvironments in in-vitro cellular models.

Introduction

Macrophages are large innate immune cells that play critical roles in inflammatory responses and tissue remodeling. They are highly plastic in their ability to adopt distinct phenotypes in response to specific cues from their microenvironments, broadly adopting either a "pro-inflammatory" (M1) or "anti-inflammatory" (M2) phenotype. The pro-inflammatory phenotype is associated with immediate response to infection, while the anti-inflammatory phenotype is involved in fibrosis and tissue remodeling. One mechanism by which macrophages regulate these processes is through the secretion of matrix metalloproteinases (MMPs), which are a large family of enzymes responsible for degrading extracellular matrix (ECM) components. Macrophages secrete MMPs to physically clear pathways to allow for cell migration and infiltration at tissues to sites of infection. Additionally, macrophages utilize MMPs to degrade ECMs when damaged to allow space for tissue remodeling. MMP-2 is an MMP subtype that is primarily responsible for the degradation of type IV collagen, the tough primary structural component of the cellular basement membrane. It is also responsible for breaking down denatured collagen (gelatin) produced as a byproduct of degradation of the initial extraneous layers of the extracellular matrix. MMP-2 can degrade or activate the inflammatory cytokine IL-1β, as well as chemokines CXCL12, CCL7, and CCL2, further displaying its important and complex role in mediating bodily inflammation (Hannocks et al., 2019).

The dysregulation of MMP-2 is strongly linked to involvement in multiple organ systems as well as clinical pathologies due to its role in weakening the structural integrity of the ECM. MMP-2 overexpression is shown to progress multiple cardiovascular diseases, worsen diabetes complications, intensify renal diseases, facilitate tumor invasion and metastasis and increase risk for stroke, dementia, and autoimmunity (Wolosowicz et al., 2024).

In the context of autoimmune diseases, which are characterized by chronic inflammation, elevated circulatory MMP-2 levels have been heavily implicated in rheumatoid arthritis, systemic lupus erythematosus, and multiple sclerosis (Chang et al., 2008) (Avolio et al., 2003). Vitamin D and E2 are synergistic hormones that can upregulate each other in vitro, best known in their protective synergistic effects against osteoporosis (Liel et al., 1999). Their cooperation has been of increased interest in autoimmune studies; for example, female-specific E2 and vitamin D synergy has been observed in a mouse model of multiple sclerosis, and vitamin D supplementation has been shown to upregulate estrogen synthesis in breast cancer cells (Nashold et al., 2009) (Krishnan et al., 2010). Despite these findings on cooperative effects between E2 and vitamin D, their combined effects on macrophage remodeling activity remain poorly understood.

Of interest in these diseases are also a number of steroid hormones, including vitamin D and 17β-Estradiol (E2). Although vitamin D is best recognized as a regulator of calcium and bone health, recent evidence has explored its significant and diverse role as an immunomodulator. Vitamin D directly modulates the inactive and active forms of MMP-2 in uterine fibroid cells and affects macrophage polarization to pro-inflammatory and anti-inflammatory states (Halder et al., 2013) (Stachowicz-Suhs et al., 2024), yet macrophage MMP-2 responses to vitamin D remain unknown. Likewise, 17β-Estradiol (E2) is a female sex hormone that plays an important role in chronic inflammation, and also directly modulates MMP-2 responses (Nilsson et al., 2006) in other cell models. However, its role in shaping macrophage-specific responses to Vitamin D has been underexplored, especially in the context of sex karyotype (XX vs XY) and polarization state (pro- vs anti-inflammatory).

Although MMP-2 mRNA expression has been reported to be higher in pro-inflammatory than in anti-inflammatory macrophages, the effects of macrophage polarization on MMP-2 activity and their sex-specific differences have not been previously studied (Jager et al., 2015). Using MMP-2 as a marker of macrophage-initiated inflammation and tissue remodeling, the following study explores the immunomodulatory effects of vitamin D, by itself and in tandem with E2, on the ECM-degrading potential of J774A.1 (XX) and RAW264.7 (XY) murine macrophage cell lines, comparing for sex differences across pro-inflammatory and anti-inflammatory phenotypes.

Aims and Hypotheses

AIM #1: Characterize the effects of phenotype polarization (pro-inflammatory vs anti-inflammatory) on MMP-2 activity in J774A.1 (XX) and RAW264.7 (XY) murine macrophages.

Hypothesis 1.1: Pro-inflammatory polarization will yield higher MMP-2 activity than anti-inflammatory polarization across both cell lines. Additionally, a sex-based difference will be observed between RAW264.7 (XY) and J774A.1 (XX) macrophage MMP-2 response to polarization.

AIM #2: To determine sex differences in dose-dependent responses of MMP-2 activity to Vitamin D in macrophage cell lines.

Hypothesis 2.1: Vitamin D treatment will reduce MMP-2 activity in a dose-dependent manner in both cell lines, with sex-based differences observed between the two cell lines.

AIM #3: Determine the modulatory role of 17β-estradiol (E2), alone and in combination with Vitamin D, on MMP-2 activity in pro- and anti-inflammatory macrophage phenotypes.

Hypothesis 3.1: E2 supplementation will modulate MMP-2 activity in a phenotype- and cell-line dependent manner. Co-treatment with Vitamin D will further affect MMP-2 activity in such a manner.

Materials and Methods

RAW264.7 (XY) and J774A.1 (XX) murine macrophage cell lines were cultured in 96-well plates. A preliminary assay was performed to determine optimize cell seeding densities; RAW264.7 cells were plated at 4.7×10^3 cells/cm², and J774A.1 cells were plated at 6.3×10^3 cells/cm². Cells were allowed 24 hours for attachment to each well bottom.

Following adherence, cells were treated with either 100 nM lipopolysaccharide (LPS) and 10 nM interferon-γ (IFN-γ) to induce a "pro-inflammatory" state or 20 nM interleukin-4 (IL-4) to induce an "anti-inflammatory" state. Cells were then allowed 48 hours for polarization into their respective states. Both pro- and anti-inflammatory cells were treated with hormone-free media, low or high (5 nM or 100 nM) VD or E2 dosages, or a combination of both (5 nM E2 + 5 nM VD, 100 nM E2 + 5 nM VD, 5 nM E2 + 100 nM VD, or 100 nM E2 + 100 nM VD). Cells were treated for 24 hours, and cell supernatant samples were isolated from each well.

MMP-2 activity was quantified using gelatin zymography following the 24-hour treatment period. Proteins were separated by molecular weight using Novex 10% Gelatin Gels. Samples within each treatment group were pooled and run in triplicate, with 25 μg of protein loaded per sample. Samples were prepared with Tris-Glycine SDS Sample Buffer and run in 1X

Tris-Glycine SDS Running Buffer. Electrophoresis was performed using the XCell SureLock Mini-Cell system at 125 V and 0.03 A for 2 hours. Gels were then rinsed in 1X Renaturing Buffer for 30 minutes to restore enzymatic activity and subsequently incubated in 1X Developing Buffer overnight at 37°C. Gels were stained with Imperial Protein Stain for 1 hour and washed with deionized water. Gels were imaged using an Odyssey CLx imaging system, and the integral of the sample intensity curve was measured in ImageJ. Each measurement was normalized by dividing the area under the curve by the gel's respective MMP-2 control activity. Statistical significance within each figure panel was assessed using one-way ANOVA followed by Tukey's post-hoc multiple-comparisons test in GraphPad Prism. A p-value of < 0.05 was considered statistically significant. Statistical comparisons should be interpreted as technical comparisons within pooled treatment groups rather than as fully independent biological replication, as samples were pooled prior to loading.

Results

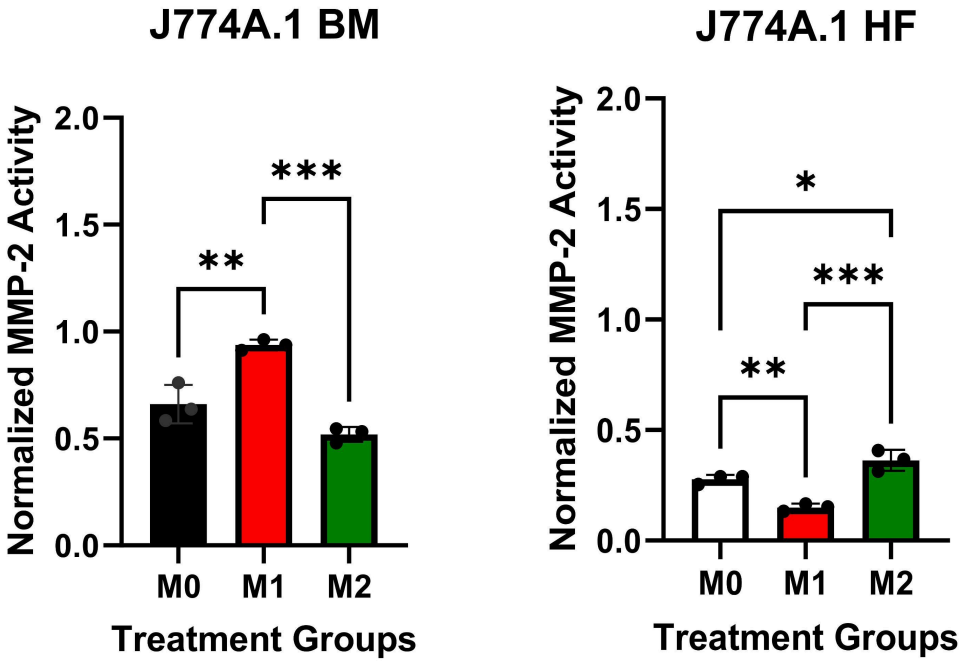

Figure 1: Effect of macrophage polarization and hormone-stripping on MMP-2 activity in J774A.1 (XX) macrophages. MMP-2 activity was quantified via gelatin zymography for M0 (unpolarized), M1 (pro-inflammatory), and M2 (anti-inflammatory) phenotypic states. Cells were cultured in basal media (BM) versus hormone-free (HF) media. Activity was normalized to a respective MMP-2 control. Statistical significance was determined via one-way ANOVA with Tukey's post-hoc test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

In basal media for female-derived J774A.1 macrophages, polarization to a pro-inflammatory (M1) state increased MMP-2 activity, while polarization to an anti-inflammatory (M2) state decreased activity as compared to the unpolarized (M0) state. Across M0, M1, and M2 polarizations, hormone-stripping resulted in significantly reduced MMP-2 activity as compared to their respective basal media counterparts. Conversely, in hormone-free media, polarization to an M1 state decreased MMP-2 activity, while polarization to an M2 state increased MMP-2 activity as compared to both the M0 and M1 states.

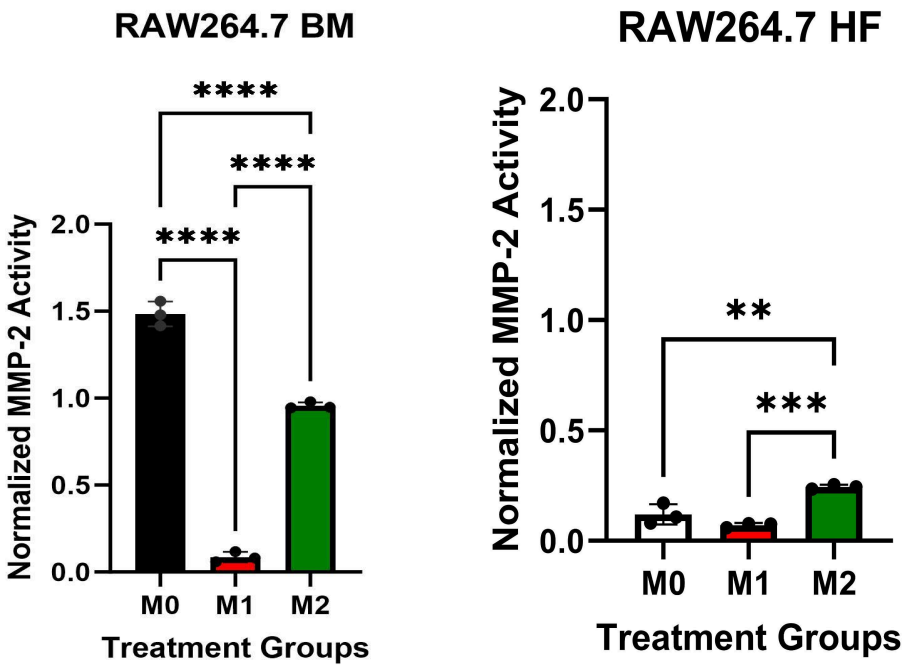

Figure 2: Effect of macrophage polarization and hormone-stripping on MMP-2 activity in RAW264.7 (XY) macrophages. Normalized MMP-2 activity was quantified via gelatin zymography for M0 (unpolarized), M1 (pro-inflammatory), and M2 (anti-inflammatory) phenotypic states. Cells were cultured in basal media (BM) versus hormone-free (HF) media . Activity was normalized to a respective MMP-2 control. Statistical significance was determined via one-way ANOVA with Tukey's post-hoc test (** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$) .

Male-derived RAW264.7 macrophages exhibited a different response to polarization than did J774A.1 cells. In basal media, polarization to both M1 and M2 states significantly decreased MMP-2 activity as compared to the M0 state, with the effect much more pronounced in M1 cells. Additionally, M2 polarization increased MMP-2 activity as compared to the M1 state. In hormone-free media, polarization to an M1 state had no effect on MMP-2 activity, while polarization to an M2 state increased activity as compared to M0 and M1 states. Like J774A.1 cells, a hormone-free environment broadly reduced the cells' overall MMP-2 activities across all three phenotypes as compared to basal media.

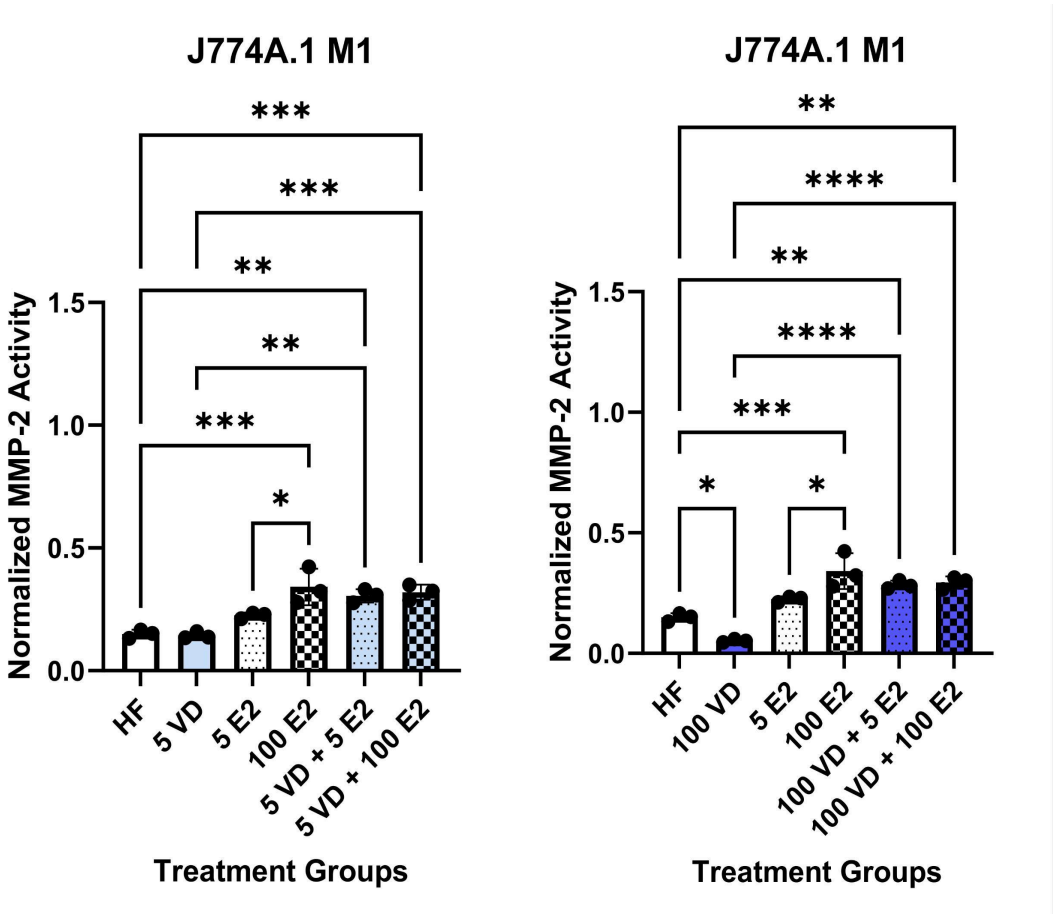

Figure 3: Combinatorial effects of Vitamin D and 17β-Estradiol (E2) on MMP-2 activity in M1-polarized J774A.1 macrophages. Normalized MMP-2 activity in female-derived J774A.1 cells polarized to a pro-inflammatory (M1) state. Cells were treated with low (5 nM, left panel) or high (100 nM, right panel) doses of Vitamin D (VD), 17β-Estradiol (E2), or co-treatments in hormone-free conditions. Activity was normalized to a respective MMP-2 control. Statistical significance was determined via one-way ANOVA with Tukey's post-hoc test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$) .

In female-derived J774A.1 macrophages polarized to the pro-inflammatory (M1) state, low Vitamin D treatment (5 nM) had no significant effect on MMP-2 activity in hormone-free conditions. However, co-dosing 5 nM Vitamin D-treated cells with either 5 nM or 100 nM of E2 significantly increased MMP-2 activity compared to solely 5 nM Vitamin D-treated cells and the hormone-free group.

In contrast to the low-dose treatment, high Vitamin D treatment (100 nM) significantly suppressed MMP-2 activity in hormone-free conditions, further diminishing the activity seen at lower doses. Co-dosing with E2 again displayed a rescue-like effect; dosing 100 nM Vitamin D-treated cells with 5 nM or 100 nM of E2 significantly increased MMP-2 activity, compared to solely 100 nM Vitamin D-treated cells and the hormone-free group. Notably, co-dosing 100 nM E2 with 100 nM Vitamin D increased MMP-2 activity compared to 100 nM Vitamin D alone, but displayed no significant difference from the activity of 100 nM E2 treatment by itself, which increased MMP-2 activity as compared to 5 nM E2 treatment by itself.

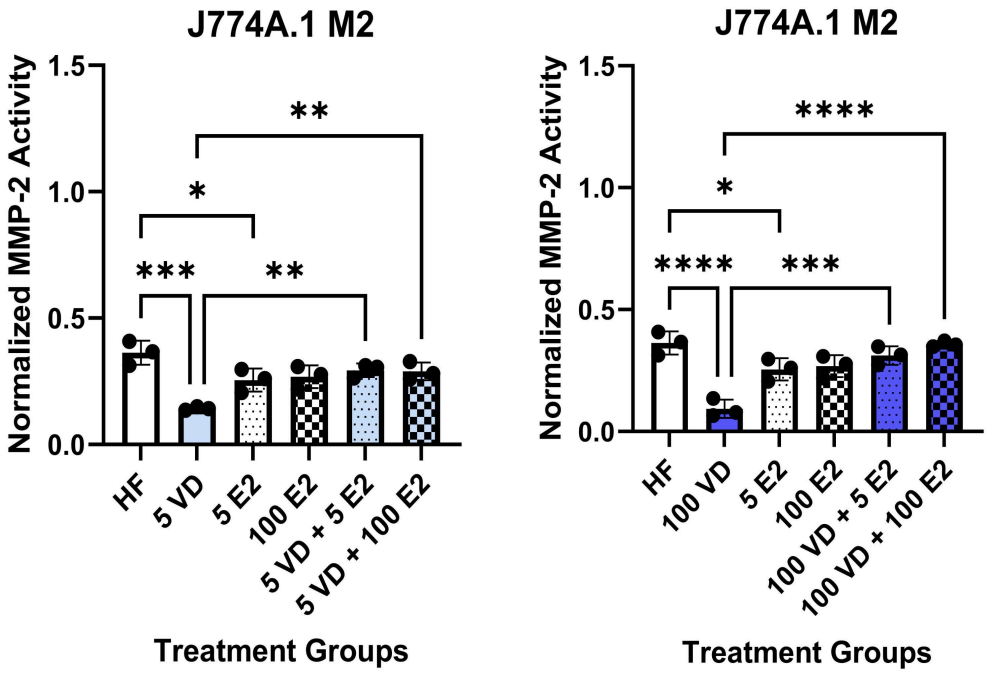

Figure 4: Combinatorial effects of Vitamin D and 17β-Estradiol (E2) on MMP-2 activity in M2-polarized J774A.1 macrophages. Normalized MMP-2 activity in female-derived J774A.1 cells polarized to an anti-inflammatory (M2) state. Cells were treated with low (5 nM, left panel) or high (100 nM, right panel) doses of Vitamin D (VD), 17β-Estradiol (E2), or co-treatments in hormone-free conditions. Activity was normalized to a

respective MMP-2 control. Statistical significance was determined via one-way ANOVA with Tukey's post-hoc test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

In the anti-inflammatory (M2) state, J774A.1 macrophages displayed divergent responses to co-dosing compared to their M1 counterparts. Co-dosing with 5 nM or 100 nM of E2 again displayed a rescue-like effect as compared to the 5 nM Vitamin D baseline, significantly elevating MMP-2 activity. However, dosing cells with 5 nM Vitamin D significantly suppressed MMP-2 activity compared to the hormone-free state, and neither the 5 nM VD + 5 nM E2 nor the 5 nM VD + 100 nM E2 co-dosing groups had any significant effects on MMP-2 activity as compared to the hormone-free group.

Treatment with 100 nM Vitamin D again significantly reduced MMP-2 activity under hormone-free conditions in M2 cells, in similar fashion to their M1 counterparts. The addition of low (5 nM) or high (100 nM) E2 dosages restored MMP-2 activity in 100 nM Vitamin D-treated cells. Notably, the co-dosed groups (100 VD + 5 E2, 100 VD + 100 E2) once again had no significant effects on MMP-2 activity as compared to the hormone-free group, a different response than M1 cells but similar to the low-dose Vitamin D group for M2 cells. In M2 cells, there was no significant difference in MMP-2 activity between the 5 nM E2 group and the 100 nM E2 group.

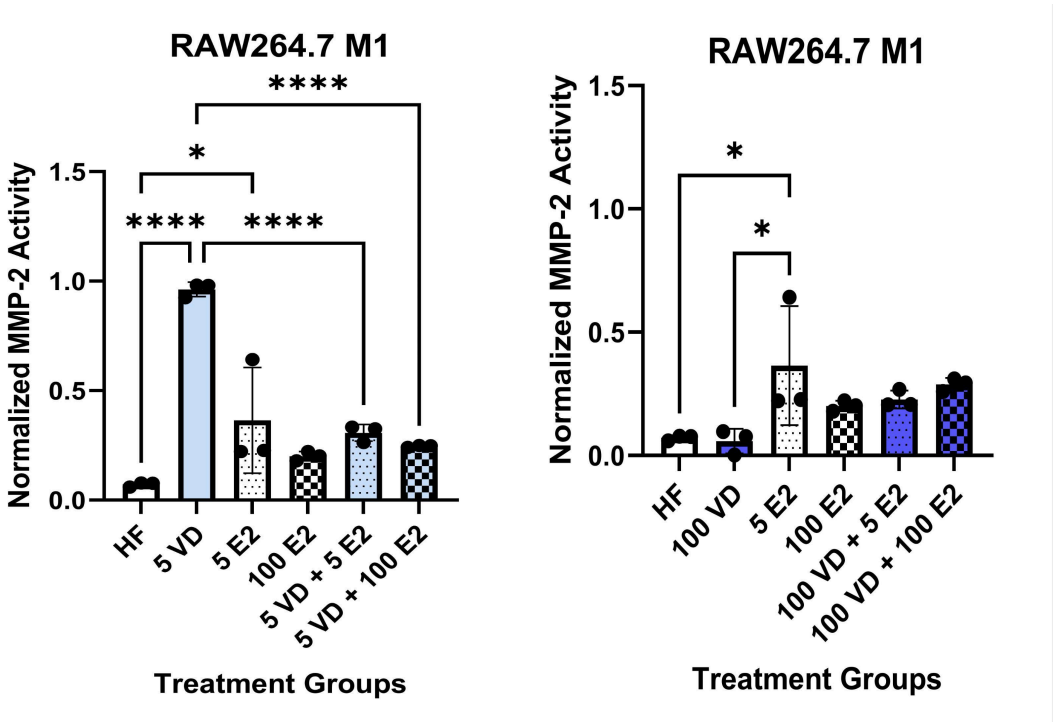

Figure 5: Combinatorial effects of Vitamin D and 17β-Estradiol (E2) on MMP-2 activity in M1-polarized RAW264.7 macrophages. Normalized MMP-2 activity in male-derived RAW264.7 cells polarized to a pro-inflammatory (M1) state. Cells were treated with low (5 nM, left panel) or high (100 nM, right panel) doses of Vitamin D (VD), 17β-Estradiol (E2), or co-treatments in hormone-free conditions. Activity was normalized to a respective MMP-2 control. Statistical significance was determined via one-way ANOVA with Tukey's post-hoc test (* $p < 0.05$, **** $p < 0.0001$).

Male-derived RAW264.7 cells displayed significant sex-differences in their responses to Vitamin D compared to J774A.1 cells. In the M1 state, 5 nM Vitamin D treatment displayed a notable and highly distinct spike of MMP-2 activity compared to the hormone-free state. Co-dosing this baseline with 5 nM or 100 nM E2 displayed significant decreases in MMP-2 activity.

Treatment with the higher 100 nM Vitamin D dose did not significantly affect MMP-2 activity in M1 RAW264.7 cells compared to hormone-free conditions. Co-dosing groups with

high Vitamin D (100 nM VD + 5 nM E2 and 100 nM VD + 100 nM E2) also displayed no significant difference in activity from the baseline hormone-free group. A significant increase in MMP-2 activity was observed in 5 nM E2 treatments, but not 100 nM E2 treatments, as compared to cells in hormone-free conditions.

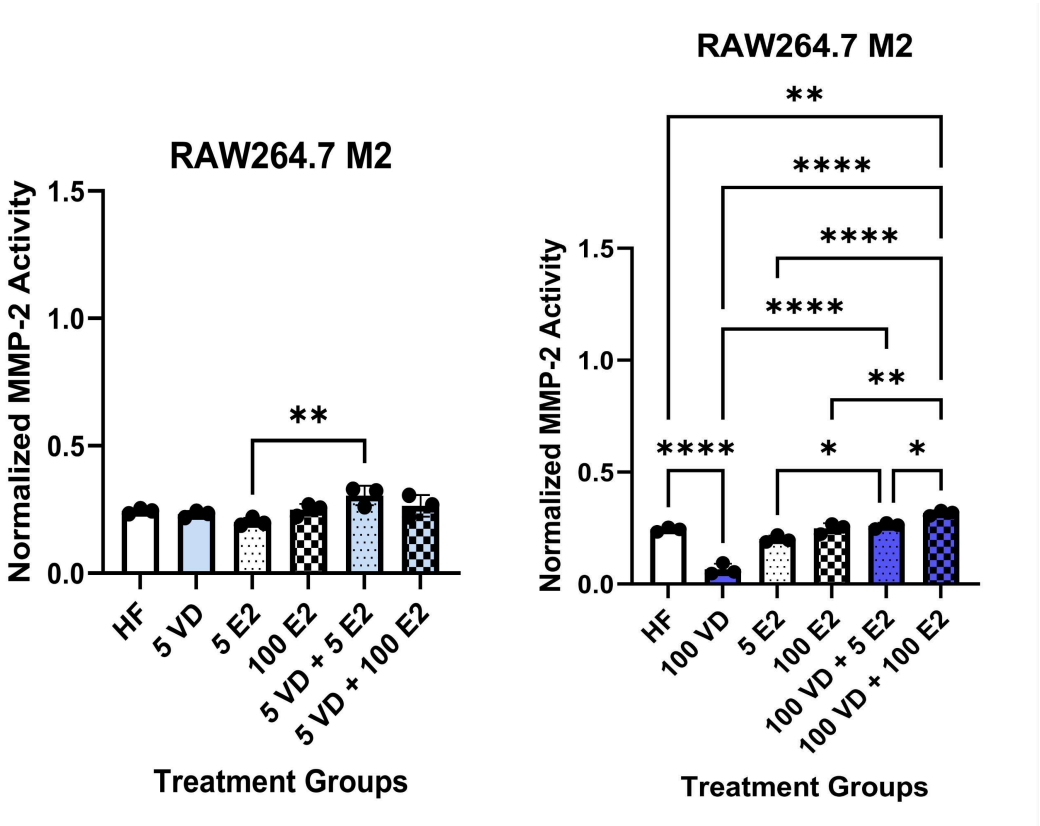

Figure 6: Combinatorial effects of Vitamin D and 17β-Estradiol (E2) on MMP-2 activity in M2-polarized RAW264.7 macrophages. Normalized MMP-2 activity in male-derived RAW264.7 cells polarized to an anti-inflammatory (M2) state. Cells were treated with low (5 nM, left panel) or high (100 nM, right panel) doses of Vitamin D (VD), 17β-Estradiol (E2), or co-treatments in hormone-free conditions. Activity was normalized to a respective MMP-2 control. Statistical significance was determined via one-way ANOVA with Tukey's post-hoc test (** $p < 0.01$, **** $p < 0.0001$).

Notably, no significant differences were observed in any of the co-treatment groups as compared to both 5 nM Vitamin D treatments and hormone-free environments in M2 RAW264.7 macrophages. A significant increase in MMP-2 activity was observed in treating 5 nM E2-treated cells with 5 nM of Vitamin D.

Treatment with 100 nM Vitamin D again significantly suppressed MMP-2 activity compared to the hormone-free state, a behavior that remained consistent across both cell lines and phenotypes. The rescue-like effect of co-treatments with 5 nM and 100 nM E2 was again observed as compared to the 100 nM VD and hormone-free baseline, similarly to M1 J774A.1 cell behavior. Notably, a significant increase in MMP-2 activity occurred in high co-dosing (100 nM Vitamin D + 100 nM E2) as compared to the 100 nM VD and hormone-free baselines, in addition to 5 nM and 100 nM E2-treated groups and the 100 nM Vitamin D + 5 nM-E2 treated group. Lastly, a significant increase in MMP-2 activity was observed in treating 100 nM E2-treated cells with 100 nM of Vitamin D.

Discussion

Overall, the results of this study display that macrophage MMP-2 activity is influenced by polarization state, endocrine context, cell line sex derivation, and hormone dose. Rather than behaving as a uniform marker of macrophage phenotype, MMP-2 activity changed depending on whether cells were cultured in basal or hormone-free conditions, if they were female-derived J774A.1 (XX) or male-derived RAW264.7 (XY), and whether they were exposed to Vitamin D, E2, or both. These findings suggest that macrophage ECM-remodeling behavior is shaped by multiple interacting biological variables rather than macrophage phenotype alone.

A central finding is that the hormonal microenvironment strongly affects MMP-2 activity. Hormone-stripping broadly reduced overall MMP-2 activity across macrophage polarization states in both RAW264.7 and J774A.1 cells, showing that baseline media conditions can have a strong influence on macrophage behavior. This further emphasizes the importance of controlling and documenting the hormonal microenvironment in cell culture research, as common cell culture components such as phenol red can have estrogen-like effects on cells, which could influence other inflammatory readouts from macrophages (Berthois et al., 1986). Therefore, future studies should control and report their cell culture hormonal microenvironment to ensure accurate findings.

The results also show that the sex derivation of these macrophage cell lines can influence behavior beyond traditional “M0/M1/M2” polarization expectations. In basal media, J774A.1 cells displayed higher MMP-2 activity in the M1 state relative to M0 and reduced activity in M2, consistent with the expectation that pro-inflammatory macrophages exhibit higher ECM-degrading behaviors (Moon et al., 2025). However, RAW264.7 cells in basal media exhibited the highest MMP-2 activity in the M0 state with a significant reduction in M1, displaying that sex may underscore traditional “M0 vs M1 vs M2” effects. This means that Hypothesis 1.1 was only supported in J774A.1 cells under basal conditions, but not in RAW264.7 cells. These results are particularly important for future research in a field which traditionally, has dominantly utilized the male-derived RAW264.7 cell-line in research (Veintimilla et al., 2025). Therefore, cellular sex-derivation should be controlled and documented in future studies to ensure accurate ECM-remodeling readouts.

Under hormone-free conditions, high-dose Vitamin D (100 nM) generally suppressed MMP-2 activity across cell lines and M1 and M2 phenotypes. This observation is consistent with

past studies in other cell types showing that Vitamin D can broadly reduce MMP-2 activity (Halder et al., 2013). However, this effect was not always linear. Notably, the RAW264.7 M1 response to low-dose vitamin D (5 nM) deviated sharply from a simple dose-response suppression model, showing a strong spike in MMP-2 activity relative to hormone-free controls. This suggests that macrophage responses to Vitamin D may depend on dose, activation state, and cell line-specific signaling. Therefore, Hypothesis 2.1 was only partially supported in that suppression was observed at a high Vitamin D dose, but not as a linear dose-response across all conditions. Further verification with independent biological replicates and assays is necessary to verify this low-dose RAW264.7 M1 phenomenon.

Co-dosing treatments with 17β-Estradiol (E2) showed that E2 frequently counteracted vitamin D–associated suppression, where co-treatment increased MMP-2 beyond suppression by low- and high-dose Vitamin D in J774A.1 cells polarized to the M1 and M2 phenotypes. In RAW264.7 cells, E2 effects were also dose- and phenotype-dependent, including reductions of the low-dose Vitamin D spike in M1 cells. These findings indicate that Hypothesis 3.1 was supported in that E2 altered macrophage responses, and suggest that E2 effects on MMP-2 activity appear to depend on cell type, dose, and the surrounding hormonal context.

These findings are especially relevant to autoimmune disease because macrophages contribute directly to tissue remodeling and inflammatory signaling. Dysregulated MMP-2 activity could contribute to autoimmune pathology by altering extracellular matrix degradation and prolonging inflammatory responses. The sex-dependent and hormone-dependent differences observed may therefore help explain why autoimmune diseases affect women much more in prevalence and severity. Additionally, this work supports a stronger consideration of sex in future in vitro inflammatory models to ensure a more accurate interpretation of macrophage responses.

There are some notable limitations to this study. First, these experiments were done using immortalized murine macrophage cell lines in vitro, which may not fully represent behavior of human macrophages. Also, samples were pooled for analysis before zymography, which could hide some variability between technical replicates. Lastly, MMP-2 activity was measured through gelatin zymography alone, which does not directly distinguish between changes in gene expression or inhibition. Future studies should include more replicates to decrease variability and human-derived cells for more relevant results. Observing additional markers of macrophage inflammation may also help in understanding macrophage responses more thoroughly.

In conclusion, this study demonstrates that macrophage MMP-2 activity is shaped by an interaction between polarization state, endocrine context, cellular sex-derivation, and hormone dose within J774A.1 and RAW264.7 macrophage models. High-dose Vitamin D generally suppressed MMP-2 activity, while E2 often modified this effect in a cell-line-specific manner. These findings highlight the need to account for sex and hormonal environment when studying macrophage biology and support the use of more sex-aware in vitro models in future autoimmune research.

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