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Sex-based Differential Regulation of Bacterial-induced Bone Resorption

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Abstract

Background and Objective—Periodontal disease pathogenesis is comprised of the complex inflammatory immune response to oral bacterial dysbiosis. Like other inflammatory diseases, there is sexual dimorphism evident in periodontal diseases. During periodontitis, inflammatory chemokines direct neutrophils to migrate to the site of infection to neutralize the pathogen. Interestingly, these same chemokines are also involved in regulating pathogen-induced osteoclast formation. Previous reports show differences in bone turnover and lymphocyte recruitment between sexes. We hypothesize that chemokine expression is differentially regulated by sex and thus result in differential osteoclast formation.

Methods—Male and female mice were utilized to isolate neutrophils based on expression of Ly6G-specific, as well as defined osteoclast progenitors (dOCPs). Cells were stimulated with LPS (100ng/ml) then analyzed for neutrophil infiltration and gene expression. dOCPs were primed: M-CSF (25ng/ml), RANKL (50ng/ml), then stimulated with LPS. Osteoclasts were enumerated via TRAP stain and mRNA isolated for gene expression analysis via qPCR.

Results—In response to LPS, male neutrophils *in vitro* respond with increased chemokine expression and significantly more osteoclast formed in response to LPS compared to females.

Conclusions—Findings support observations in humans regarding a sexual dimorphism in oral bacterial infections of alveolar bone loss. Males have a strong inflammatory response to bacterial infection, resulting in increased inflammatory microenvironment, reduced pathogenic bacteria clearance and increased osteoclast-driven bone loss in response to differential expression of key chemokines.

Keywords

oral infections; osteoclastogenesis; chemokines; neutrophils

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Introduction

Sex-based differences in disease are a consequence of genetic differences attributable to X-chromosome inactivation, differences in steroid hormone expression, and differences in anatomy, gender or life experiences(1). Sexual dimorphism is quite evident in the innate immune system. In general, men mount a more aggressive acute inflammatory response to microbial pathogens than women. Consistent with this concept, women have diminished proinflammatory responses compared to men in standardized endotoxemia models (2, 3).

Oral infectious diseases, including periodontal diseases and endodontic infections, lead to the destruction of the alveolar bone that supports the dentition. The current paradigm of periodontal and endodontic bone loss supports the predominate role of host inflammatory response for tissue destruction (4-6). Large epidemiological studies indicate males have a higher degree of periodontal disease susceptibility and progression/severity than females after controlling for all major covariates (3). Clinical and experimental evidence indicates that men exhibit a heightened innate inflammatory response to bacterial infections and injury compared to women.

During periodontal and periapical disease initiation, neutrophils are critical mediators of inflammation and are the first cells to migrate to the local site of infection (6, 7). Once at the infected site, neutrophils function to secrete pro-inflammatory cytokines and reactive oxygen species to clear the pathogen and perpetuate the host immune response (7). One of the key aspects of bacterial-driven bone loss is persistent inflammation driven by infiltrating leukocytes in response to infection. Sex differences in the neutrophil recruitment and chemokine expression between male and female mice have been observed in other infection models (8). This suggests that protective effects noted in females may extend beyond sex hormone differences, and could reflect distinct and differential immune response to pathogens among the sexes.

Under inflammatory conditions, chemokines CXCL1 and CXCL2, expressed by both tissue cells and other activated neutrophils, engage their receptor CXCR2 and activate neutrophils in an autocrine manner (9, 10). In a murine model of *A. actinomycetemcomitans* oral infection, CXCL1 is the first chemokine to be up regulated and maintained throughout infection (11). Under acute conditions, once recruited to infected tissues, cytokines and chemokines released locally activate these cells leading toward subsiding infection.

While necessary for immune cell recruitment and clearance of the pathogen, the prolonged expression of inflammatory cytokines and chemokines in the oral infectious microenvironment leads to collateral tissue damage and subsequent alveolar bone loss. In fact, multiple studies have recently shown that these same cytokines and chemokines can influence pathogen driven osteoclastogenesis (12, 13). Osteoclasts are the bone resorptive cells of hematopoietic lineage that form in response to differentiating cytokines including macrophage-colony stimulating factor (M-CSF) and receptor activator of NF- κ B Ligand (RANKL) (14). Notably, the inflammatory periodontal microenvironment can support osteoclast formation, resulting in pathogenic bone loss (15). Others and we showed that chemokines CXCL1 and CXCL2 are required to drive LPS-induced osteoclast formation

and that blocking their common receptor (CXCR2) blunted pathogenic osteoclast formation (12, 13). While the role of these chemokines during pathogenic osteoclast formation is becoming better established, it is not known if differences inherently exist in the inflammatory microenvironment and subsequent osteoclast formation between sexes.

We have reported that male mice had significantly more bone loss than females in response to pre-clinical oral commensal bacterial infection (16). In the current study, we explore the role of bacterial-driven neutrophil activity and subsequent osteoclastogenesis between sexes using in vitro models of bacterial-driven osteoclast formation and address potential molecular mechanisms that govern sex-dependent susceptibility in oral disease.

Materials and Methods

Animals and Cell Isolation

The Institutional Animal Care and Use Committee (IACUC) at the Medical University of South Carolina approved all experimental protocols. Male and female, age-matched mice (8-12 week age range; 5 per group), on a mixed C57/129 background, were obtained for peripheral blood (PB) by cardiac puncture and primary bone marrow cells (BM) as previously described (17). PB samples were run on a Scil Vet ABC complete blood counter (CBC) to determine distribution of lymphocytes, granulocytes and monocytes (Scil Vet, Gurnee, IL). Cells (PB and BM) were immunophenotyped using makers against T cells (CD4), B cells CD45R (B220), granulocytes (Gr1) and monocytes (CD11b) and CD115 (Colony-Stimulating Factor 1 Receptor). All antibodies were obtained from Miltenyi Biotec (Auburn, CA) and attached to fluorophores. Following immunophenotyping analysis, Gr1⁺ granulocytes (Ly6G⁺) were obtained from whole BM using antibodies conjugated to magnetic beads. Osteoclast progenitors were isolated from both male and female BM cells as previously described (17). Briefly, using the AutoMACS cell sorter (Miltenyi Biotec Inc., San Diego, CA, USA), BM cells were separated by expression of integrin CD11b into CD11b^{hi}, CD11b^{lo} and CD11b⁻ fractions, where the CD11b^{lo/-} fractions are negative for lineage markers including CD3, CD45R, Gr1, CD11c and positive for CD115.

Neutrophil Culture and Treatments

Naïve isolated granulocytic neutrophils obtained from bone marrow (Ly6G⁺) were plated at 1×10^6 cells/ml in growth media and stimulated with *A. actinomycetemcomitans*-derived LPS (100ng/ml) for 4 hours. LPS was isolated and purified as previously described (18). In additional experiments, cells were pre-treated with small molecule inhibitors against p38 (SB203580) and JNK (SP600125) each at 5mM, 30-minutes prior to stimulation with LPS.

Osteoclast Differentiation Assays

Isolated cells were grouped as CD11b^{hi} (macrophage lineage), CD11b^{lo} (defined osteoclast progenitor (dOCP)) and CD11b⁻ (naïve progenitor). Cells were seeded at 5×10^4 cells per well in 96-well plates and up to 2×10^6 in 6-well plates for osteoclast formation/function and gene expression assays, respectively. Seeded cells were plated in priming media as previously described (17). Briefly, cells were plated in alpha-MEM media (10% Hyclone FBS depleted of estradiol, 1% P/S) supplemented with 10ng/ml M-CSF (R&D systems)

overnight. 24hrs later, adherent cells were stimulated with 25ng/ml M-CSF and 50ng/ml RANKL (R&D systems) in fresh media. Cells were allowed to prime in osteoclastogenic media for 48 hours, then stimulated with 50ng/ml LPS for 48- and 96-hours and harvested for downstream analysis. TRAP staining was performed in cultured bone marrow cells using a leukocyte acid phosphatase kit (Sigma Aldrich). Osteoclasts were defined as multinucleated TRAP-positive cells (with ≥ 3 nuclei). Data were collected semi-automatically via an Olympus BX61 microscope and Visiopharm software.

mRNA Expression Analysis (Chemokine Array and qPCR)

For chemokine analysis of isolated neutrophils, mRNA was isolated using the Qiagen RNeasy micro-RNA isolation kit as described by the manufacturer. RNA was measured for integrity using a BioAgilent analyzer. Samples sizes of at least 3 biological replicates with a RNA integrity number (RIN) of 8 or higher were used in these studies. A minimum of 100µg of sample RNA was converted to cDNA using the SABiosciences mouse inflammation array kit according to manufacturer's protocol. cDNA from male and female Ly6G⁺ neutrophils was added to pre-coated plates and evaluated for inflammatory gene expression. Values were normalized to internal housekeeping genes and internal positive and negative controls as directed by the manufacturer.

For confirmation as well as additional mechanistic studies, qPCR was performed using mRNA isolated from cells (both Ly6G⁺ neutrophils and dOCs) treated with LPS alone or in the presence of MAPK inhibitors again using the RNeasy Kit according to the manufacturer's specifications as previously described (17). Amplified cDNA was then probed using amplicon primers obtained from ABI including Cxcl1, Cxcl2, Cxcr2, Cxcr4, Nos2, Il10, Nfya1 and Tm7sf4 (DC-STAMP) mRNAs. *Gapdh* was used as endogenous loading control for normalization. Normalized amplicon values were expressed as fold change compared the unstimulated or M-CSF/RANKL-primed treated controls, respectively.

In vivo analysis

To perform immunohistochemistry (IHC) on *in vivo* samples, male and female tissue sections from our previously published oral commensal model of bacterial-driven bone loss (16). Briefly, 10-week old male and female mice were anesthetized using intraperitoneal injection of Ketamine (80mg/kg) and xylazine (10mg/kg) in sterile phosphate-buffered saline after induction with inhalational isoflurane. For experimental groups, lower first molars were accessed and pulps exposed under surgical operating microscope (Olympus Highlight 3100/SZ61; Olympus Imaging America, Center Valley, PA) with a dental handpiece (Aseptico; Woodinville, WA) and 1/4 round bur. Access allowed penetration of #6 endodontic hand file to the mesial and distal root canals. The pulp was then left exposed to the oral environment for eight weeks. Euthanization occurred by CO₂ asphyxiation. Mandibles were harvested and sectioned into two halves at the midline for analysis. Control mice were mixed gender and did not receive exposure. To identify neutrophils, a neutrophil specific marker; anti-Ly6G IgG (R&D systems,) was utilized. IHC was developed using DAB substrate. Matched IgG isotype was used as an experimental control and non-infected oral tissues sections were used as negative controls. Tissues were counterstained with hematoxylin to highlight immune cells. Samples were visualized under light microscopy at

40 and 60× magnification for inflammatory infiltrate and neutrophil specific staining. For neutrophil enumeration, samples were analyzed by a trained examiner blinded to the identity of the samples and scored based on inflammatory infiltration and positive IHC staining for each marker using a 0-3 ordinal scoring system. Inflammation/IHC was scored on a 0–3 scale (0, no inflammation or IHC staining; 1, minimal inflammation (occasional scattered granulocytes and leukocytes) or 5-15% IHC staining; 2, mild-moderate inflammation (scattered granulocytes with patchy infiltrates) or 20-50% IHC staining; and 3, severe inflammation (multiple extensive areas with abundant granulocytes and marked infiltrates) or >50% IHC staining adjacent to root apex.

Statistical Analysis

Data were analyzed with GraphPad Prism software, version 5.0. To compare values between two groups, an unpaired t-test followed by a post-hoc analysis. Comparison of values between multiple groups was measured using two-way ANOVA analysis followed by Bonferroni's multiple comparison tests. For measures requiring an ordinal scale, a Mann-Whitney test was employed to analyze the non-parametric data. Results were expressed as mean $\pm$ SD of at least three (n=3) biological replicates for each experiment. A *P* value of less than 0.05 was considered to be statistically significant.

Results

Sex differentially regulates neutrophil recruitment in response to bacterial infiltrate

Previously published reports from our lab have shown male mice exhibited enhanced bone loss and inflammatory infiltration in a bacterial-induced bone loss murine model compared to females (16). Based on these observations and the known involvement of neutrophils in response to infection, we wanted to characterize the cellular infiltrate in response to oral bacterial infection. Figure 1A shows that in response to oral commensal bacterial infection, a large number of infiltrating cells were positive for a neutrophil-specific marker (Ly6G). Enumeration of inflammatory infiltrate (Figure 1B) suggests that male mice may have more ($P=0.051$), albeit not quite reaching significance, infiltrating immune cells compared to female mice. In Figure 1C, we report that males have nearly double the amount of Ly6G⁺ neutrophil infiltration compared to matched females ($P=0.05$).

Granulocytic neutrophil immunophenotype is not differentially regulated by sex in peripheral blood or bone marrow

To further dissect the distribution of granulocytes in the bone marrow as well as in peripheral blood, flow cytometry was employed to look at monocyte versus granulocyte distribution (Figures 2A and 2B). As the data shows, female and male mice have similar percentage of distribution of monocytes/granulocytes. Next, in bone marrow (Figures 2C and 2D) we show that there is a similar distribution of macrophages (CD11b⁺GR1⁻) between sexes. After confirming distribution of granulocytes and monocytes in both male and female mice and confirming that no significant differences exist, we next aimed to isolate these cells for further *ex vivo* experimentation. Using a pre-established sorting method, Ly6G⁺ granulocyte cell enrichment of naïve granulocytes from bone marrow

achieved greater than 95% purity following magnetic sorting (data not shown). Cells from these isolations were used for gene expression analysis.

Sex influences chemokine and cytokine gene expression in neutrophils in response to bacterial pathogen stimulation

Based on the observation that basal distribution of neutrophils does not differ between sexes, we next aimed to investigate if these cells may differentially respond to LPS stimulation. Data presented in Table 1 summarizes the key findings from Ly6G⁺ cells isolated and stimulated with LPS for 4 hours. Here, female and male mRNA is expressed in treated cells relative to their own unstimulated controls. These results illustrate that males exhibited a significant increase in chemokines *Cxcl1*, *Cxcl2*, *Cxcl3*, and *Cxcl10* (3.26, 9.8, 6.5 and 82.07-fold, respectively) compared to females. Conversely, we showed that males, compared to females, expressed significantly less chemokine receptors *Cxcr2*, *Cxcr3* and *Cxcr4* (−2.69, −38.09 and −3.74-fold, respectively).

Male neutrophils express increased chemokines and inflammatory genes in response to bacterial pathogenic stimulation

Given the substantial differences in chemokine expression between sexes revealed from our array analysis, we next aimed to validate the findings using qPCR. Furthermore, we sought to investigate the contribution of the p38 and JNK signaling pathways in chemokine gene expression as multiple studies have implicated the role of MAPK activity on increased production of these cytokines in response to oral bacterial infections (13, 20, 21), Figure 3A-E highlights mRNA expression in response to LPS between sexes as well as the role of p38 and JNK in the activation of these key chemokines. Results expressed in Figure 3A reveal that *Cxcl1*, that encodes a ligand for CXCR1 and CXCR2, was not significantly different in response to LPS between sexes. However, we found that with the addition of a p38 inhibitor, *Cxcl1* expression, which encodes a ligand for CXCR2, was blunted in females and unchanged in males, resulting in a significant difference in expression between the sexes ($P<0.0001$). Addition of JNK inhibitor blunted expression of *Cxcl1* in both groups. In Figure 3B our results validate the observation that males express significantly more *Cxcl2* than females (>4-fold) in response to LPS. With the addition of p38 and JNK inhibitors, we showed a marked reduction in expression of *Cxcl2* in both populations ($P<0.0001$, respectively). Looking next at *Cxcl12*, the neutrophil egress signal and ligand for CXCR4, (Figs. 3C) we found that no significant differences exist alone or in the presence of inhibitors. In Figure 3D we observed no significant differences in *Cxcr2* expression between sexes. Moreover, this receptor's expression was not influenced by addition of the MAPK inhibitors used above. Finally, the expression of *Cxcr4*, the cognate receptor for CXCL12, showed that males express less (2-fold) of this receptor than females ($P<0.05$). Interestingly, expression of *Cxcr4* was reduced in females in response to both p38 and JNK inhibitors.

Table 1 shows that compared to females, males have enhanced expression of mRNAs encoding IL1 β , IL-6 and TNF- α (5.2, 3.86 and 8.54-fold, respectively). Using qPCR to measure other markers expressed by neutrophils that were not present on the array, we measured expression of mRNAs encoding IL-10 and iNOS in these cells between sexes. Data in Figure 4 reveal that in response to LPS stimulation, males express significantly more

(>11-fold) IL-10 than matched females ($P<0.0001$). Remarkably, addition of both p38 and JNK inhibitors reduced expression by about 50% but did not abrogate the differences between sexes. In addition, iNOS mRNA expression in males was significantly higher (>10-fold) compared to females in response to LPS. Using p38 inhibitors and JNK inhibitors actually reduced expression of iNOS and rescued this hyper-inflammatory activity between sexes (p38 inhibitor data only).

Bacterial pathogen-induced osteoclast formation is differentially regulated by sex

Based on recent findings from our group that select chemokines drive osteoclast formation (21), we next aimed to demonstrate that sex differences affected LPS-driven osteoclast formation. Supplemental Figure 1 shows the immunophenotype of osteoclast progenitors between male and female populations. Enumeration of this data revealed that the true osteoclast progenitor population ($CD3^{-}CD45R^{-}Gr1^{-}CD11b^{lo}CD115^{+}$) were reduced nearly 50% in male cells ($P<0.05$). To determine if these results influenced osteoclastogenesis, dOCP derived from male and female mice were treated as previously described (21). When subpopulations were enumerated (Figure 5A), we found male mature macrophage cells had significantly higher TRAP scoring than females ($P<0.0001$). Conversely, female dOCP populations ($CD11b^{lo}$ and $CD11b^{-}$) had significantly more TRAP⁺ mononuclear osteoclast precursors cells than males ($P<0.001$ and $P<0.0001$, respectively). Figure 5B depicts the number of osteoclasts formed in each population following 48 and 96-hours of LPS stimulation. As the results show, all male populations yielded significantly more the amount of osteoclasts than matched female-sorted cells (all at least $P<0.01$) following LPS stimulation. In Figure 5C, we found that at both times (48- and 96-hours), the male macrophage ($CD11b^{hi}$) and $CD11b^{-}$ fractions only had significantly more nuclei/ osteoclast than females ($P<0.0001$ and $P<0.001$, respectively).

Defined osteoclast male progenitor populations express more osteoclastogenic genes compared to females

In order to determine if the increased number of osteoclasts observed in males and increased size observed in females was the result of differences in genes involved in osteoclast formation and fusion, we analyzed expression of *Nfatc1* and *Tm7sf4* (DCSTAMP), respectively, over the treatment period. Figure 6A reveals that all sorted male populations ($CD11b^{hi/lo/-}$) expressed significantly more *Nfatc1* than female counterparts at 48-hours post LPS stimulation ($P<0.05$, $P<0.01$, $P<0.01$, respectively). By completion of the experiment, *Nfatc1* values were significantly decreased in all populations regardless of sex. Expression of the master regulator of osteoclast fusion, *Tm7sf4* (DCSTAMP) shows that the male dOCP^{lo} and dOCP⁻ populations express significantly more of the gene than female counterparts (Figure 6B; $P<0.05$, each).

Discussion

While not completely understood, it has long been observed that a difference in susceptibility, progression and presentation of bacterial diseases exists between the sexes. The current study supports the notion of a sexual dimorphism in response to pathogenic infection and goes further to try and elucidate the differences between males and females

driving infectious bone loss. By far the most interesting finding reported in this study is the increased number of osteoclast formed *in vitro* in male versus female dOCs in response to bacterial-driven infection. This finding is in contrast to previously findings that gonadectomy resulted in increased osteoclast formation and bone loss (22). However, the discrepancy between previous reports and the current findings is likely due to the concept that females have a diminished proinflammatory response to bacterial infection and that males generally mount a more aggressive inflammatory response to these same microbes (23).

In the absence of all sex hormones, it is likely that proinflammatory differences will perturb the entire system and therefore are not a clear representation of true sex differences in response to infection. Nevertheless, we are able to demonstrate that in all defined osteoclast precursor populations; males express more DC-STAMP, a key regulator of osteoclast fusion gene in response to priming and LPS stimulation. These results suggest that in addition to LPS, additional factors in the inflammatory microenvironment are likely influencing the differences in size and number of osteoclasts formed between sexes.

In addition to differences in osteoclast formation, this study reveals sex differences in chemokine expression in response to bacterial stimulation, which contribute to the inflammatory microenvironment and thus, bacterial-induced osteoclast formation. Recently published research from our lab has shown that in response to LPS, dOC cells express CXCL1, CXCL2 and their cognate receptor CXCR2. LPS-induction of these chemokines following priming with M-CSF and RANKL was shown to induced osteoclast formation (21). These chemokines were verified to be involved through the use of CXCR2 blocking antibodies as well as recombinant chemokine ligand induced osteoclast formation assays. Also, while CXCL1 and CXCL2 were both effective in inducing osteoclast, CXCL2 was more effective and generated larger osteoclasts. Interestingly, a more recent study showed that even in response to commensal bacteria, CXCL2 expression was increased, leading to increased neutrophil recruitment and periodontal disease development (24). In the current study, we show that in neutrophils stimulated with LPS, CXCL2 is significantly increased in males compared to females. This finding is in line with previous work which shows that in females, estrogen reduced CXCL2 expression in monocytes treated with LPS (25). Interestingly, CXCL1 was up-regulated in response to LPS but was not differentially regulated by sex. Taken together, these data indicate that CXCL2 produced by neutrophils may be key to understanding differences in osteoclast formation and bone loss between sexes.

Previous research has shown that MAPK signaling is essential in recruitment and activation of monocytes and neutrophils (26). To try and manipulate the signaling involved in neutrophil activation and egress, we employed the use of p38 and JNK small molecule inhibitors prior to stimulation with LPS. Our data are consistent with previous studies, indicating that JNK signaling is involved in regulation of CXCL1 and CXCL2 (27, 28). Administration of JNK inhibitors blunted CXCL1 and CXCL2 expression, suggesting that JNK signaling regulate these genes independent of sex. Multiple studies have shown that p38 MAPK is essential for CXCL1 and CXCL2 signaling and subsequent neutrophil recruitment (29-31). In congruence with these studies, administration of the p38 inhibitor

reduced expression of *Cxcl1* mRNA in females but actually increased *Cxcl1* mRNA expression in males. Addition of p38 inhibitor blunted expression of *Cxcl2* mRNA expression in both females and male neutrophils. Intriguingly, p38 and JNK signaling did not affect expression of CXCR2, the cognate receptor to CXCL1 and CXCL2.

In addition to increased chemokine production, it is likely that the uncontrolled recruitment seen in male mice may allow for persistent inflammation thus activating other systems including the macrophage/dOCP population, driving them toward osteoclasts. Supporting this notion is the observation that male-derived neutrophils expressed more inflammatory cytokines including IL-6 and TNF α . In response to LPS stimulation, males had more iNOS and IL-10 compared to females. In the presence of p38 and JNK inhibitors, it was shown that for IL-10, expression was blunted in both sexes but more so in females. For iNOS expression, it was shown that both JNK and p38 reduced expression in both sexes; however, p38 alone regulated differences observed between sexes. Supporting the idea of increased inflammation leads to increased osteoclast formation, Lee et al reported that NO produced by iNOS stimulated the survival of osteoclasts produced in response to inflammatory stimuli (32) whereas the enhanced expression of IL-10 suppresses inflammatory driven osteoclastogenesis through a mechanism of feedback inhibition (33). In summation, these data support the concept that while males can recruit more neutrophils in response to infection, their inflammatory response is exacerbated; leading to more collateral damage, i.e. bone loss.

Under the current paradigm of neutrophil egress from the bone marrow reserve, it is known that CXCL12 functions as the retention signaling and upon stimulation, the ratio of CXCR4 to CXCL12 increases in favor of CXCR4, thus promoting egress. Recently, and in support of our findings, Scotland et al investigated at the role of sex and immune cell infiltration in response to acute infection using a peritonitis model (8). In that study, they noted that the females acute inflammatory response was blunted due to reduced neutrophil recruitment compared to matched male mice. Specifically, they found that CXCL12 as well as CXCR4 were up significantly in female mice compared to males, thus preventing egress. To help explain why there may be more cellular infiltrate in male tissues exposed to the oral commensal bacterial infection, we isolated neutrophils and looked at their capacity for egress based on CXCL12 and CXCR4 expression in response to LPS. We found that while *Cxcl12* mRNA was not different between sexes, but *Cxcr4* was reduced by nearly half in males, thus supporting egress as a plausible explanation of the observed greater neutrophil infiltration in response to oral infection. Moreover, we found the effect of p38 and JNK inhibition did not influence *Cxcl12* expression. However, while p38 was shown to not influence *Cxcl12* gene expression, data indicates that disrupted p38 signaling leads to a reduction in chemotaxis in leukocytes (34) and thus may influence neutrophil migration. In this study, *Cxcr4* expression was dependent on p38 and JNK signaling, suggesting the possibly reducing neutrophil chemotaxis. This finding is interesting as *Cxcr4* expression is upstream of p38 and JNK signaling. It has been shown that the CXCR4/CXCL12 signaling axis up-regulates p38 expression (35). Given this finding, it is apparent that signaling feedback may be influencing neutrophil egress and immune plasticity.

With respect to observed differences in sexual dimorphism, CXCL10 and CXCR3 expression is most striking. Female neutrophils treated with LPS saw a modest increase in *Cxcl10* (6-fold) and decrease in *Cxcr3* (3-fold) mRNA levels. In stark contrast, male neutrophils had a sharp reduction (–38-fold) in *Cxcr3* and a large increase (82-fold) in *Cxcl10* relative to control. These findings are of interest as the CXCR3-CXCL10 signaling axis has been implicated in neutrophil mediated pathogenesis. Importantly, excessive CXCL10 can contribute to osteoclast-driven bone loss by up-regulating RANKL (36). Our results, in conjunction with previous findings that LPS induces CXCL10 which in turn induces osteoclast formation suggests, that excessive CXCL10 secreted by male neutrophils into the inflammatory microenvironment may be a contributor to the excessive bone loss seen in males (37).

Based on our results, we conclude that in response to oral infection, male neutrophils can more easily egress to the sight of infection, produce greater amounts of inflammatory cytokines and chemokines to preferentially activate infiltrating dOCP as they enter the microenvironment, creating more osteoclasts when compared with females. Together, these findings highlight that key chemokine ligands are differentially regulated by sex and could be studied further for targeted therapies to reduce inflammation and inflammatory-driven bone loss in oral bacterial diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Neutrophil infiltration and inflammation is greater in males in response to infection. Male and female mice (n=5/group) were subject to endodontic surgery by root canal exposure allowing for colonization of oral bacteria into the periapical region. **(A)** Inflammatory infiltration was measured using H&E staining. Neutrophil-specific infiltration was measured using a Ly6G-specific antibody and subsequent IHC staining. **(B)** Quantitation of inflammatory score **(C)** Quantitation of neutrophil infiltration relative to inflammatory score. Pictures are representative independent observations and graphical analysis is depicted as mean and SD of an ordinal pathological scaling system staining scale. B=Alveolar bone adjacent to root apex; PA= Periapical connective tissue (*P<0.05)

Figure 2.

Male and female mice do not exhibit a different granulocytic immunophenotype in peripheral blood or bone marrow. Female and male cells were isolated from PB and BM and subjected to flow cytometric analysis prior to stimulation with LPS. **(A)** Females (left) and male (right) PB was analyzed for distribution of monocytes (CD11b+Gr1⁻), granulocytes (CD11b+Gr1⁺) and neutrophils (CD11b-Gr1⁺). **(B)** Enumeration of flow cytometric findings from each population. **(C)** Female (left) and male (right) BM-derived cells were analyzed for monocytes (CD11b+Gr1⁻), granulocytes (CD11b^{hi}Gr1⁺) and neutrophils (CD11b^{lo/-}Gr1⁺) and non-granulocytic cells (CD11b⁻Gr1⁻). **(D)** Enumeration of flow cytometric findings between populations and sexes. Flow cytometric and graphical data are representation of at least n =4 biological replicates. Graphical data is depicted as mean and SD.

Figure 3. p38 and JNK MAPK inhibitors regulate autocrine chemokine sex differences. Female and Male isolated neutrophils were subjected to LPS stimulation (100ng/ml) alone or in the presence of p38 (SB203580) and JNK (SP600125) inhibitors and harvested for RNA and subsequent gene expression. (A) *Cxcl1* expression (B) *Cxcl2* expression. (C) *Cxcl12* expression. (D) *Cxcr2* expression. (E) *Cxcr4* expression. Graphical data is depicted as mean and SD fold-change of at least n=3 biological replicates, relative to untreated control. (*P<0.05, **** P<0.0001)

Figure 4.

Sex differentially regulates inflammatory cytokine expression in response to pathogenic LPS stimulation. Male and female isolated granulocytes were subject to stimulation with LPS (100ng/ml, 4hrs) or vehicle alone or in the presence of p38 (SB203580) and JNK (SP600125) inhibitors and harvested for RNA and subsequent gene expression. Samples were measured for expression of *II10* (A) and *Nos2* (B). Graphical data is depicted as mean fold change and SD of at least n=3 biological replicates. (** P<0.01, ***P<0.001, ****P<0.0001)

Figure 5.

Pathogen-driven osteoclastogenesis is differentially regulated by sex. Female and male sorted monocytes and dOCPs were primed with M-CSF and RANKL and subjected to pathogen stimulation with 50ng/ml LPS for up to 96-hours. Female-derived and male-derived osteoclasts from Day 0 (primed), Day 2 (48-hour LPS) and Day 4 (96-hour LPS) from sorted monocytic populations were utilized for analysis. **(A)** Enumeration of pre-osteoclast following 48-hours of priming in each population. Cells were measured for relative TRAP positivity using an ordinal scoring system. **(B)** Enumeration of the number of

osteoclast formed per field of view between sexes and from each sorted population at Days 2 and 4 post-LPS stimulation. (C) Enumeration of the number of nuclei per osteoclast between sexes from each sorted population at Days 2 and 4 post-LPS stimulation. Data is representative of the average of each well from at least n=6 experimental and biological replicates. (*P<0.05, ** P<0.01, ***P<0.001, ****P<0.0001)

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Figure 6.

Osteoclast-specific gene expression is differentially regulated by sex. Male and female monocyte and dOCP-sorted populations were plated, primed and subjected to osteoclast differentiation in the presence of LPS stimulation as described. Cells were harvested for mRNA expression of Osteoclastogenic genes following priming and 48- and 96-hours post LPS stimulation. (A) *Nfatc1* expression in male and female-sorted populations. (B) DC-STAMP (*Tm7sf4*) expression in male and female-sorted populations. Gene expression was normalized to internal loading and housekeeping control and plotted as a fold-change

relative to Day 0 treatment. Graphic data is representative of at least n=4 independent observations. (*P<0.05, ** P<0.01)

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Table 1
Male vs Female Inflammatory PCR Array Summary From Isolated Neutrophils

Gene ID	Function	Female Tx vs Con	Male Tx vs Con
<i>C5ar1</i>	Neutrophil Migration and Recruitment	1.10	1.02
<i>Ccl4</i>	Neutrophil Derived Inflamm. Chemottractant	-1.53	3.06
<i>Ccl5</i>	Neutrophil Act., Inflamm. Assoc. w/ Inflamm. Infiltration	1.34	18.40
<i>Ccr1</i>	Involvement in Neutrophil Migration	1.41	-5.10
<i>Cxcl10</i>	Neutrophil Chemotaxis, Binds w/ Cxcr3	1.47	82.07
<i>Cxcl11</i>	Neutrophil Chemotaxis, Binds w/ Cxcr3	1.15	2.08
<i>Cxcl12</i>	Neg. Reg. of Neutrophil Chemotaxis	-1.82	-3.54
<i>Cxcl1</i>	Neutrophil Chemottractant, Binds to Cxcr2	-2.53	3.26
<i>Cxcl2</i>	Neutrophil Chemottractant, Binds to Cxcr2	-3.39	9.80
<i>Cxcl3</i>	Neutrophil Chemottractant	1.03	6.50
<i>Cxcl9</i>	Assoc. w/ Cxcl11 function, Act. By TLR ligand	2.18	1.68
<i>Cxcr2</i>	Neutrophil Act., Chemotaxis	-1.02	-2.69
<i>Cxcr3</i>	Interact. with Cxcl 10 for Chemotaxis	-1.18	-38.09
<i>Cxcr4</i>	Neg. Reg. of Neutrophil Chemotaxis	1.11	-3.74
<i>Cxcr7</i>	Assoc. w/ Cxcr4	-1.89	3.96
<i>Il1b</i>	Encodes IL-1 β , Inflamm. Cytokine	1.66	5.20
<i>Il6</i>	Encodes IL-6, Inflamm. Cytokine	1.30	3.86
<i>Itgam</i>	Encodes for CD11b, Migration/Adhesion	1.51	-3.75
<i>Itgb2</i>	Encodes for CD11c, Migration/Adhesion	1.02	-1.72
<i>Mapk14</i>	encodes for p38 MAPK, Inflamm. Signaling	-1.28	-1.93
<i>Tnf</i>	Encodes for TNF- α , Inflamm. Cytokine	-2.34	8.54
<i>Xcl1</i>	Pos. Reg. of Neutrophil Chemotaxis	-1.16	2.84

mRNA gene expression from male and female murine neutrophils (L6G⁺) stimulated with 100ng/ml *A. actinomycetemcomitans* LPS, 4-hours post isolation. Values represent fold change relative to the untreated control from each sex. Increased gene expression over 2-fold is highlighted in red and is positive. Reduced gene expression below 2-fold is highlighted in blue and is negative.