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Sexually-divergent differentiation and inflammatory response to osteoclasts

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SEXUALLY-DIVERGENT DIFFERENTIATION AND INFLAMMATORY RESPONSE OF OSTEOCLASTS

A Thesis

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By

Lilijanna L. Cummings

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INTRODUCTION

Osteoporosis affects about 1 in 3 women and 1 out of 5 men over the age of 50[1]. During osteoporosis, bone is broken down faster than it is replaced leading to bone fragility and increased risk for fracture[2,3]. A decline in estrogen is the main event for most cases of osteoporosis in post-menopausal women due to its regulatory role of resorption in bone remodeling [4]. Previous work comparing resorption between male and female-derived osteoclasts of people of varying ages found that the resorption activity of males less than 50 years old was significantly higher than females of the same age. Exceeding 50 years of age, however, the resorption activity of males decreased while resorption in females increased[5] .The process of breaking down bone matrix and replacing it with new bone matrix is called bone remodeling and occurs as a normal physiological process in response to minor fractures or breaks in order to maintain bone health and sustain mineral homeostasis[6,7]. The cell types involved in this process are osteoblasts (OB), osteoclasts (OC), and osteocytes. OB's and osteocytes are derived from the mesenchymal stem cell lineage, responsible for cells of the body such as connective tissue, cartilage, and fat. Osteoclasts, however, are derived from the hematopoietic stem cell lineage, alongside blood cells and cells of the immune system. OB's lay down new bone matrix following the work of OC's breaking down old bone matrix under the regulation of osteocytes in conjunction with cytokines. Too much osteoclastic activity and bone will become very fragile as seen in diseases such as osteoporosis.

Osteoclast precursor cells (OPC's) originate in the bone marrow, are macrophage like, and able to act as part of the innate immune system if not signaled to differentiation[8]. Two important cytokines for osteoclasts are macrophage colony stimulating factor (MCSF), important for survival and proliferation of osteoclast precursor cells, and receptor activator of nuclear factor kB ligand (RANKL) that drives differentiation and fusion of precursors into osteoclasts as

demonstrated in Figure 1 [9]. Genes specific to the differentiation of fusion of the osteoclast precursor cells include nuclear factor activated T cell 1 (NFATC1) the master differential regulator, calcitonin receptor (CALCR) to regulate bone formation, cathepsin K (CTSK) used as a secreted protease to break down matrix, and matrix metallopeptidase 9 (MMP9), a secreted enzyme. These are often measured to provide evidence for osteoclastic activity.

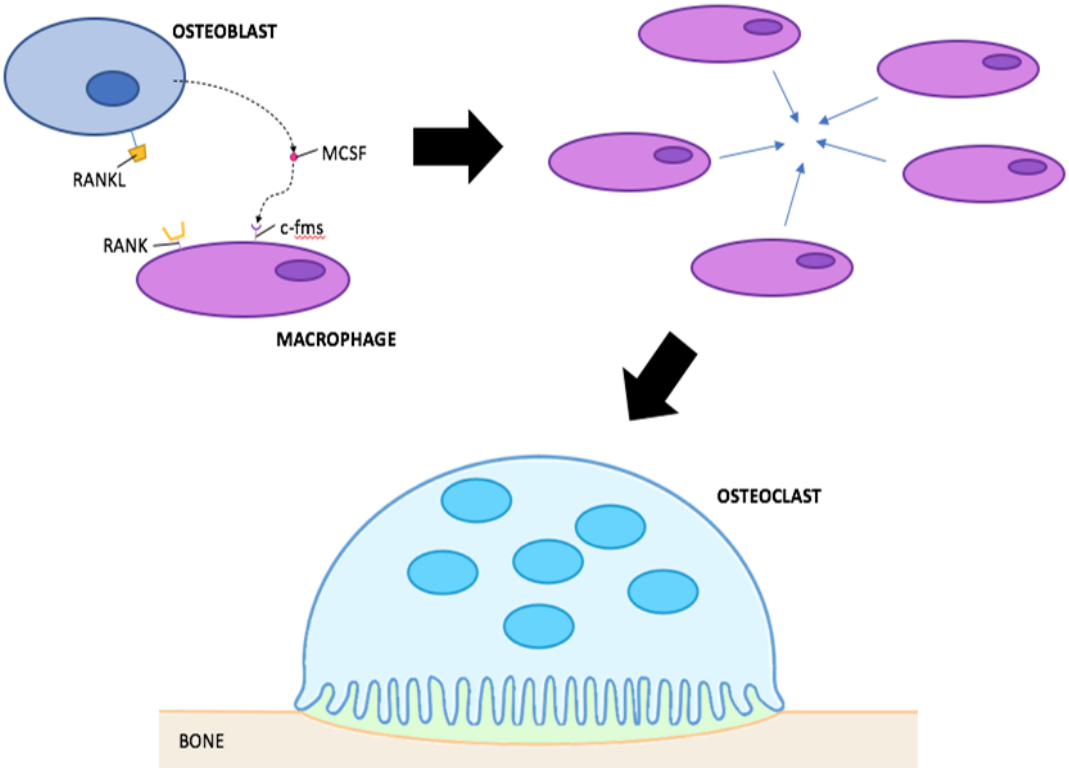


Figure 1. Osteoclastogenesis require two signals, MCSF and RANKL. MCSF acts on receptor c-fms to simulate proliferation and growth of macrophages. Additional signal from RANKL drives the differentiation and fusion of osteoclast precursor cells, resulting in a large multinucleated osteoclast.

RANKL is a part of the Tumor Necrosis Factor (TNF) superfamily, closely related to immune functions as well as, activates an important pro-osteoclast transcription factor: nuclear factor kappa light chain enhancer of activated B cells (NFkB)[10]. NFkB can also be activated as a

result of binding of a pathogen recognition receptor (PRR) [11,12.].Toll like receptors (TLR) are a large family of membrane bound PRR's used by macrophages and dendritic cells to recognize pathogen associated molecular patterns (PAMP's) and induce a proinflammatory response such as expression of cytokines interleukin 1 beta (IL-1b) and TNF as well as enzymes like cyclooxygenase2 (COX2)[13]. Osteoclast precursor cells express TLR's due to their heritage and macrophage likeness. Stimulation of the TLR's on osteoclast precursor cells after exposure to RANKL will drive the cells into osteoclastogenesis[14]. This is thought to be a result of the pro-osteoclastogenic transcription factor NFkB[15]. Examples of TLR agonist include lipopolysaccharide (LPS) found in gram-negative bacteria, viral RNA, and zymosan found in fungus. Each TLR agonist is recognized by a specific variant of TLR. Without previous exposure to RANKL, OPC's will act as a part of the innate immune system provoking a proinflammatory response. A committed OPC with previous RANKL exposure, can continue osteoclastogenesis independent of RANKL if exposed to a TLR agonist, such as LPS[11].

Sex as a biological variable for disease has become increasingly important in the research community, such as recent observation of sex-dependent adverse effects of pharmaceutical drugs on skeletal integrity [16]. For decades it has been standard procedure to culture cells of male mice, but current studies have shown there can be significant difference in the behavior of male versus female derived cells[17]. In 2019, Zarei et al suggested a sexually divergent sensitivity in the NFkB pathway and hypothesized it was dependent on estrogen. Though variance in cell signaling can be influenced by estrogen, they found the difference observed in the NFkB pathway was independent of estrogen signaling[18]. Previous research in our lab has shown a significant difference in expression of TLR's between male and female derived cells[19]. While still in the naïve state, no exposure to RANKL, there is a higher expression of TLR in male derived OPC's. In mature osteoclast's, the opposite was true, TLR expression was lower in male derived cells. However, associated domains to TLR's such as myeloid differentiation factor 88 (MyD88), that have been shown to influence TLR induced

osteoclastogenesis, did not show a significant sex-dependent gene expression difference[20]. Induction of osteoclast differentiation with LPS, in absence of RANKL, has been shown to produce larger osteoclasts than RANKL osteoclastogenesis, though this has not been studied in a sex-dependent manner.

This study focuses on TLR2 for zymosan recognition, TLR3 for double stranded RNA recognition, and TLR4 for LPS. Polyinosinic:polycytidylic acid (PolyI:C) is a syntetic analog of double-stranded RNA and is used as a TLR3 agonist for this study. The purpose of this study is to investigate sexually divergent responses to three different inflammatory signals (LPS, zymosan, and double-stranded RNA) in naïve and committed osteoclast precursors with respect to osteoclast differentiation, induction of osteoclast and inflammatory genes, and production of the inflammatory cytokine Tumor Necrosis Factor (TNF).

METHODS

Animal care and use

For each replicate, 2 C57BL/6 mice (1 male 1 female) between the ages of 2 and 9 months old were used as a source of bone marrow macrophages. This study consisted of three replications for a total use of 6 mice. All mice were euthanized in accordance with the University Institutional Animal Care and Use Committee by CO_2 inhalation for 10 minutes followed by cervical dislocation.

Isolation of osteoclast precursor cells

Femurs and tibias were removed, and bone marrow macrophages were flushed with medium composed of (Millipore-Sigma, M0894), 2g sodium bicarbonate (Fisher Scientific, S233-500), 20mM L-glutamine (Millipore-Sigma, G7513), 10% heat-inactivated fetal bovine serum (Atlas Biologicals, F0500-D)) into tissue culture treated dishes at 5% CO_2 and 37 degrees Celsius overnight.

Activation and differentiation of osteoclasts

24 hours post culture, the osteoclast precursor cells suspended in medium were collected and plated in six, 3mL dishes with the addition of MCSF. Cells are cultured for three days at 37 degrees Celsius and 5% CO_2. On the second day culture medium with MCSF is refreshed. After 36 hours the cells are lifted from the plates using 500µL of Accutase and tapping the side of the dish to loosen the cells. After two minutes, 500µL of medium is added, and with vigorous pipetting the cells are lifted and counted using hemocytometry. In a 96 well plate, cells are plated at 100k cells per well in 200µL of medium with MCSF. At this point, half of the male and female derived cells will stay naïve while the other half get 50µL/mL of RANKL to prime the cells for differentiation. Cells are cultured for another 48 hours at 37 degrees Celsius with 5% CO_2.

The naïve osteoclast precursor cells will have one control group that only gets MCSF, and three concentrations of each treatment: Poly I:C, LPS, and zymosan. The RANKL treated osteoclast precursors had a control group of continual RANKL and three groups of each treatment concentration. After treatments were applied for 16 hours at 37 degrees Celsius and 5% CO_2, RNA was collected for PCR. Cells used for staining continued to differentiate for an additional 8 to 16 hours until differentiated.

Treatments: PolyI:C, LPS, and Zymosan

Male and female osteoclasts were seeded into a 96-well culture plate (ThermoFisher, 142475) at a density of 10,000 cells/ml in 200 µL of nutrient medium containing 35ng/mL M-CSF and half with100ng/mL recombinant RANKL. PolyI:C, the activator of TLR3, was serial diluted in cell culture medium to make three concentrations; 100ng/µL, 1 µg/µL and 10µg/µL[21]. LPS, an exclusive activator of TLR4, was serial diluted to make three varying concentrations at 100 ng/µL, 1000ng/µL, and 1µg/mL[22]. Zymosan, TLR2 agonist, was also diluted to create concentration of 1 µg/µL 10 µg/µL, and 100 µg/µL[23].

The cells were incubated for 48 hours at 5% CO_2 and 37 degrees Celius. After 16 hours, RNA was lysed and after 48 hours cells were fixed and stained.

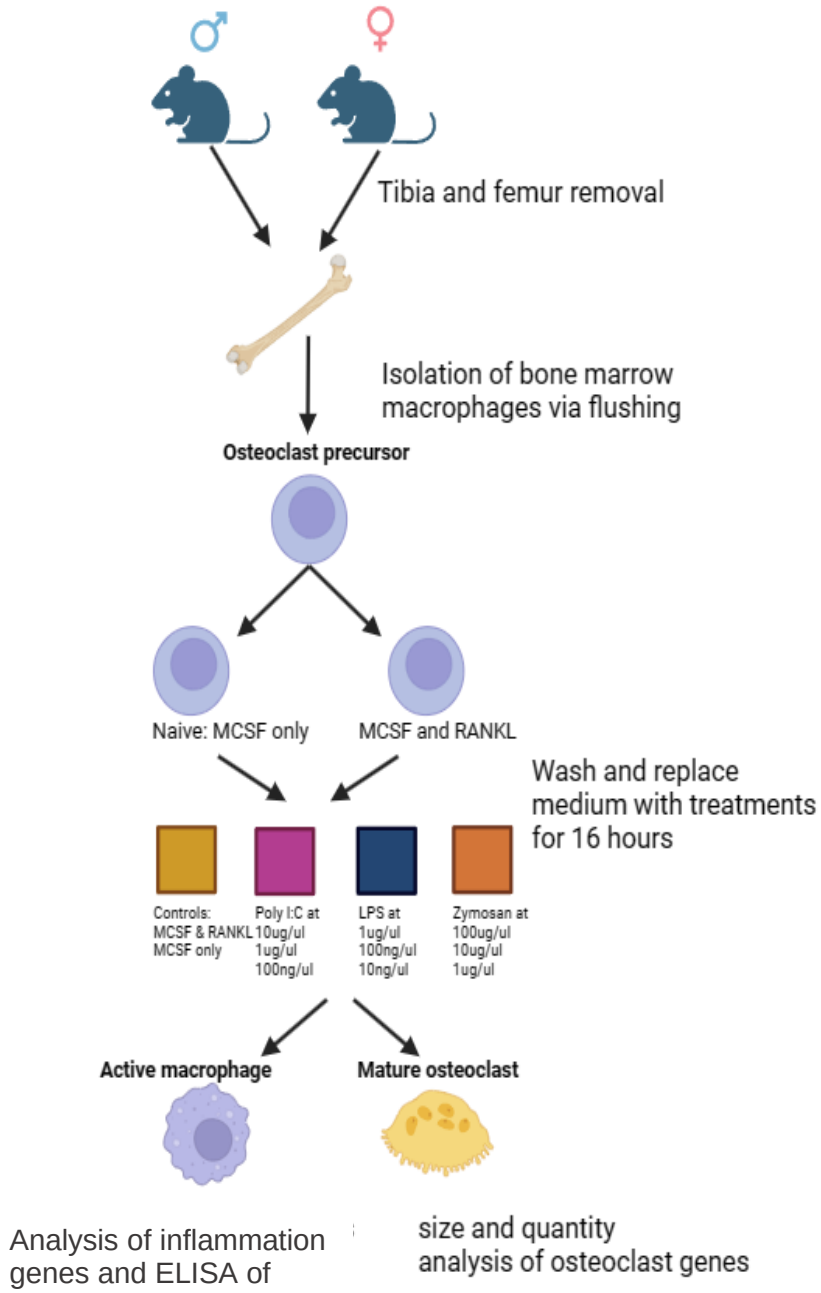


Figure 2. Graphical abstract of workflow. Osteoclast precursor cells are isolated from the tibia and femur of male and female mice. Half the cells of each sex were pre-committed to osteoclastogensis with treatment of RANKL for 48 hours. All cells were treated with either their respective control and each of the concentrations of Poly I:C, LPS, and zymosan. RNA was collected from both groups for RT-qPCR analysis. Culture medium was collected from the activated macrophages for ELISA TNF analysis. The mature osteoclasts were stained for size and quality analysis.

RNA Extraction and Quantitative PCR

cDNA was generated from extracted RNA samples using SuperScript IV VILO Master Mix (ThermoFisher, 11756050) according to supplier instructions and diluted to an effective starting RNA concentration of 5ng/µL. Each qPCR sample consisted of 10µL Luna Universal qPCR Master Mix (New England BioLabs, M3003S), 2µL of 2µM primer mix, and 8µL cDNA template. Reactions were run with a Bio-rad real-time PCR system. Relative gene expression was calculated using the 2-Δ ΔCT method using 18s rRNA as an endogenous loading control. The following genes were measured as inflammatory markers via PCR: tumor necrosis factor (TNF) and cyclooxygenase-2 (Cox2). Nuclear factor of activated T-cells, cytoplasmic 1 (Nfatc1), calcitonin receptor (Calcr), and cathepsin K (Ctsk) were measured as osteoclast markers.

ELISA

Culture medium was collected from naïve and committed osteoclast cells. Using mouse TNF-alpha ELISA protocol, protein expression was calculated for each treatment.

Quantification of osteoclast size

A full view of each well at 10x was taken using EVOS Images. Osteoclasts with >3 nuclei were counted using ImageJ software (https://imagej.nih.gov/ij/). Osteoclast quantity was averaged across the three iterations of the experiment. These same fields were used to measure osteoclast size (μm^2) using ImageJ. Ten of the largest osteoclasts were measured and averaged across three iterations of the experiment.

Statistical Analysis

For all RT-qPCR, delta delta CT was used to get the relative gene expression for each sample. The relative expression was used to find the area under the curve (AUC) for each treatment concentration. For cell size, 10 cells from each sample were measured using ImageJ and averaged before calculating AUC. ELISA concentration of TNF was used by measuring optical density and fitting it to a standard line of known concentrations, then calculating AUC for each concentration within the treatments. To calculate the AUC, we used the equation below:

$$AUC_1 = \frac{x_1 + x_2}{2} \cdot conc._{high-low}$$

Using R, version 4.1.2, (https://www.R-project.org) for all data sets, the total AUC was added for each treatment group, the average was taken across replicates. A three way ANOVA was run to look at effects of sex and treatment on AUC in groups treated with and without RANKL. A pairwise comparison to look at sex across treatment was used to calculate the p-values to test the null hypothesis, there is no difference between the male and female group.

RESULTS

Activation of inflammatory pathways

To address if the difference in TLR expression of naïve and committed OPC's correlates to a difference in TLR activity, cells were treated with PAMP's and downstream expression of TLR genes was measured. After 16 hours of treatment, osteoclast precursor cells were treated with one of the three TLR agonists, cells held physical characteristics of activated macrophages. Levels of inflammation-associated genes are measured from these activation treatment groups. It was only in the zymosan treatment we observed a clear pattern in COX2 gene expression between male-derived and female-derived OPC's. In cells that received no RANKL treatment, naive OPC's,

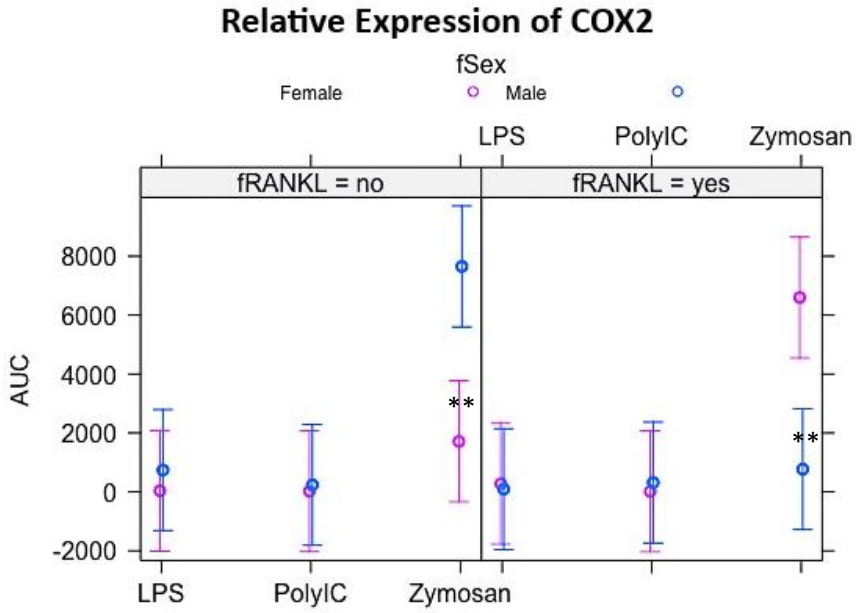


Figure 3: Expression of COX2 in naïve (no RANKL) and committed osteoclast (RANKL) cells measured using RT-qPCR. AUC was calculated for each treatment across concentration. Error bars represent 95% confidence interval. P-value for naive male vs female: LPS (0.62), PolyI:C (0.62), zymosan (0.0003). Male vs Female committed: LPS (0.89), Poly I:C (0.83), zymosan (0.0004). *=p<0.05, **=p<0.001

there is no significant difference in expression in the PolyI:C and LPS treatments. For committed precursor cells, treated with RANKL, there is more of an expression among the females only in the zymosan treatment (Figure 3). This does not provide strong

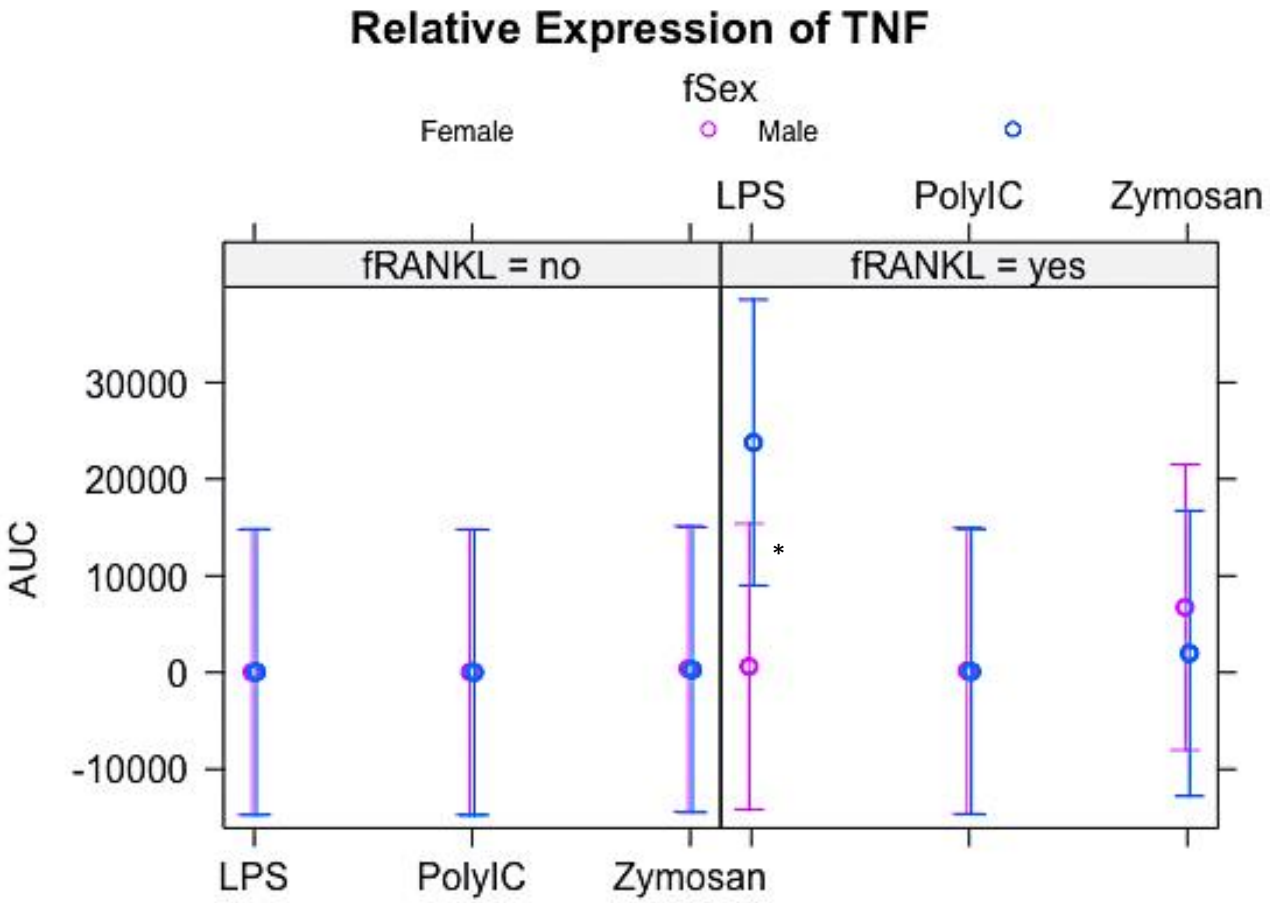


Figure 4: Relative concentration of TNF in naïve (no RANKL) and committed (RANKL) osteoclast cells measured from RT-qPCR. AUC was calculated for each treatment for comparison. . Error bars represent 95% confidence interval P-values for male vs female naïve: LPS (0.99), Poly (0.99), and Zymosan (0.99).P-values for male vs female committed osteoclasts: LPS (0.03), Poly(0.99), and Zymosan(0.62). *=p<0.05, **=p<0.001

evidence to support our hypothesis that naive osteoclast precursor cells will have a higher expression among the male-derived than the female, increasing inflammation

gene expression. Only in the case of the zymosan treatments, female expression was significantly (P<0.05) higher than male derived cells.

The expression of TNF in committed and naïve OPC did not follow as clear a pattern as COX2. TNF expression exhibited no difference between male or female derived cells across all treatment in the naïve state. Contrary to what we expected,

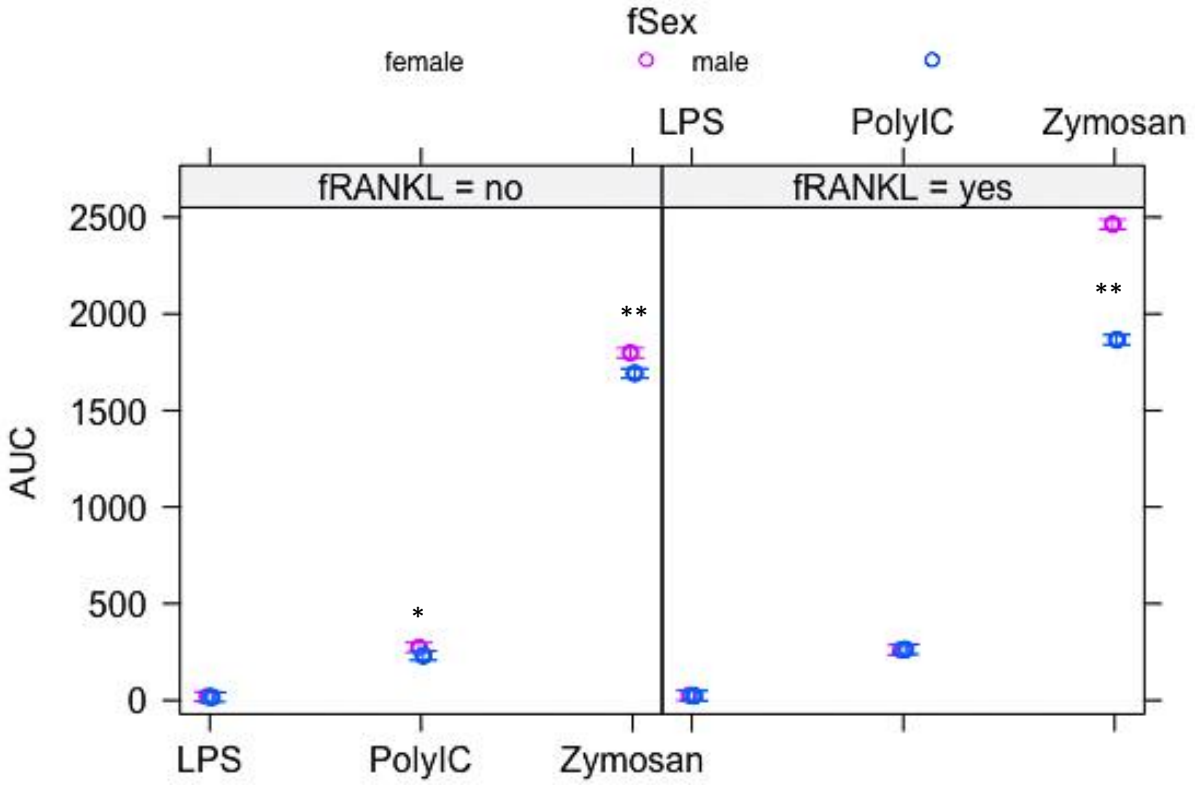


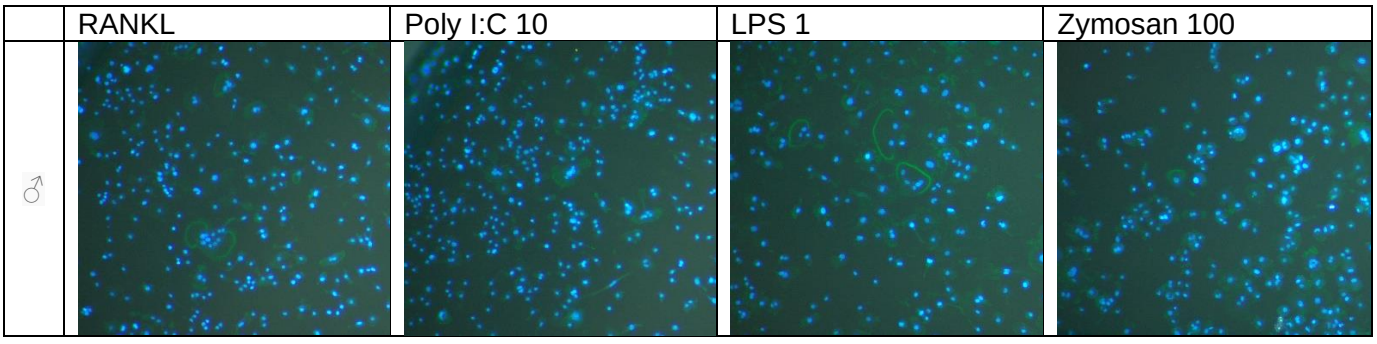
Figure 5: Relative concentration of TNF in naïve (no RANKL) and committed (RANKL) osteoclast cells measured from ELISA. AUC was calculated for each treatment for comparison. Error bars represent 95% confidence interval P-values for male vs female naïve: LPS (0.83), Poly (0.02), and zymosan (<0.001).P-values for male vs female committed osteoclasts: LPS (0.90), Poly(0.87), and zymosan(<0.0001). *=p<0.05, **=p<0.001

expression of TNF in LPS treatments showed the opposite and the male derived cells had significantly higher expression after commitment to osteoclastogenesis than female OPC's (Figure 4).

Additional analysis of TNF in serum, using Elisa, had results similar to what we expected to see (Figure 5). While Male expression of TNF did not show to be higher in the naïve state than the female TNF expression, interestingly enough the naïve female TNF concentration was significantly higher than male TNF levels in two of the three treatment groups. In the committed osteoclast state the females continued to show levels of TNF, but only significantly higher in the zymosan treatments.

Osteoclastogenesis

To further support that the difference in expression of TLR's of male and female derived osteoclasts has a functional significance, size of osteoclast and key osteoclast activity genes were measured. OPC's treated with RANKL for 48 hours to commit them to osteoclastogenesis followed by 24 hours of each of the three treatments and their respective concentrations until the OPC's were mature. Cells were fixed and stained with actin (phalloidin) and nuclei (Dapi) staining. *Figure 6* shows images of male and female derived cells at the highest concentration of each treatment (Poly I:C 10 µg, LPS at 1 µg, and zymosan at 100 µg). Osteoclasts are apparent in all images but notably the female-derived cells each time showed larger and more osteoclasts than the male-derived cells in all the treatments. Quantification of osteoclasts supports this clear pattern of larger developed female derived osteoclasts when driven by TLR stimulation though no significant difference was found in any of the treatments (Figure 7).



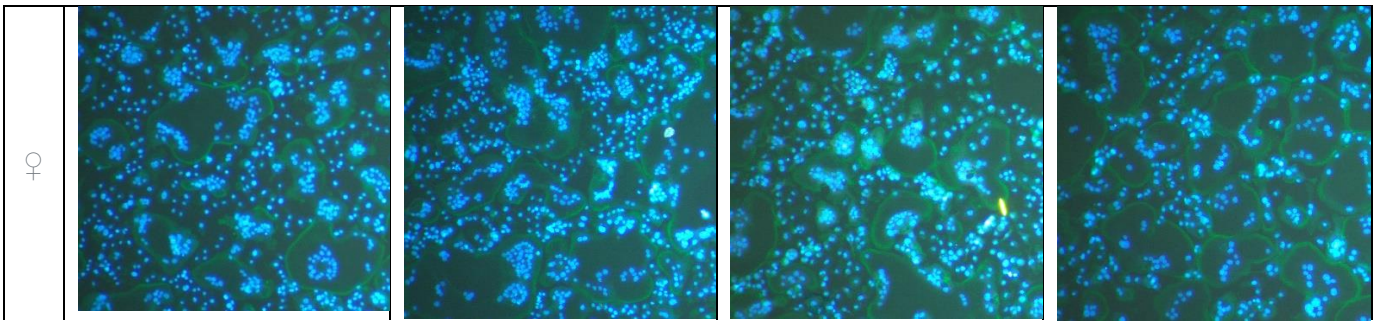


Figure 6:Male and female derived osteoclasts stained with Phalloidian (green) and Dapi (Blue).

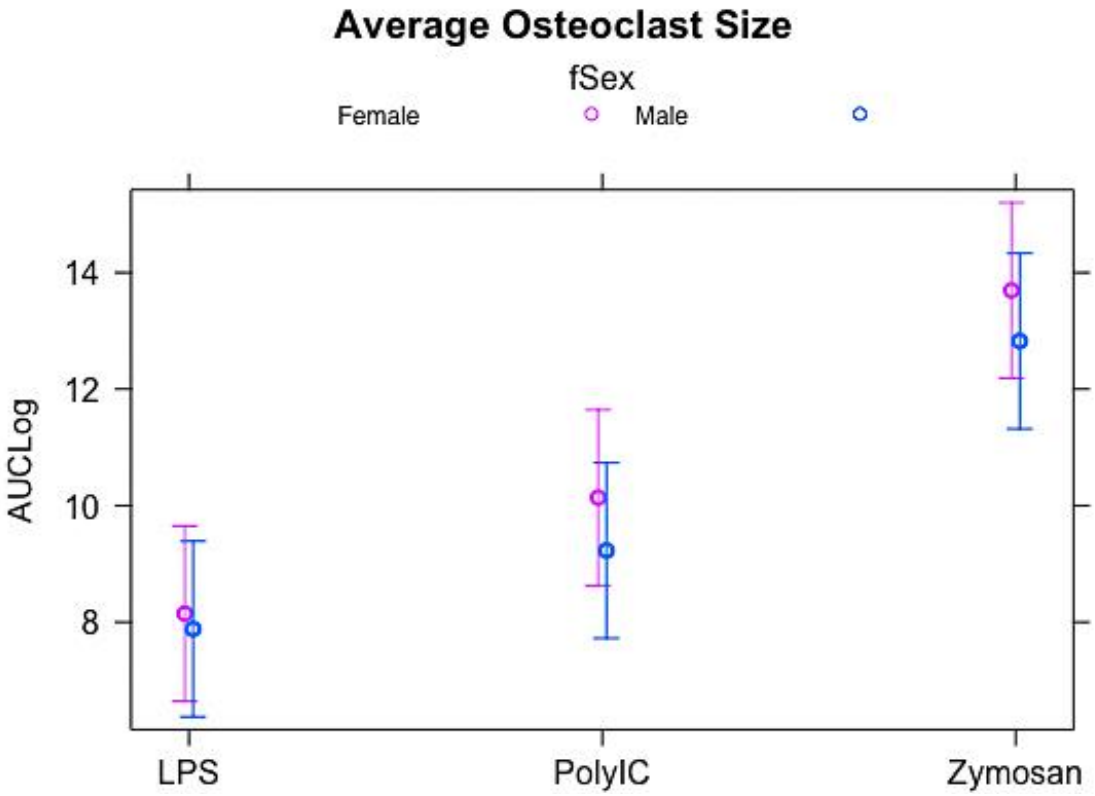


Figure 7: Average osteoclast size. AUC was calculated for each treatment for comparison. Error bars represent 95% confidence interval. P-values for male vs female osteoclasts: LPS (0.99), Poly(0.92), and zymosan(0.26). *=p<0.05, **=p<0.001

We hypothesized that other osteoclastogenic genes to follow the same pattern as COX expression between male and female derived committed OPC's. CALCR

demonstrated the least responsiveness to treatments, and there were no significant differences attributable to sex (Figure 8). NFATc1 (Figure 9) and CTSK (Figure 10) showed modest, but more consistent upregulation in response to inflammatory signals, and, consistent with our hypothesis, female cells demonstrated a greater response than males. Most notable is expression of CTSK in the zymosan treatments, female derived cells show a significant difference in expression compared to the male derived cells. This supports the idea that female-derived committed osteoclasts are more sensitive to PAMP's via expression of TLR.

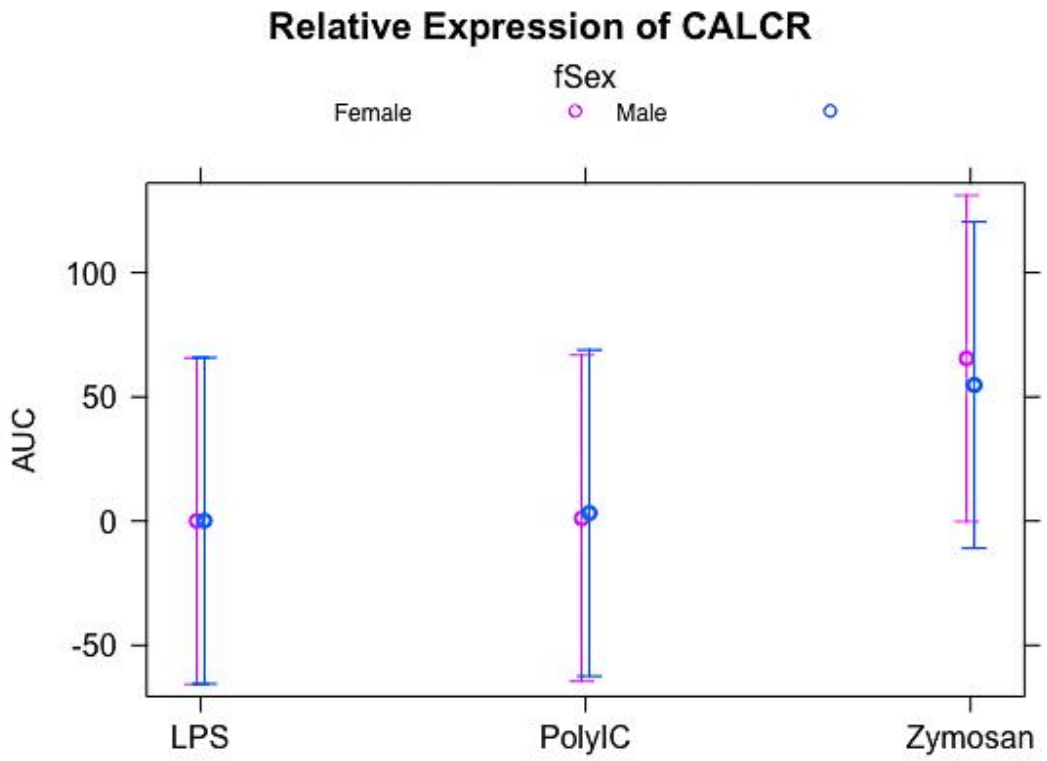


Figure 8: Relative expression of CALCR in committed (RANKL) osteoclast cells measured from RT-qPCR. AUC was calculated for each treatment for comparison. Error bars represent 95% confidence interval. P-values for male vs female committed osteoclasts: LPS (0.99), Poly(0.96), and zymosan(0.85). *=p<0.05, **=p<0.001

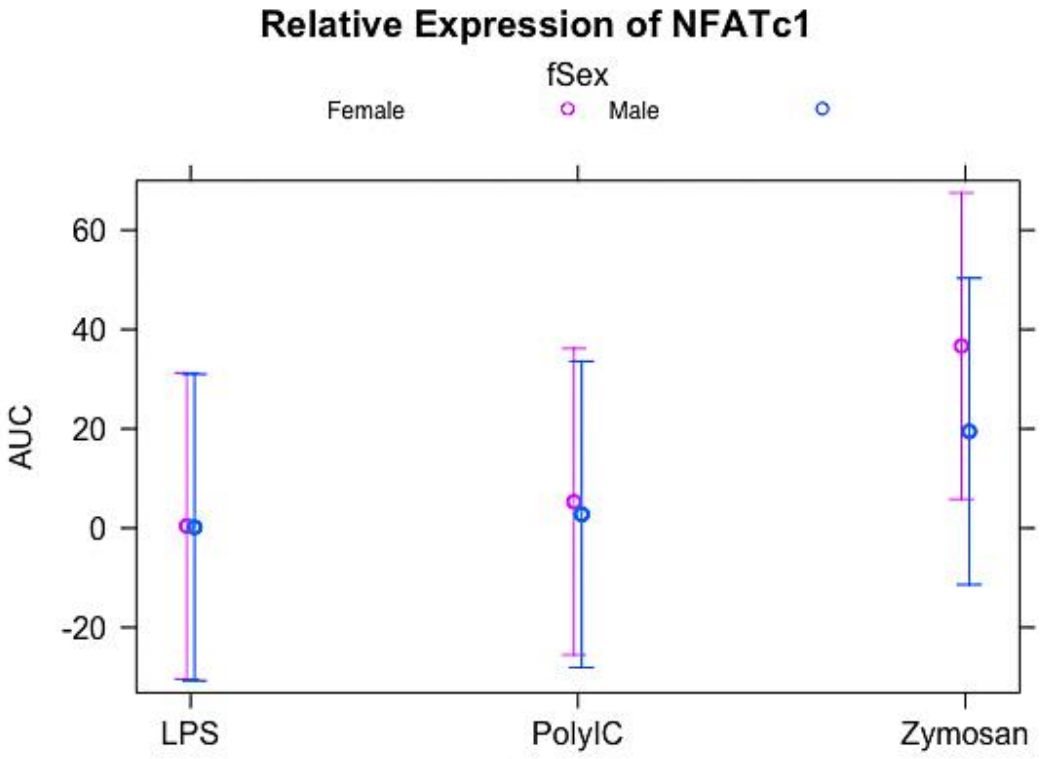


Figure 9: Relative expression of NFATc1 in committed (RANKL) osteoclast cells measured from RT-qPCR. AUC was calculated for each treatment for comparison. Error bars represent 95% confidence interval. P-values for male vs female committed osteoclasts: LPS (0.98), Poly(0.90), and zymosan(0.40). *=p<0.05, **=p<0.001

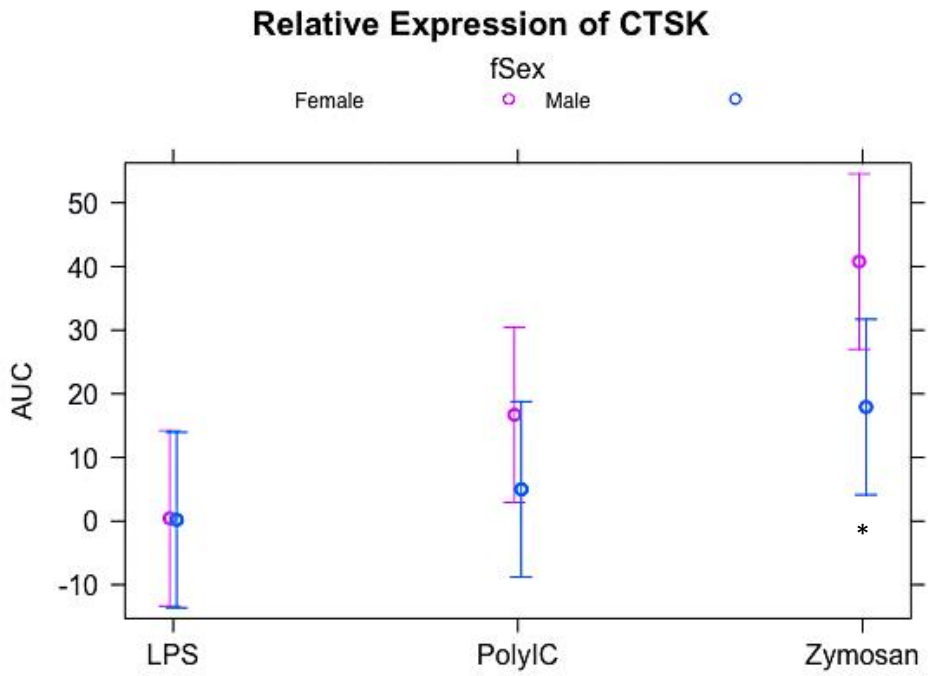


Figure 10: Relative expression of CTSK in committed (RANKL) osteoclast cells measured from RT-qPCR. AUC was calculated for each treatment for comparison. Error bars represent 95% confidence interval. P-values for male vs female committed osteoclasts: LPS (0.97), Poly(0.21), and zymosan(0.02). *=p<0.05, **=p<0.001

DISCUSSION

We found higher sensitivity of toll-like receptors in male naïve osteoclast precursor cells relative to females. AUC analysis of relative expression of COX2 shows a pattern similar to what we expected to see after seeing patterns of differing expression in TLR from genetic sequencing. The significant value recorded for male versus female COX2

expression in the zymosan could be due to the increased sensitivity the OPC's had to zymosan. Not many studies have been done to look at the effects of zymosan on osteoclast development, the potency of the treatment could have been more than that of PolyI:C or LPS. Across all parameters measured, zymosan shows higher gene expression and produces larger osteoclasts. Along with COX2, TNF was also measured as an inflammatory marker. The relative expression of TNF as well as TNF protein concentration in serum showed no clear patterns except in the LPS treatment of committed cells. This could be due to the rate TNF is expressed, after stimulation of TLR it would take about 36 hours to get robust TNF expression, our data collected samples around the 16-hour mark. Poly I:C and zymosan showed a clear pattern of high TNF protein levels in female derived naïve OPC's and, only in zymosan, continued on the same pattern in the committed state.

Due to an increased expression of TLR's on female derived osteoclast, we expected to see an increase in sensitivity of the female derived cells on TLR driving osteoclastogenesis. Visually there were much larger osteoclasts in the female derived cells. Figure 6 is a great visualization of the osteoclast size compared across the highest dose of each treatment. Statistically, the female derived cells consistently showed larger size osteoclasts than male derived cells, though not significantly different. Increased replication could strengthen this pattern furthering the idea that female committed OPC's are at a higher sensitivity than males.

To further look at TLR induced osteoclastogenesis in the context of sexual divergence, we measured key osteoclast specific genes, NFATc1, CTSK and CALCR. The expression of CALCR and NFATc1 did show females producing a higher expression of both genes across treatment but not significantly enough to confidently support our hypothesis. CTSK showed a much clearer pattern of higher gene expression in female derived cells in PolyI:C and zymosan treatments. This pattern paired with the patterns we observed in COX2 is evidence that sexual divergent expression of TLR's does have an

effect on osteoclast development. More research is needed to see if there is a significant effect on osteoclast function as well. With most of the data showing the pattern we expected to see without significant p values, an increase in replications could have been helpful to ensure the patterns recorded are representative.

To further investigate this in light of sexual variance, we observed differentiation and measured osteoclast size, to test sensitivity of TLR derived signals between committed male and female osteoclasts. In agreement with our genetic expression data, we found that females form larger osteoclasts in response to TLR signaling but not necessarily osteoclast specific gene expression. This increased sensitivity seen in the females agrees with the higher expression of TLR's recorded previously in our lab. Other studies have found effects of Poly I:C on osteoblasts cells, promoting expression of the osteoclast's differentiation gene Tnfsf11, suggesting that *in vivo* TLR effects could be much greater[24]. Emerging work has suggested the importance of adaptive and innate immune system components and their contribution to proinflammatory disease[25,26]. Srivasta et al. has proposed a term "immunoporosis" to further stress the importance of immune cells in the progression and pathogenesis of osteoporosis[27].

Our findings suggest that females are at greater risk for elevated inflammatory related osteoclast activity as a result of greater sensitivity to bacterial, viral, and fungal signaling. In the context of inflammatory bone disease, women are at a much higher risk due to the increased sensitivity of osteoclastogenesis via TLR's. Rheumatoid arthritis is one example where inflammation in the bone leads to bone loss. In cases like chronic periodontitis, where bacteria is the leading cause of erosion of the bone, women could be at a much higher risk for faster developing cases. Although there is no data to support a sexual variance in either of these diseases, it would be interesting to compare the rate of disease progression between men and women. Enhanced osteoclast activation through TLR's would in turn pre-dispose women to higher rates of bone loss following surgery or

trauma. Although the exposure of bone during surgery is transient and antibiotics are locally applied as a means to avoid infection, antibiotics merely prevent the proliferation of bacteria without removing pre-existing bacterial structural components. A common mechanism by which antibiotics inhibit bacterial replication is through disruption of the bacterial membrane. This in turn releases bacterial endotoxins, such as LPS, into the surrounding environment, which pose the risk of subsequently modulating host cell behavior. Thus it is important that research continues to look at sex as a biological variable for disease after observation of sex-dependent inflammation and differentiation response of osteoclasts.

REFERENCES

1. Johnston, C. B. & Dagar, M. Osteoporosis in Older Adults. *Medical Clinics of North America* vol. 104 873–884 Preprint at https://doi.org/10.1016/j.mcna.2020.06.004 (2020).
2. David Roodman, G. & Silbermann, R. Mechanisms of osteolytic and osteoblastic skeletal lesions. *Bonekey Rep* **4**, (2015).
3. Kazuhiko Matsuoka, Kyoung-ae Park, Masako Ito, Kyoji Ikeda & Sunao Takeshita. Osteoclast-Derived Complement Component 3a Stimulates Osteoblast Differentiation. *Journal of Bone and Mineral Research* vol. 29 1519–1521 Preprint at https://doi.org/10.1002/jbmr.2276 (2014).
4. Kim, H. N. *et al.* Estrogens decrease osteoclast number by attenuating mitochondria oxidative phosphorylation and ATP production in early osteoclast precursors. *Sci Rep* **10**, (2020).
5. Jevon, M. *et al. Gender-and age-related differences in osteoclast formation from circulating precursors*. *Journal of Endocrinology* vol. 172 http://www.endocrinology.org (2002).
6. Teitelbaum, S. L. Osteoclasts: What do they do and how do they do it? in *American Journal of Pathology* vol. 170 427–435 (American Society for Investigative Pathology Inc., 2007).
7. Delgado-Calle, J. & Bellido, T. Osteocytes and Skeletal Pathophysiology. *Curr Mol Biol Rep* **1**, 157–167 (2015).
8. Liu, J. *et al.* Molecular mechanism of the bifunctional role of lipopolysaccharide in osteoclastogenesis. *Journal of Biological Chemistry* **284**, 12512–12523 (2009).
9. Feng, X. RANKing intracellular signaling in osteoclasts. *IUBMB Life* vol. 57 389–395 Preprint at https://doi.org/10.1080/15216540500137669 (2005).
10. Maruyama, K., Kawagoe, T., Kondo, T., Akira, S. & Takeuchi, O. TRAF family member-associated NF-κB activator (TANK) is a negative regulator of osteoclastogenesis and bone formation. *Journal of Biological Chemistry* **287**, 29114–29124 (2012).
11. Zeng, X. zhou *et al.* Artesunate attenuates LPS-induced osteoclastogenesis by suppressing TLR4/TRAF6 and PLCγ1-Ca2+-NFATc1 signaling pathway. *Acta Pharmacol Sin* **41**, 229–236 (2020).
12. Xiao, Y. *et al.* Cellular study of the LPS-induced osteoclastic multinucleated cell formation from RAW264.7 cells. *J Cell Physiol* **235**, 421–428 (2020).
13. Souza, P. P. C. & Lerner, U. H. Finding a toll on the route: The fate of osteoclast progenitors after toll-like receptor activation. *Frontiers in Immunology* vol. 10 Preprint at https://doi.org/10.3389/fimmu.2019.01663 (2019).
14. Yao, Y. *et al.* The Macrophage-Osteoclast Axis in Osteoimmunity and Osteo-Related Diseases. *Frontiers in Immunology* vol. 12 Preprint at https://doi.org/10.3389/fimmu.2021.664871 (2021).
15. Ahmadzadeh, K. *et al.* Multinucleation resets human macrophages for specialized functions at the expense of their identity. *EMBO Rep* **24**, (2023).
16. Uddin, S. M. Z. *et al.* Methylphenidate regulation of osteoclasts in a dose- A nd sex-dependent manner adversely affects skeletal mechanical integrity. *Sci Rep* **8**, (2018).
17. Lorenzo, J. Sexual Dimorphism in Osteoclasts. *Cells* vol. 9 Preprint at https://doi.org/10.3390/cells9092086 (2020).
18. Zarei, A. *et al.* Manipulation of the Alternative NF-κB Pathway in Mice Has Sexually Dimorphic Effects on Bone. *JBMR Plus* **3**, 14–22 (2019).

19. Keever, A. L., Collins, K. M., Clark, R. A., Framstad, A. L. & Ashley, J. W. RANK signaling in osteoclast precursors results in a more permissive epigenetic landscape and sexually divergent patterns of gene expression. *PeerJ* **11**, (2023).
20. Sato, N. *et al.* MyD88 but not TRIF is essential for osteoclastogenesis induced by lipopolysaccharide, diacyl lipopeptide, and IL-1α. *Journal of Experimental Medicine* **200**, 601–611 (2004).
21. Cui, W. Y. *et al.* Identification and characterization of Poly(I:C)-induced molecular responses attenuated by nicotine in mouse macrophages. *Mol Pharmacol* **83**, 61–72 (2013).
22. Fang, H. *et al. Lipopolysaccharide-Induced Macrophage Inflammatory Response Is Regulated by SHIP 1*. *The Journal of Immunology* vol. 173 http://journals.aai.org/jimmunol/article-pdf/173/1/360/1187026/360.pdf (2004).
23. Young, S. H., Ye, J., Frazer, D. G., Shi, X. & Castranova, V. Molecular Mechanism of Tumor Necrosis Factor-α Production in 1→3-β-Glucan (Zymosan)-activated Macrophages. *Journal of Biological Chemistry* **276**, 20781–20787 (2001).
24. Tominari, T. *et al.* Endosomal TLR3 signaling in stromal osteoblasts induces prostaglandin E2–mediated inflammatory periodontal bone resorption. *Journal of Biological Chemistry* **298**, (2022).
25. Zupan, J., Jeras, M. & Marc, J. Osteoimmunology and the influence of pro-inflammatory cytokines on osteoclasts. *Biochemia Medica* vol. 23 43–63 Preprint at https://doi.org/10.11613/BM.2013.007 (2013).
26. Frase, D., Lee, C., Nachiappan, C., Gupta, R. & Akkouch, A. The Inflammatory Contribution of B-Lymphocytes and Neutrophils in Progression to Osteoporosis. *Cells* vol. 12 Preprint at https://doi.org/10.3390/cells12131744 (2023).
27. Srivastava, R. K. & Sapra, L. The Rising Era of “Immunoporosis”: Role of Immune System in the Pathophysiology of Osteoporosis. *Journal of Inflammation Research* vol. 15 1667–1698 Preprint at https://doi.org/10.2147/JIR.S351918 (2022).

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