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Research Article

Androgen Exhibits a Protective Role Against Focal Erosions in Murine TNF-induced Inflammatory Arthritis

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Abstract

Rheumatoid arthritis (RA) is characterized by erosive pathology associated with joint inflammation and a sexual dimorphism with increased prevalence in females. Here, we aim to determine whether androgen is protective against inflammatory-erosive disease in TNF-transgenic (TNF-Tg) mice.

Wild-type (WT) and TNF-Tg male mice underwent sham (WT, n = 3; TNF-Tg, n = 7) or orchiectomy (WT, n = 3; TNF-Tg, n = 7) surgery at 1 month old to remove androgen production confirmed by serum testosterone concentration. Cohorts of orchiectomized TNF-Tg males were treated with either 5α-dihydrotestosterone (.025 mg/day) (n = 3) or placebo (n = 3) via subcutaneous pellet insertion. Weekly clinical measures, along with mid-hindpaw bone volumes and ankle histology at 3 months old were evaluated for all groups.

Orchiectomies in TNF-Tg males significantly decreased serum testosterone ($P < .05$), weight gain ($P < .001$), and mid-hindpaw bone volumes ($P < .05$) in comparison to sham TNF-Tg mice. The cuboid bone also had increased synovitis by histology with the loss of androgen ($P < .05$). Treatment of orchiectomized TNF-Tg males with 5α-dihydrotestosterone protected against the changes in weight gain ($P < .01$) and bone erosion ($P < .05$) associated with decreased osteoclast number in the cuboid ($P < .01$).

In the TNF-Tg model of chronic inflammatory arthritis, androgen is protective in erosive disease. The loss of endogenous androgen significantly accelerated the progression of inflammatory-erosive arthritis in male TNF-Tg mice to a similar severity as age-matched female mice. In addition, treatment with exogenous androgen prevented this observed bone loss in orchiectomized TNF-Tg males. Overall, androgen delays and limits bone erosion even in the presence of active inflammation and future studies are warranted to elucidate the associated mechanisms.

Key Words: mouse model, arthritis, androgen, bone erosion, inflammation

Abbreviations: CAIA, collagen antibody-induced arthritis model; CIA, collagen-induced arthritis model; CT, computed tomography; H&E, hematoxylin and eosin; NAVLATINT, navicular and lateral intermediate cuneiform; orchx, orchiectomy; RA, rheumatoid arthritis; TNG-Tg, TNF-transgenic; TRAP, tartrate-resistant acid phosphatase; WT, wild-type; ZIA, zymosan-induced arthritis model.

Rheumatoid arthritis (RA) is a chronic immune-mediated inflammatory disorder that develops from overexpression of pro-inflammatory cytokines in the joint, leading to destructive joint inflammation [1-3]. RA and other disorders with autoimmune arthritis, including psoriatic arthritis, juvenile idiopathic arthritis, and ankylosing spondylitis, are characterized by the sequelae of bone erosions mediated by the inflammatory environment of the joints [4-7]. In RA, the inflamed synovium develops into an invading pannus with increased cytokine signaling toward production and activation of bone resorbing osteoclasts that create focal periarticular erosions, which serve as markers of severe disease [4, 5, 8]. These inflammatory erosions can be a result of various factors that influence disease, 1 of which is the female-predominant sexual dimorphism common to autoimmune disorders.

Despite the remarkable relevance to clinical disease prevalence and progression for autoimmune conditions, including RA, systemic lupus erythematosus, Sjogren disease, and systemic sclerosis, the etiology of these pronounced sex differences remain unclear [9]. However, it is known that immune cells express receptors for sex hormones, which can mediate their inflammatory activity [10]. Multiple models of inflammatory-erosive arthritis have been found to exhibit sexual dimorphism, including the collagen-induced (CIA), collagen antibody-induced (CAIA), and zymosan-induced (ZIA) arthritis mouse models [11-13], with evidence supporting that these differences are related to sex hormones [13-17]. Androgen, the predominant sex hormone in males, promotes anti-inflammatory effects on immune cells, establishing the potential to ameliorate inflammatory activity [18, 19]. In RA, androgen levels have also been found to be lower in patients, including males, compared to healthy individuals [20-22]. However, data regarding clinical response of erosive arthritis to androgen therapy are limited; 1 small study of 7 males with RA suggested equivocal response with improved tender joint count but unchanged erythrocyte sedimentation rate and erosions were not evaluated [23]. Another study in female patients with RA also found improvement in erythrocyte

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sedimentation rate, pain, and a clinical health questionnaire [24]. Sex hormones do affect bone remodeling, a process dysregulated in inflammatory arthritis, with androgen found to stimulate bone formation and inhibit bone loss [25-27]. However, the specific role of androgen in an inflammatory erosive environment, such as RA, has not been determined.

The TNF-transgenic (TNF-Tg; line 3647) mouse is a model of chronic inflammatory-erosive arthritis that displays a remarkable sexual dimorphism [28]. TNF-Tg mice overexpress the human TNF-α transgene, leading to severe synovitis, pannus formation, and bone erosions, and we found that female TNF-Tg mice have earlier onset and more accelerated disease than males [28, 29]. In TNF-Tg males, inflammatory erosive disease is significantly delayed in comparison to female mice, that show erosive disease by age 3 months [28, 30]. Based on this difference between sexes, we hypothesized that androgens delay focal bone erosions even in an inflammatory environment. Here, we investigated the role of androgens on the progression of inflammatory-erosive arthritis in TNF-Tg mice with removal of endogenous androgen production via orchiectomy and treatment with exogenous androgen.

Materials and Methods

Animals

All animal research was approved by the University of Rochester Medical Center University Committee for Animal Resources. The 3647 TNF-Tg mice overexpress 1 copy of the human TNF-α gene [29]. The 3647 line was originally obtained from Dr. George Kollias (Institute of Immunology, Alexander Fleming Biomedical Sciences Research Center, Vari, Greece) [31, 32] and has since been maintained at the University of Rochester on a C57BL/6J background. Wild-type (WT) littermates were used as controls.

Orchiectomies and Pellet Insertions

Orchiectomy or sham control aseptic surgeries were performed on 1-month-old male TNF-Tg (sham, n = 7; orchiectomy, n = 7) and WT (sham, n = 3; orchiectomy, n = 3) mice under isoflurane anesthesia. In all orchiectomies, both testes were removed, preventing any endogenous androgen production, as mice do not produce adrenal androgen [33]. Sham procedures included the initial incision in the scrotum but no removal of testes. Incisions were closed with 4-0 monofilament suture. Age-matched TNF-Tg females (n = 5) were compared to these groups. In cohorts with exogenous androgen replenishment, pellets containing 1.5 mg of DHT, the active form of testosterone, or placebo (Innovative Research of America) were inserted subcutaneously in the back immediately following orchiectomies (DHT, n = 3; placebo, n = 3). Pellets released .025 mg of DHT or placebo per day for 60 days. Intact TNF-Tg males (n = 3) were compared to these cohorts. Orchiectomized and placebo-treated animals were housed separately from sham and DHT-treated animals. Mice of the appropriate genotype were randomly divided for treatment. A total of 33 animals was used for analysis. After euthanization and collection, mouse identifications were blinded for analysis. Previous studies comparing WT and TNF-Tg mice determined the initial sample sizes [34]. Because TNF-Tg mice typically develop an arthritis phenotype at age 2 months, surgery was performed at age 1 month [29]. DHT treatment was used to avoid the possible aromatization of testosterone and to supplement the loss of androgen with orchiectomy [35]. Previous validated studies using DHT pellets in mice determined the dosage [36-39].

Serum Collection and Analysis

Sham (n = 4) and orchiectomized TNF-Tg mice (n = 5) were euthanized at age 3 months and blood was harvested by abdominal aortic puncture. Blood was held at room temperature for 30 minutes, then centrifuged at 1500*g* for 10 minutes, and the supernatant was collected. Serum was analyzed with a serum testosterone ELISA (Catalog #582701, RRID: AB_2895148) following the manufacturer's instructions. Before assay, all serum samples were extracted with diethyl ether to reduce interference with serum components such as proteins and lipids. Concentrations above the limit of detection in the ELISA for our orchiectomized mice were attributed to possible cross-reactivity or nonspecific binding that is common in hormone immunoassays [40-44]. Because mice do not have the enzyme CYP17 in their adrenals, it is unlikely that production and conversion of peripheral androgen is occurring to normal physiological levels [33, 45].

Weight and Paw Scoring

Mice (sham/orchiectomized TNF-Tg/WT, n = 3; placebo- and DHT-treated orchiectomized TNF-Tg, n = 3; TNF-Tg female, n = 5) were weighed at age 1 month before surgery as a baseline, then weighed weekly until euthanasia at age 3 months. The baseline and final weights were used as a clinical measure of general health. Paw inflammation was subjectively scored weekly from age 1 month (before surgery) until age 3 months on a scale of 0 to 3 (averaged weekly per mouse), with higher scores indicating worse paw swelling as a clinical measure.

Micro-computed Tomography Imaging and Analysis

Hindpaws of 3-month-old live mice (sham/orchiectomized WT, n = 3; sham/orchiectomized TNF-Tg, n = 7; placebo- and DHT-treated orchiectomized TNF-Tg, n = 3; TNF-Tg females, n = 5; intact TNF-Tg males, n = 3) were imaged by micro-computed tomography (micro-CT; VivaCT 40, Scanco USA, Inc.) under isoflurane anesthesia. DICOMs were exported to Amira (V2020.2, Thermo Fisher Scientific, USA) for analysis with a semiautomated segmentation method [46]. The bone volumes of the cuboid, navicular and lateral intermediate cuneiform (NAVLATINT), and talus were compared among groups and were the primary outcome measures. The NAVLATINT exhibits variable fusion [46]; therefore, the sum of the volumes was measured. The TNF-Tg mouse model experiences asymmetric disease in which the severity of the disease differs between each limb. This has been previously validated in measuring the bone volume of these mice [47]. Both limbs from TNF-Tg mice have also been previously studied as independent datapoints to measure disease [28, 48-52]. Therefore, both hindpaws for each mouse was analyzed as an independent datapoint.

Histology

Ankles from 3-month-old mice (sham TNF-Tg, n = 4; orchiectomized TNF-Tg, n = 5; placebo- and DHT-treated orchiectomized TNF-Tg, n = 3) were collected and fixed in 10% formalin for 3 days, followed by decalcification, histology processing, paraffin embedding, and sectioning (5 μm) onto microscope slides (Center for Musculoskeletal Research Histology Core

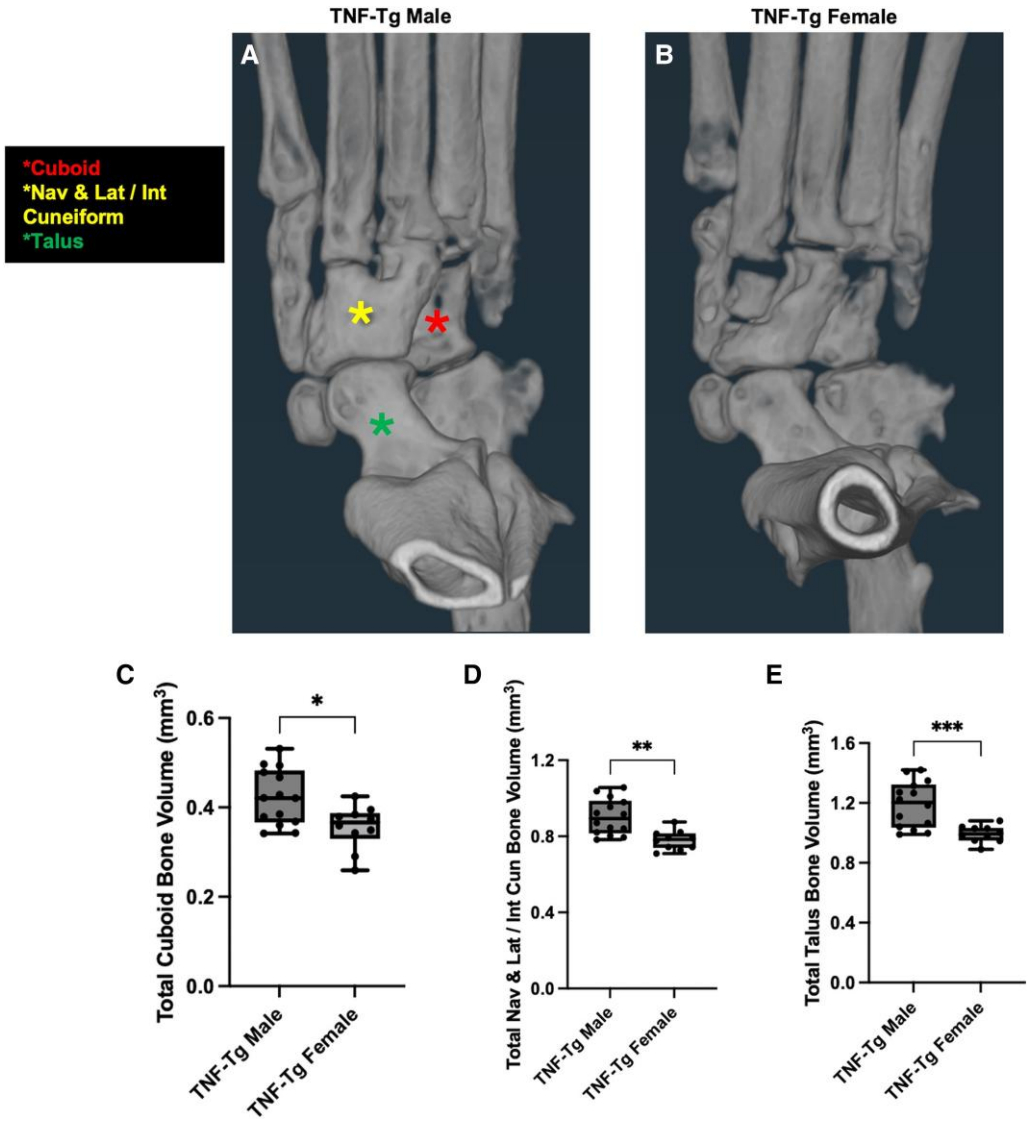

Figure 1. Female TNF-Tg mice have significantly less bone volume than male TNF-Tg mice. The hindpaws of TNF-Tg male (n = 7) and female (n = 5) mice were imaged with micro-computed tomography (micro-CT) at age 3 months. Representative 3-dimensional-rendered images of a TNF-Tg male (A) and TNF-Tg female (B) exhibit the mid-hindpaw bones compared between sexes, including the cuboid (A, red/right asterisk), navicular and lateral intermediate cuneiform (A, yellow/left asterisk), and talus (A, green/lower asterisk). The total bone volume of the cuboid (C), navicular and lateral intermediate cuneiform (D), and talus (E) bones was compared between males and females, with females having a significantly lower bone volume than males in all analyzed bones. Statistics: unpaired *t*-tests (C-E); $*= P < .05$, $**= P < .01$, $***= P < .001$.

Facility, University of Rochester Medical Center). Cuboid and talus sections were stained with hematoxylin and eosin (H&E; synovium) or tartrate-resistant acid phosphatase (TRAP; osteoclasts) with .08% Fast Green counterstain. Stained slides were scanned with an Olympus VS120 at 20× objective magnification, and synovial and TRAP+ area were quantified using Visiopharm (V2021.07, Visiopharm A/S, Denmark), as previously described [28]. Each datapoint represents 1 paw.

Statistics

Statistics, including *t*-tests and 1- or 2-way ANOVA with Tukey or Sidak multiple comparisons, were performed as appropriate in GraphPad Prism (V10.2.0, GraphPad Software, USA). Continuous data using parametric analysis were confirmed to have normal distribution with Shapiro-Wilk tests. Outliers were defined using the interquartile range method [53, 54]. This method determined that values 1.5 × interquartile range above the upper quartile and below the lower quartile were excluded from analysis. One paw in the micro-CT analysis for DHT-treated orchiectomized mice and 1 paw in the histology analysis for placebo-treated orchiectomized mice were outliers and excluded from analysis. No other exclusions were made. Values are reported as mean ± SD.

Results

TNF-Tg Mice Females Exhibit Accelerated Erosive Arthritis

Micro-CT analyses of 3-month-old TNF-Tg male and female mice were used to determine the difference in bone volume as a measure of erosion between sexes. The bones of the mid-hindpaw, the cuboid (Fig. 1A, red asterisk), NAVLATINT

(Fig. 1A, yellow asterisk), and talus (Fig. 1A, green asterisk) were measured to compare total bone volumes. Representative 3-dimensional-rendered images of a TNF-Tg male (Fig. 1A) and female (Fig. 1B) exhibit erosive bone loss occurring, most notably in the female. Female TNF-Tg mice were also found to have significantly more erosions in the cuboid (TNF-Tg male $= .42 \pm .04$ mm^3, TNF-Tg female $= .36 \pm .05$ mm^3, $P < .05$), NAVLATINT (TNF-Tg male $= .90 \pm .09$ mm^3, TNF-Tg female $= .78 \pm .05$ mm^3, $P < .01$), and talus (TNF-Tg male $= 1.19 \pm .153$ mm^3, TNF-Tg female $= .99 \pm .05$ mm^3, $P < .001$) compared to TNF-Tg males (Fig. 1C-E).

Orchiectomized TNF-Tg Male Mice Exhibit Exacerbated Erosive Arthritis Compared to Sham Controls

To investigate the role of androgen loss in TNF-Tg erosive arthritis, we performed orchiectomies and sham control surgeries on 1-month-old WT and TNF-Tg male mice. Successful orchiectomy (orchx) was confirmed by significantly lower serum testosterone (sham TNF-Tg $= 193.4 \pm 127.3$ pg/mL, orchx TNF-Tg $= 58.14 \pm 22.88$ pg/mL, $P < .05$) (Fig. 2A). Measures of serum testosterone in orchiectomized mice above the limit of detection is a result of known cross-reactivity with other steroids in serum [40-44]. Cohorts with little to undetectable levels of androgen (orchiectomized males and TNF-Tg females) had significantly smaller final weights than cohorts with higher androgen production (sham WT and TNF-Tg males) (sham TNF-Tg $= 26.36 \pm 2.14$ g, orchx TNF-Tg $= 20.31 \pm 1.39$ g, $P < .05$) (Fig. 2B). There were no significant differences among TNF-Tg cohorts in weekly paw deformation scores (Fig. 2C). Micro-CT images displayed the bones compared among cohorts (Fig. 2D-H). Analysis of mid-hindpaw bones exhibited reduced bone volumes in orchiectomized TNF-Tg males compared to sham (cuboid: sham TNF-Tg male $= .42 \pm .04$ mm^3, orchx TNF-Tg male $= .37 \pm .03$ mm^3, $P < .05$; NAVLATINT: sham TNF-Tg male $= .90 \pm .09$ mm^3, orchx TNF-Tg male $= .82 \pm .04$ mm^3, $P < .05$; talus: sham TNF-Tg male $= 1.19 \pm .153$ mm^3, orchx TNF-Tg male $= 1.06 \pm .08$ mm^3, $P < .05$) (Fig. 2I-K). Notably, there were no differences in mid-hindpaw bone volumes between orchiectomized TNF-Tg males and TNF-Tg females, whereas both were decreased compared to sham TNF-Tg males (TNF-Tg female cuboid: $.36 \pm .05$ mm^3; NAVLATINT: $.78 \pm .05$ mm^3; talus: $.99 \pm .05$ mm^3, $P < .01$, $P < .001$, 1-way ANOVA). There was also no significant bone loss between orchiectomized and sham WT males.

Orchiectomy Leads to More Severe Disease in the Ankles of TNF-Tg Mice

To further evaluate the potential mechanisms of severe bone erosions in orchiectomized TNF-Tg mice, H&E (Fig. 3A-B, H-I; synovial inflammation) and TRAP stains (Fig. 3C-D, J-K; osteoclast number) were assessed in ankle bones. Orchiectomized TNF-Tg mice had significantly greater total synovial area (sham TNF-Tg $= 246\,421 \pm 39\,289$ μm^2, orchx TNF-Tg $= 356\,784 \pm 66\,264$ μm^2, $P < .05$) and synovial nuclei area (sham TNF-Tg $= 42\,273 \pm 7664$ μm^2, Orchx TNF-Tg $= 71\,905 \pm 19\,585$ μm^2, $P < .05$) than sham TNF-Tg mice in the cuboid (Fig. 3E-F). We also analyzed the talus bone (Fig. 3L-M), and although there were increases in measures of synovial inflammation in orchiectomized mice, it was not significant. TRAP+ area was not significantly different between groups in either bone (Fig. 3G and N) at 3 months.

Androgen Replacement Protects Against Bone Loss in Orchiectomized TNF-Tg Mice

The influence of androgen on inflammatory erosive disease was evaluated by androgen or placebo replacement via subcutaneous pellets in orchiectomized TNF-Tg mice. The final weight at age 3 months of the orchiectomized mice treated with DHT was significantly greater than orchiectomized mice that received placebo (orchx TNF-Tg with placebo $= 19.97 \pm .25$ g, orchx TNF-Tg with DHT $= 25.77 \pm 2.12$ g, $P < .01$) (Fig. 4A). There were no significant differences in average paw deformation score between the placebo- and DHT-treated cohorts (Fig. 4B). Hindpaw micro-CT at age 3 months including a cohort of intact TNF-Tg males for comparison (Fig. 4C-E) showed marked bone loss in the placebo-treated cohort that was not present with DHT treatment. The placebo-treated orchiectomized mice have the lowest total bone volume between all cohorts, and the DHT-treated orchiectomized mice are protected from that loss (Fig. 4F-I) in the cuboid (orchx TNF-Tg with placebo $= .34 \pm .03$ mm^3, orchx TNF-Tg with DHT $= .43 \pm .02$ mm^3, $P < .01$), NAVLATINT (orchx TNF-Tg with placebo $= .78 \pm .04$ mm^3, orchx TNF-Tg with DHT $= .87 \pm .03$ mm^3, $P < .01$), and talus (orchx TNF-Tg with olacebo $= .97 \pm .09$ mm^3, orchx TNF-Tg with DHT $= 1.10 \pm .06$ mm^3, $P < .05$) bones.

DHT-treated Orchiectomized Mice Are Protected From Erosive Disease

Ankle histology of the placebo-treated and DHT-treated orchiectomized TNF-Tg mice was also evaluated by H&E (Fig. 5A-B, H-I) and TRAP staining (Fig. 5C-D, J-K) of the cuboid and talus bones. There was no significant difference between groups in total synovial area or synovial nuclei area in the cuboid and talus (Fig. 5E-F, L-M). Notably, however, DHT-treated mice were found to have a significantly less TRAP+ area in the cuboid than placebo-treated mice (orchx TNF-Tg with placebo $= 8030 \pm 2866$ μm^2, orchx TNF-Tg with DHT $= 3289 \pm 1206$ μm^2, $P < .01$) (Fig. 5G).

Discussion

To our knowledge, this study is the first to show that androgen limits erosive disease in a TNF-mediated chronic inflammatory-erosive mouse model of RA, which has an established sexual dimorphism wherein males have delayed disease progression compared to females [28]. As androgens are known to be anti-inflammatory and inhibit bone loss [18, 19, 25-27], we hypothesized that androgens are protective in inflammatory-erosive arthritis. We demonstrated that lack of androgen increased inflammatory erosive disease in male TNF-Tg mice, whereas androgen treatment was protective and delayed the bone resorptive activity enhance by TNF-mediated inflammation. Overall, these results indicate that androgen may have a significant role in mitigating inflammatory-erosive arthritis.

Orchiectomy prevents male mice from producing endogenous androgen [33] because sex organs are the only source of androgen in mice, unlike humans with the capacity for androgen production in the adrenals. Lack of androgen leads to several changes in mice, including reduction in muscle mass that is a biological effect from surgery [55, 56]. Orchiectomies of TNF-Tg and WT mice corroborated these findings with significantly reduced testosterone and final weights compared to sham cohorts. Regarding bone

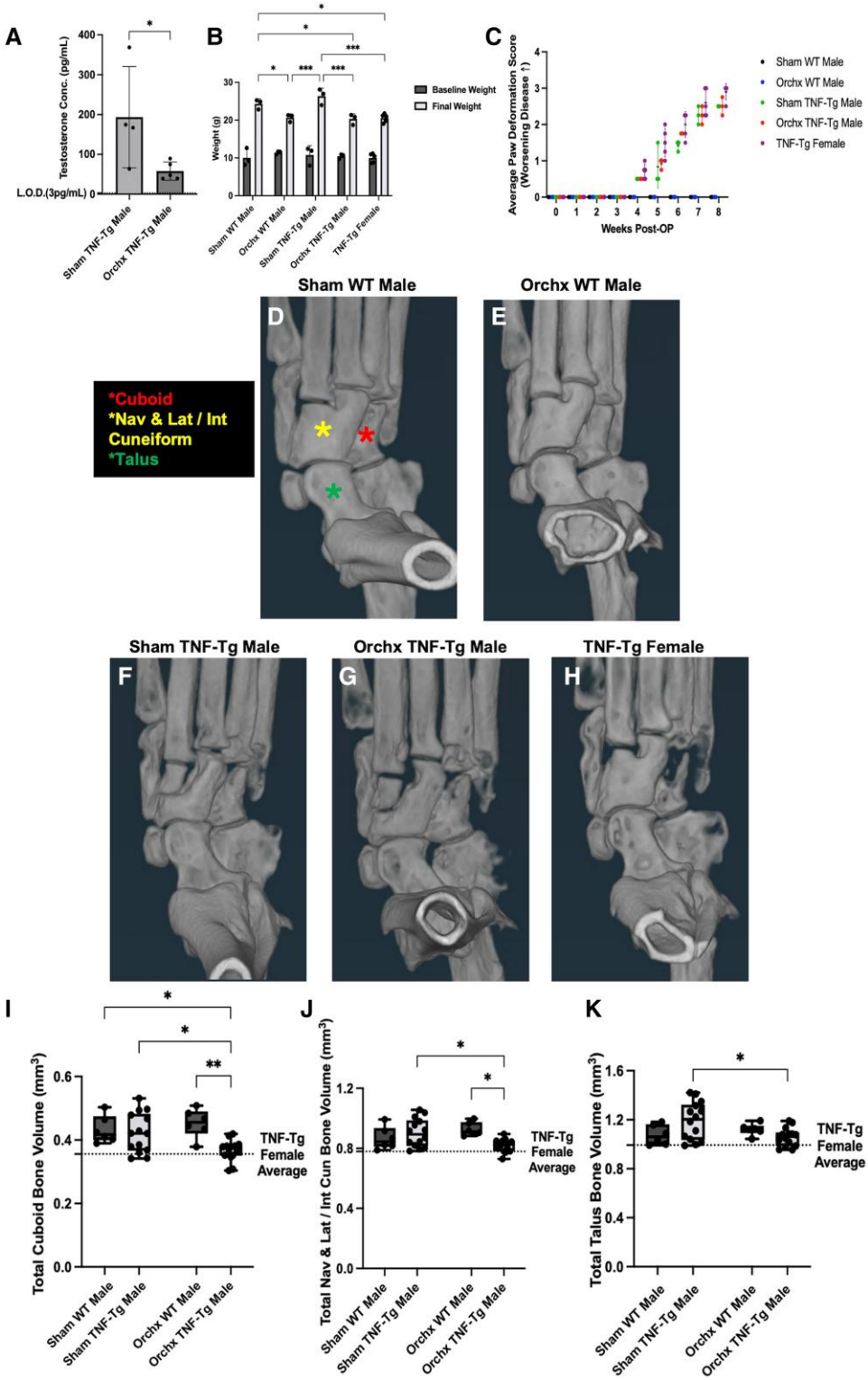

Figure 2. Orchiectomized TNF-Tg male mice develop accelerated bone erosions. Orchiectomies and sham control surgeries were performed on 1-month-old WT and TNF-Tg male mice (n = 3-7 mice/group) and mice were aged to 3 months old. Same-age TNF-Tg female mice (n = 5) were also compared to male cohorts. Serum testosterone concentrations in sham and orchiectomized TNF-Tg mice confirmed decreased endogenous androgen after orchiectomy (A). Weight and paw deformation score were compared among cohorts (B-C). Micro-CT images of a 3-month-old sham WT (D), orchiectomized (orchx) WT (E), sham TNF-Tg (F), orchx TNF-Tg (G), and TNF-Tg female (H) mouse display the bones analyzed: the cuboid (D, red/right asterisk), navicular and lateral intermediate cuneiform (D, yellow/left asterisk), and talus (D, green/lower asterisk). Total bone volume was compared among sham and orchx cohorts (I-K), with orchx TNF-Tg mice exhibiting accelerated erosions compared to sham TNF-Tg mice. The dashed line illustrates the average bone volume of TNF-Tg females for each bone. Statistics: unpaired *t*-test (A), 2-way ANOVA with Tukey multiple comparisons (B-C, I-K). Significant comparisons between WT and TNF-Tg cohorts are not shown in the average paw deformation score. LOD, limit of detection. $*P < .05$; $**P < .01$; $***P < .001$.

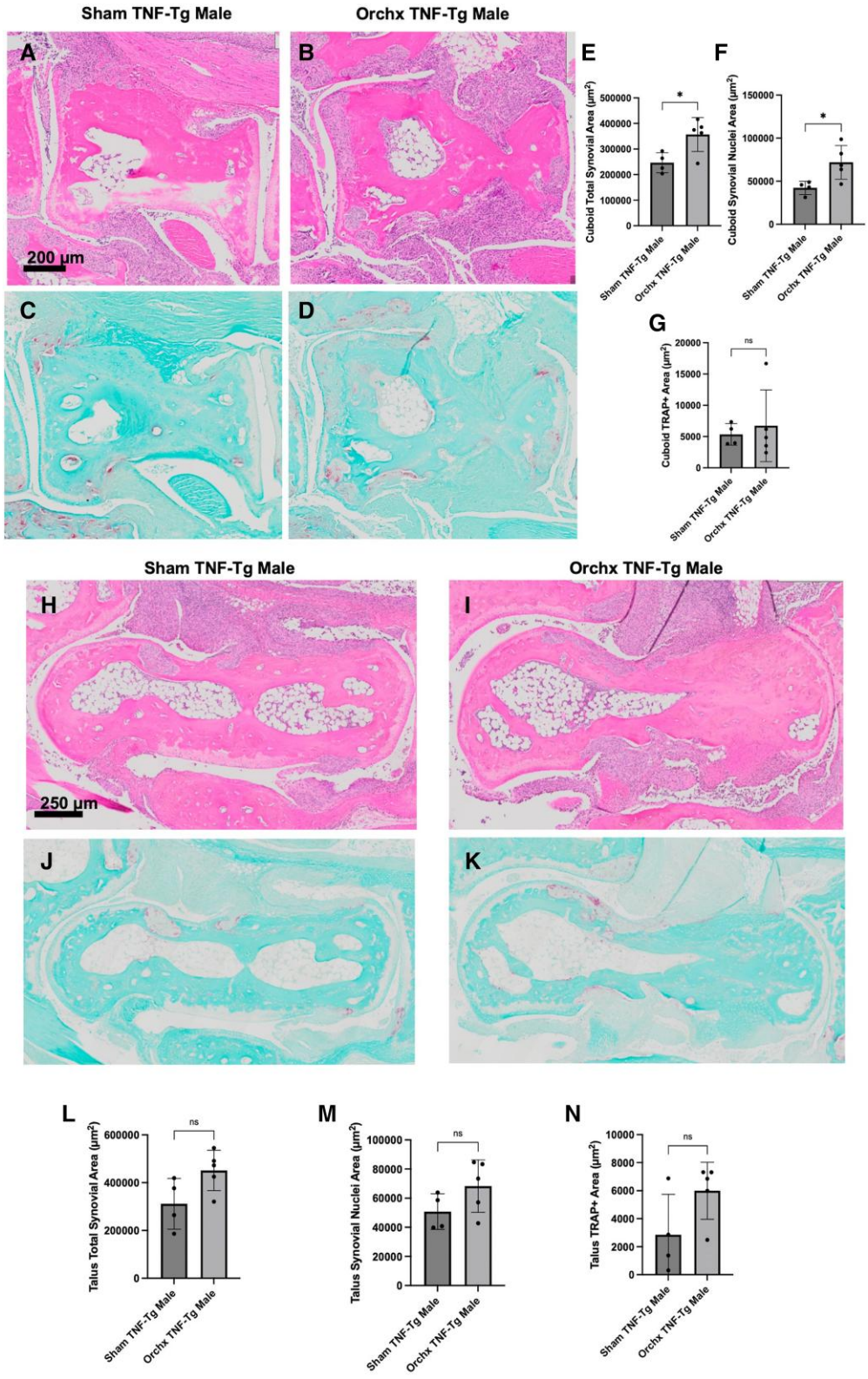

Figure 3. Inflammatory erosive disease is more severe in the ankles of orchiectomized TNF-Tg mice. Cuboid and talus sections of 3-month-old orchx and sham TNF-Tg mice (n = 4-5 mice/group) were H&E (A-B and H-I) and TRAP-stained (C-D and J-K) to display synovial inflammation and osteoclast number for histomorphometric analysis. Total synovial area, synovial nuclei area, and TRAP+ area were quantified (E-G and L-N) and showed greater inflammation in the cuboid of orchx mice. Statistics: unpaired *t*-tests (E-G, L-N); ns, not significant; $^{*}P < .05$.

loss, orchiectomized TNF-Tg mice had significantly less mid-hindpaw bone volume than sham TNF-Tg males and was comparable to age-matched TNF-Tg females. In addition, although there is variability with bone volume changes, there is no significant difference between sham and orchiectomized WT mice, especially because these mice do not develop

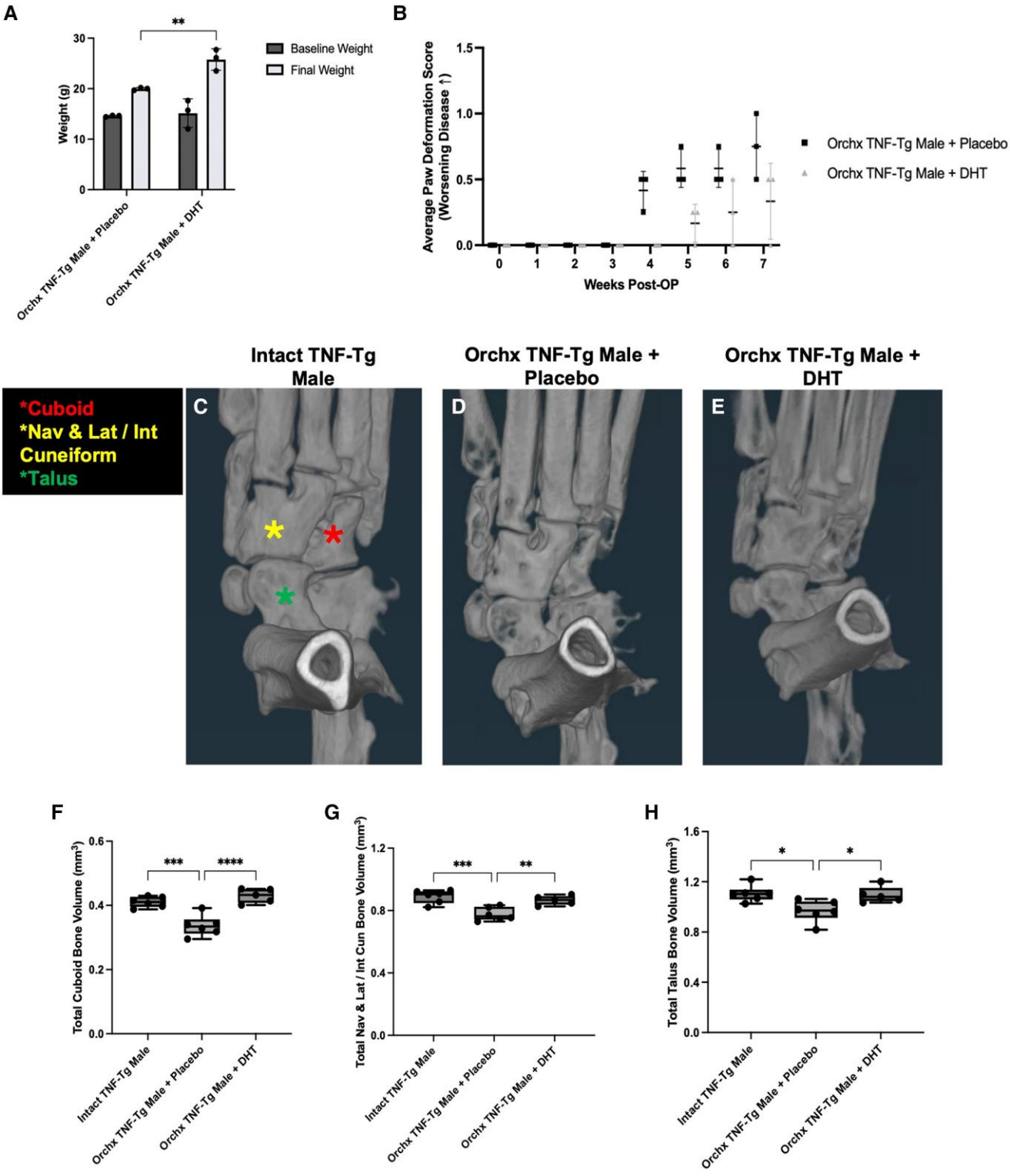

Figure 4. DHT treatment decreases erosions in orchiectomized TNF-Tg mice. Orchiectomies followed by subcutaneous implantation of either a DHT or placebo pellet were performed on 1-month-old TNF-Tg male mice (n = 3 mice/group). The baseline and final weights along with weekly paw deformation scores were compared between groups (A-B). Micro-CT scans of hindpaws were taken at age 3 months and compared with age-matched intact TNF-Tg male mice (n = 3). Representative 3-dimensional-rendered images of an intact TNF-Tg (C), placebo-treated orchx TNF-Tg (D), and DHT-treated orchx TNF-Tg male (E) show the mid-hindpaw bones analyzed: cuboid (C, red/right asterisk), navicular and lateral intermediate cuneiform (C, yellow/left asterisk), and talus (C, green/lower asterisk). Total bone volumes compared among cohorts (F-H), showed DHT-treated orchx mice have greater bone volumes than placebo-treated orchx mice. Statistics: 2-way ANOVA with Sidak multiple comparisons (A-B), 1-way ANOVA with Tukey multiple comparisons (F-H); $^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$; $^{****}P < .0001$.

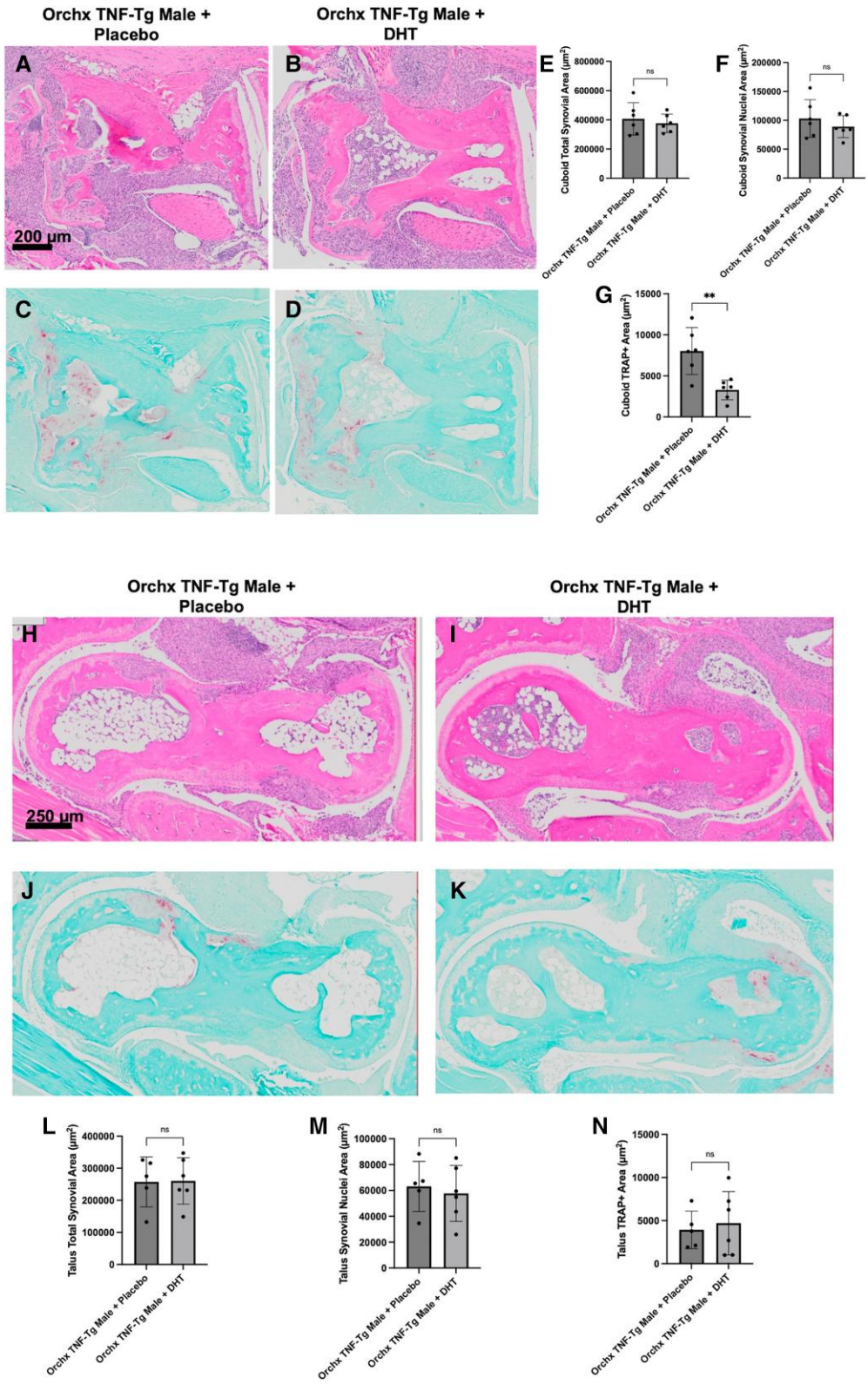

Figure 5. DHT-treated orchiectomized TNF-Tg mice have less severe erosive disease. Cuboid and talus sections were stained and analyzed to quantify total synovial area, synovial nuclei area, and TRAP+ area between placebo-treated and DHT-treated orchiectomized male TNF-Tg mice (n = 3 mice/group). Representative images of H&E and TRAP-stained cuboid from a placebo-treated orchiectomized TNF-Tg male (A and C) and DHT-treated orchiectomized TNF-Tg male (B and D), display inflammatory erosive disease with significantly decreased osteoclast burden in the DHT-treated cohort (E-G). The talus was also stained and quantified in both cohorts (H-N). Statistics: unpaired *t*-tests (E-G, L-N); ns, not significant; $^{**}P < .01$.

inflammatory erosive arthritis. Together, these findings provide evidence that the removal of androgen in TNF-Tg males accelerated inflammatory erosive disease similar to female mice.

The exacerbation of inflammatory erosive arthritis was validated by histology, wherein orchiectomized TNF-Tg mice had significantly greater synovial inflammatory infiltrate

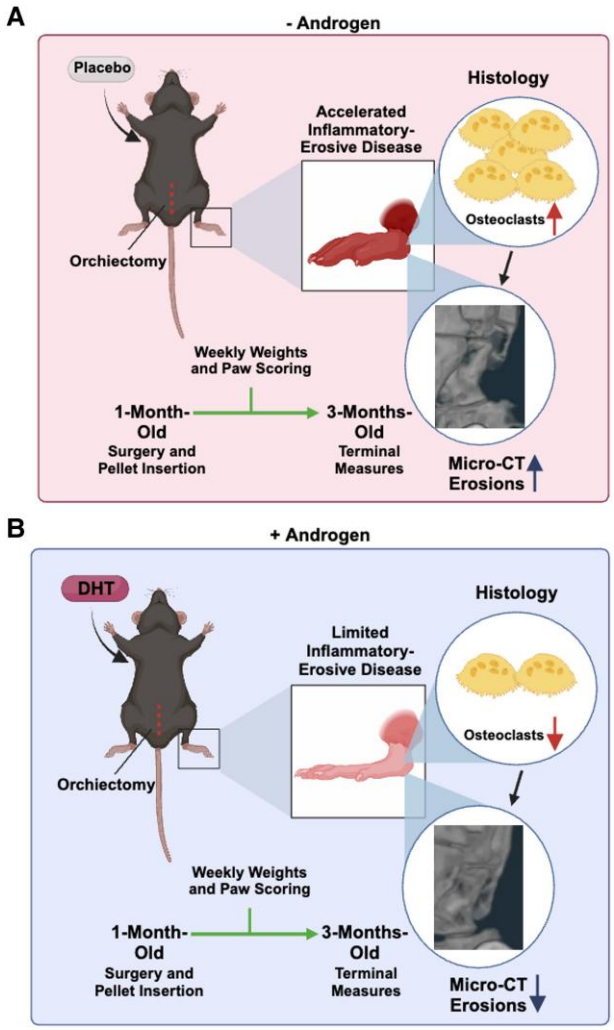

Figure 6. Androgen mitigates inflammation-mediated erosions. TNF-Tg male mice were orchiectomized to remove endogenous androgen and received a placebo pellet or active exogenous androgen (DHT) at age 1 month. Mice underwent weekly clinical measures for 3 months, followed by micro-CT of hindpaws and tissue collection for histology. Placebo-treated mice (similar to TNF-Tg females) had accelerated erosive disease in which inflammation stimulates the production of osteoclasts that resorb bone (A). In contrast, DHT-treated TNF-Tg mice had decreased erosions (B). Figure was created with BioRender.com.

than sham mice at the cuboid bone surface. Analysis of the talus bone also exhibited greater values of synovial inflammation in orchiectomized mice, although not significantly different, possibly because of the increased variability in orchiectomized mice. However, there was no associated change in TRAP staining, which is an indirect measure of osteoclast number but not total resorptive activity. In RA, synovial thickening and pannus invasion into bone contribute to both osteoclast number and activity [4, 5, 8]. Therefore, it is possible that osteoclast resorptive activity was more affected, leading to the bone loss noted by micro-CT. Although prior studies have shown that higher testosterone levels are associated with decreased TNFα in clinical analysis and that androgen can reduce in vitro TNF-α expression [18, 57], similar levels of TNF-α in sera were found between male and female TNF-Tg mice [28] and between orchiectomized and sham TNF-Tg mice in a separate cohort (data not shown), indicating that the action of androgen may negatively regulate, and therefore delay, erosions independent of usual inflammatory pathways. The importance of our findings is that it is the first to show that in a TNF-mediated inflammatory environment, androgen effects have a modulating effect on bone erosions. We believe this novel finding will require further analysis to elucidate the relationship between TNF and androgens.

Orchiectomy also prevents the production of other steroids, including estrogen, which is produced by converting testosterone with the enzyme aromatase. To determine the specific effect of androgen on inflammatory-mediated erosions, we treated cohorts of orchiectomized TNF-Tg mice with exogenous androgen, specifically DHT, which cannot be converted to estrogen. Weight comparisons exhibited the biological effect of androgen on weight, with the DHT-treated cohort having a significantly greater final weight than the placebo-treated group. In addition, the removal of androgen also reduces the mass of the prostate and other male reproductive organs in mice [58, 59]. This was seen in these cohorts of mice, with placebo-treated mice having little to no visible prostate, whereas intact TNF-Tg male and DHT-treated mice exhibited typical prostate tissue (data not shown). In micro-CT analysis, the bone volumes of DHT-treated orchiectomized mice were significantly greater than placebo-treated orchiectomized mice, with the replenishment of androgens protecting against bone erosions. In histology analysis, DHT-treated groups showed a slight decrease in mean synovial inflammation measures at the cuboid and talus bone surface, although not statistically significant. However, TRAP+ area of the cuboid was significantly reduced in the DHT-treated cohort, suggesting that androgen may decrease erosions even when there is less effect on inflammation. This result is not displayed in the talus as the effect of surface erosions may be more prevalent in smaller bones, such as the cuboid, at earlier timepoints. Androgen, specifically DHT, has been found to suppress osteoclast formation through inhibition of differentiation in precursor cells [60]. Taken together, these results demonstrate that in our cohort of androgen-treated TNF-Tg mice, osteoclast production is significantly reduced in comparison to androgen-depleted cohorts, therefore limiting erosive disease (Fig. 6). Further investigation into specific cellular mechanisms suggested by these findings are warranted.

Androgen and other sex hormone treatments have been discussed and are more recently being studied as potential therapies in RA and other sexually dimorphic diseases. Other mouse models of arthritis such as CIA and CAIA (male-predominant) and ZIA (female-predominant), have also undergone studies to elucidate this difference. Ovariectomized CIA mice developed worse arthritis and sex hormone treatment of male and female mice protected against disease [15, 16], whereas CAIA mice treated with 17β-estradiol had less severe arthritis but no change in disease development [14]. Interestingly, orchiectomized male ZIA mice developed a similar disease severity as female mice and ovariectomized female ZIA mice developed worse disease but had improved disease with estrogen treatment [13, 17]. Clinical trials have also demonstrated that treatment of RA male patients and RA postmenopausal female patients with testosterone shows some improved measures of disease [23, 24]. In addition, combined therapy of established inflammatory inhibitors and anti-inflammatory androgen could potentially provide significant effects on ameliorating RA [61]. However, hormone-based therapies may be difficult to implement in a standardized manner

across a population taking into careful consideration the range of ages, sexes, and gender identities impacted by RA and other autoimmune conditions. Thus, further understanding of the cellular mechanisms mediated by sex hormones are essential toward the development of precise pharmaceutical targets in RA.

A limitation of this study was the focus on androgen treatment in male mice only. This was done to study androgen effects on inflammatory erosions in the male joint microenvironment without having to control estrogen activity that occurs in the female joint. However, androgen treatment in female TNF-Tg mice may help to confirm the effects of androgen on erosive disease by determining if arthritis will be delayed similar to male mice. We also considered the young age of the mice because all terminal measures were performed at age 3 months. However, it has been found that C57BL/6J mice can reach a skeletal maturity as early as age 3 month [62]. TNF-Tg mice also develop their arthritis phenotype as early as age 2 months and can show significant bone erosions by age 3 months with no improvements as they age [28, 29, 63]. We have also not directly measured the role of estrogen or progesterone in disease progression because it is possible that female-dominant sex hormones may exacerbate disease in the TNF-Tg mouse. Previous studies examining estrogen in patients have found it challenging to determine if it leads to improvement in disease [64, 65]. In addition, the ratio of androgen to estrogen levels in human males (300:1) is significantly greater than the ratio in human females (1.5:1), indicating that androgen expression overall may have a larger impact [66-69]. Similarly, although it is possible that the little estrogen present in male mice may have a role, given that male mice have 100 times the level of androgen than estrogen, we expect a greater effect of androgen loss [70, 71]. Further, androgen-depleted males in our study developed comparable erosive arthritis to females, and this would argue that the predominant sex hormone effect is related to androgens in TNF-Tg mice. However, it is also important to consider that different sex hormones may exhibit differential effects based on the male vs female origin of the cells, where there is evidence that cells can be primed to a specific sex and therefore may react differently [72-74]. Further investigation into the specific cellular and molecular targets of sex hormones will aid in identifying androgen-mediated mechanisms that ameliorate inflammatory-erosive disease.

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Disclosures

No conflicts of interest to disclose.

Data Availability

Some or all datasets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

References

1. McInnes IB, Schett G. Cytokines in the pathogenesis of rheumatoid arthritis. *Nat Rev Immunol*. 2007;7(6):429-442.
2. McInnes IB, Schett G. The pathogenesis of rheumatoid arthritis. *N Engl J Med*. 2011;365(23):2205-2219.
3. Guo Q, Wang Y, Xu D, Nossent J, Pavlos NJ, Xu J. Rheumatoid arthritis: pathological mechanisms and modern pharmacologic therapies. *Bone Res*. 2018;6(1):15.
4. Schett G, Gravallese E. Bone erosion in rheumatoid arthritis: mechanisms, diagnosis and treatment. *Nat Rev Rheumatol*. 2012;8(11):656-664.
5. Panagopoulos PK, Lambrou GI. Bone erosions in rheumatoid arthritis: recent developments in pathogenesis and therapeutic implications. *J Musculoskelet Neuronal Interact*. 2018;18(3):304-319.
6. Ording Muller LS, Humphries P, Rosendahl K. The joints in juvenile idiopathic arthritis. *Insights Imaging*. 2015;6(3):275-284.
7. Clunie G, Horwood N. Loss and gain of bone in spondyloarthritis: what drives these opposing clinical features? *Ther Adv Musculoskelet Dis*. 2020;12:1759720X20969260.
8. Goldring SR. The final pathogenetic steps in focal bone erosions in rheumatoid arthritis. *Ann Rheum Dis*. 2000;59 Suppl 1(Suppl 1):i72-i74.
9. Whitacre CC. Sex differences in autoimmune disease. *Nat Immunol*. 2001;2(9):777-780.
10. Hoffmann JP, Liu JA, Seddu K, Klein SL. Sex hormone signaling and regulation of immune function. *Immunity*. 2023;56(11):2472-2491.
11. Schuh BM, Macakova K, Fejes A, *et al*. Sex differences in long-term effects of collagen-induced arthritis in middle-aged mice. *Front Physiol*. 2023;14:1195604.
12. Kim JR, Kim HA. Molecular mechanisms of sex-related differences in arthritis and associated pain. *Int J Mol Sci*. 2020;21(21):7938.
13. Keith RC, Sokolove J, Edelman BL, *et al*. Testosterone is protective in the sexually dimorphic development of arthritis and lung disease in SKG mice. *Arthritis Rheum*. 2013;65(6):1487-1493.
14. Nandakumar KS, Svensson L, Holmdahl R. Collagen type II-specific monoclonal antibody-induced arthritis in mice: description of the disease and the influence of age, sex, and genes. *Am J Pathol*. 2003;163(5):1827-1837.
15. Holmdahl R, Jansson L, Andersson M. Female sex hormones suppress development of collagen-induced arthritis in mice. *Arthritis Rheum*. 1986;29(12):1501-1509.
16. Wilder RL. Hormones and autoimmunity: animal models of arthritis. *Baillieres Clin Rheumatol*. 1996;10(2):259-271.
17. Inoue K, Inoue E, Imai Y. Female sex hormones ameliorate arthritis in SKG mice. *Biochem Biophys Res Commun*. 2013;434(4):740-745.
18. Traish A, Bolanos J, Nair S, Saad F, Morgentaler A. Do androgens modulate the pathophysiological pathways of inflammation? Appraising the contemporary evidence. *J Clin Med*. 2018;7(12):549.
19. Bianchi VE. The anti-inflammatory effects of testosterone. *J Endocr Soc*. 2019;3(1):91-107.
20. Cutolo M. Androgens in rheumatoid arthritis: when are they effectors? *Arthritis Res Ther*. 2009;11(5):126.
21. Gordon D, Beastall GH, Thomson JA, Sturrock RD. Prolonged hypogonadism in male patients with rheumatoid arthritis during flares in disease activity. *Br J Rheumatol*. 1988;27(6):440-444.
22. James WH. Further evidence that low androgen values are a cause of rheumatoid arthritis: the response of rheumatoid arthritis to seriously stressful life events. *Ann Rheum Dis*. 1997;56(9):566.

23. Cutolo M, Balleari E, Giusti M, Intra E, Accardo S. Androgen replacement therapy in male patients with rheumatoid arthritis. *Arthritis Rheum*. 1991;34(1):1-5.
24. Booji A, Biewenga-Booji CM, Huber-Bruning O, Cornelis C, Jacobs JW, Bijlsma JW. Androgens as adjuvant treatment in postmenopausal female patients with rheumatoid arthritis. *Ann Rheum Dis*. 1996;55(11):811-815.
25. Notelovitz M. Androgen effects on bone and muscle. *Fertil Steril*. 2002;77 Suppl 4:S34-S41.
26. Chen JF, Lin PW, Tsai YR, Yang YC, Kang HY. Androgens and androgen receptor actions on bone health and disease: from androgen deficiency to androgen therapy. *Cells*. 2019;8(11):1318.
27. Clarke BL, Khosla S. Androgens and bone. *Steroids*. 2009;74(3):296-305.
28. Bell RD, Wu EK, Rudmann CA, *et al*. Selective sexual dimorphisms in musculoskeletal and cardiopulmonary pathologic manifestations and mortality incidence in the tumor necrosis factor-transgenic mouse model of rheumatoid arthritis. *Arthritis Rheumatol*. 2019;71(9):1512-1523.
29. Li P, Schwarz EM. The TNF-alpha transgenic mouse model of inflammatory arthritis. *Springer Semin Immunopathol*. 2003;25(1):19-33.
30. Kenney HM, Chen KL, Schnur L, *et al*. High-throughput micro-CT analysis identifies sex-dependent biomarkers of erosive arthritis in TNF-Tg mice and differential response to anti-TNF therapy. *PLoS One*. 2024;19(7):e0305623.
31. Douni E, Akassoglou K, Alexopoulou L, *et al*. Transgenic and knockout analyses of the role of TNF in immune regulation and disease pathogenesis. *J Inflamm*. 1995;47(1-2):27-38.
32. Keffer J, Probert L, Cazlaris H, *et al*. Transgenic mice expressing human tumour necrosis factor: a predictive genetic model of arthritis. *EMBO J*. 1991;10(13):4025-4031.
33. van Weerden WM, Bierings HG, van Steenbrugge GJ, de Jong FH, Schroder FH. Adrenal glands of mouse and rat do not synthesize androgens. *Life Sci*. 1992;50(12):857-861.
34. Bell RD, Slattery PN, Wu EK, Xing L, Ritchlin CT, Schwarz EM. iNOS dependent and independent phases of lymph node expansion in mice with TNF-induced inflammatory-erosive arthritis. *Arthritis Res Ther*. 2019;21(1):240.
35. Swerdloff RS, Wang C. Dihydrotestosterone: a rationale for its use as a non-aromatizable androgen replacement therapeutic agent. *Baillieres Clin Endocrinol Metab*. 1998;12(3):501-506.
36. Astapova O, Minor BMN, Hammes SR. Physiological and pathological androgen actions in the ovary. *Endocrinology*. 2019;160(5):1166-1174.
37. Labruijere S, van Houten EL, de Vries R, *et al*. Analysis of the vascular responses in a murine model of polycystic ovary syndrome. *J Endocrinol*. 2013;218(2):205-213.
38. Johnson AM, O'Connell MJ, Miyamoto H, *et al*. Androgenic dependence of exophytic tumor growth in a transgenic mouse model of bladder cancer: a role for thrombospondin-1. *BMC Urol*. 2008;8(1):7.
39. Chappell NR, Zhou B, Hosseinzadeh P, Schutt A, Gibbons WE, Blesson CS. Hyperandrogenemia alters mitochondrial structure and function in the oocytes of obese mouse with polycystic ovary syndrome. *F S Sci*. 2021;2(1):101-112.
40. Tate J, Ward G. Interferences in immunoassay. *Clin Biochem Rev*. 2004;25(2):105-120.
41. Cresta F, Arcuri L, Bianchin S, *et al*. A case of interference in testosterone, DHEA-S and progesterone measurements by second generation immunoassays. *Clin Chem Lab Med*. 2021;59(7):e275-e277.
42. Stanczyk FZ, Jurow J, Hsing AW. Limitations of direct immunoassays for measuring circulating estradiol levels in postmenopausal women and men in epidemiologic studies. *Cancer Epidemiol Biomarkers Prev*. 2010;19(4):903-906.
43. Krasowski MD, Drees D, Morris CS, Maakestad J, Blau JL, Ekins S. Cross-reactivity of steroid hormone immunoassays: clinical significance and two-dimensional molecular similarity prediction. *BMC Clin Pathol*. 2014;14(1):33.
44. Koren L, Ng ES, Soma KK, Wynne-Edwards KE. Sample preparation and liquid chromatography-tandem mass spectrometry for multiple steroids in mammalian and avian circulation. *PLoS One*. 2012;7(2):e32496.
45. Missaghian E, Kempna P, Dick B, *et al*. Role of DNA methylation in the tissue-specific expression of the CYP17A1 gene for steroidogenesis in rodents. *J Endocrinol*. 2009;202(1):99-109.
46. Kenney HM, Peng Y, Chen KL, *et al*. A high-throughput semi-automated bone segmentation workflow for murine hindpaw micro-CT datasets. *Bone Rep*. 2022;16:101167.
47. Kenney HM, Peng Y, Bell RD, *et al*. Persistent popliteal lymphatic muscle cell coverage defects despite amelioration of arthritis and recovery of popliteal lymphatic vessel function in TNF-Tg mice following anti-TNF therapy. *Sci Rep*. 2022;12(1):12751.
48. Li J, Ju Y, Bouta EM, *et al*. Efficacy of B cell depletion therapy for murine joint arthritis flare is associated with increased lymphatic flow. *Arthritis Rheum*. 2013;65(1):130-138.
49. Li J, Zhou Q, Wood RW, *et al*. CD23(+)/CD21(hi) B-cell translocation and ipsilateral lymph node collapse is associated with asymmetric arthritic flare in TNF-Tg mice. *Arthritis Res Ther*. 2011;13(4):R138.
50. Li J, Kuzin I, Moshkani S, *et al*. Expanded CD23(+)/CD21(hi) B cells in inflamed lymph nodes are associated with the onset of inflammatory-erosive arthritis in TNF-transgenic mice and are targets of anti-CD20 therapy. *J Immunol*. 2010;184(11):6142-6150.
51. Bouta EM, Ju Y, Rahimi H, *et al*. Power Doppler ultrasound phenotyping of expanding versus collapsed popliteal lymph nodes in murine inflammatory arthritis. *PLoS One*. 2013;8(9):e73766.
52. Bouta EM, Kuzin I, de Mesy Bentley K, *et al*. Brief report: treatment of tumor necrosis factor-transgenic mice with anti-tumor necrosis factor restores lymphatic contractions, repairs lymphatic vessels, and may increase monocyte/macrophage egress. *Arthritis Rheumatol*. 2017;69(6):1187-1193.
53. Kwak SK, Kim JH. Statistical data preparation: management of missing values and outliers. *Korean J Anesthesiol*. 2017;70(4):407-411.
54. Schwertman NC, Owens MA, Adnan R. A simple more general boxplot method for identifying outliers. *Comput Stat Data Anal*. 2004;47(1):165-174.
55. Hooper AC, Brien TG, Lawlor PG. The effects of orchidectomy and the role of testosterone in determining the growth of male mice selected for increased body weight. *Andrologia*. 1986;18(5):509-515.
56. Davidyan A, Pathak S, Baar K, Bodine SC. Maintenance of muscle mass in adult male mice is independent of testosterone. *PLoS One*. 2021;16(3):e0240278.
57. Becerra-Diaz M, Song M, Heller N. Androgen and androgen receptors as regulators of monocyte and macrophage biology in the healthy and diseased lung. *Front Immunol*. 2020;11:1698.
58. Zhang R, Singh S, Pan C, *et al*. Rate of castration-induced prostate stroma regression is reduced in a mouse model of benign prostatic hyperplasia. *Am J Clin Exp Urol*. 2023;11(1):12-26.
59. Valkenburg KC, Amend SR, Pienta KJ. Murine prostate microdissection and surgical castration. *J Vis Exp*. 2016;111:53984.
60. Huber DM, Bendixen AC, Pathrose P, *et al*. Androgens suppress osteoclast formation induced by RANKL and macrophage-colony stimulating factor. *Endocrinology*. 2001;142(9):3800-3808.
61. Singh H, Bala S, Jain D, Jagota R, Mathur R. Spironolactone (an adjuvant therapy) in rheumatoid arthritis: a case control study. *Reumatologia*. 2018;56(2):87-91.
62. Ferguson VL, Ayers RA, Bateman TA, Simske SJ. Bone development and age-related bone loss in male C57BL/6J mice. *Bone*. 2003;33(3):387-398.
63. Kenney HM, Wood RW, Ramirez G, *et al*. Implementation of automated behavior metrics to evaluate voluntary wheel running effects on inflammatory-erosive arthritis and interstitial lung disease in TNF-Tg mice. *Arthritis Res Ther*. 2023;25(1):17.
64. Alpizar-Rodriguez D, Pluchino N, Canny G, Gabay C, Finckh A. The role of female hormonal factors in the development of rheumatoid arthritis. *Rheumatology (Oxford)*. 2017;56(9):1254-1263.

65. Cutolo M, Gotelli E. Complex role of oestrogens in the risk and severity of rheumatoid arthritis in menopause. *RMD Open*. 2023;9(2):e003176.
66. Travison TG, Vesper HW, Orwoll E, *et al*. Harmonized reference ranges for circulating testosterone levels in men of four cohort studies in the United States and Europe. *J Clin Endocrinol Metab*. 2017;102(4):1161-1173.
67. Chadid S, Barber JR, Rohrmann S, *et al*. Age-specific serum total and free estradiol concentrations in healthy men in US nationally representative samples. *J Endocr Soc*. 2019;3(10):1825-1836.
68. Braunstein GD, Reitz RE, Buch A, Schnell D, Caulfield MP. Testosterone reference ranges in normally cycling healthy premenopausal women. *J Sex Med*. 2011;8(10):2924-2934.
69. Hassan LS, Monson RS, Danielson KK. Oestradiol levels may differ between premenopausal women, ages 18-50, with type 1 diabetes and matched controls. *Diabetes Metab Res Rev*. 2017;33(2): 10.1002/dmrr.2829.
70. Nelson JF, Latham KR, Finch CE. Plasma testosterone levels in C57BL/6J male mice: effects of age and disease. *Acta Endocrinol (Copenh)*. 1975;80(4):744-752.
71. Saito T, Ciobotaru A, Bopassa JC, Toro L, Stefani E, Eghbali M. Estrogen contributes to gender differences in mouse ventricular repolarization. *Circ Res*. 2009;105(4):343-352.
72. Penaloza C, Estevez B, Orlanski S, *et al*. Sex of the cell dictates its response: differential gene expression and sensitivity to cell death inducing stress in male and female cells. *FASEB J*. 2009;23(6): 1869-1879.
73. Brundin PMA, Landgren BM, Fjallstrom P, *et al*. Expression of sex hormone receptor and immune response genes in peripheral blood mononuclear cells during the menstrual cycle. *Front Endocrinol (Lausanne)*. 2021;12:721813.
74. Klein SL. Immune cells have sex and so should journal articles. *Endocrinology*. 2012;153(6):2544-2550.