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17β-estradiol and Tamoxifen prevent gastric cancer by modulating leukocyte recruitment and oncogenic pathways in *Helicobacter pylori*-infected INS-GAS male mice

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Abstract

Helicobacter pylori infection promotes male-predominant gastric adenocarcinoma in humans. Estrogens reduce gastric cancer risk and previous studies demonstrated that prophylactic 17β-estradiol (E2) in INS-GAS mice decreases *H. pylori*-induced carcinogenesis. We examined the effect of E2 and Tamoxifen, on *H. pylori*-induced gastric cancer in male and female INS-GAS mice. After confirming robust gastric pathology at 16 weeks post-infection (WPI), mice were implanted with E2, Tamoxifen, both E2 and Tamoxifen, or placebo pellets for 12 weeks. At 28 WPI, gastric histopathology, gene expression and immune cell infiltration were evaluated, and serum inflammatory cytokines measured. After treatment, no gastric cancer was observed in *H. pylori*-infected males receiving E2 and/or Tamoxifen, while 40% of infected untreated males developed gastric cancer. E2, Tamoxifen and their combination significantly reduced gastric precancerous lesions in infected males compared to infected untreated males ($P<0.001$, 0.01 and 0.01, respectively). However, Tamoxifen did not alter female pathology regardless of infection status. Differentially expressed genes from males treated with E2 or Tamoxifen (n=363 and n=144, $Q<0.05$) associated highly with cancer and cellular movement, indicating overlapping pathways in the reduction of gastric lesions. E2 or Tamoxifen deregulated genes associated with metastasis (*PLAUR* and *MMP10*) and Wnt inhibition (*FZD6* and *SFRP2*). Compared to controls, E2 decreased gastric mRNA ($Q<0.05$) and serum levels ($P<0.05$) of CXCL1, a neutrophil chemokine, leading to decreased neutrophil infiltration ($P<0.01$). Prevention of *H. pylori*-induced gastric cancer by E2 and Tamoxifen may be mediated by estrogen signaling and is associated with decreased CXCL1, decreased neutrophil counts and downregulation of oncogenic pathways.

User-defined Keywords

Estrogen; *Helicobacter pylori*; Tamoxifen

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Keywords

Gastrointestinal cancers; stomach; Animal models of cancer; Hormonal carcinogenesis; Hormonal control; Hormone signaling and inhibitors; Immune responses to cancer; Immunomodulation

Introduction

Chronic inflammation induced by *Helicobacter pylori* is a significant risk factor for gastric cancer, the second most frequent cause of cancer-related death worldwide(1). *H. pylori* infection increases lifetime risk of developing duodenal and gastric ulcers, mucosa-associated lymphoid tissue (MALT) lymphoma, mucosal atrophy and gastric adenocarcinoma(2). Worldwide, age-standardized and cumulative incidence rates indicate that men are more likely than women to develop gastric cancer, with a 2 to 2.5-fold greater incidence at age 60(3); implying that intrinsic sex differences modulate *H. pylori*-induced carcinogenesis irrespective of other environmental factors.

Estrogens have been associated with protection against gastric cancer in women due to the decreased gastric cancer risk associated with delayed menopause, increased fertility life and hormone therapies in men and women(1, 3). Additionally, epidemiological data links anti-estrogen therapy, particularly the breast cancer drug Tamoxifen (TAM), with increased incidence rates of gastrointestinal malignancies(4–5). However, TAM is a selective estrogen receptor modulator (SERM). For example, TAM is an antagonist of estrogen signaling in estrogen receptor positive breast cancer, but acts as an agonist in the endometrium, where it increases cancer incidence in postmenopausal women(6). TAM's function in the stomach and its effect on gastrointestinal cancers, and gastric cancer in particular, is not clear; one study reports no effect(6) while others have associated TAM treatment with increased risk of gastric cancer(4–5). Due to their retrospective nature, these studies assessing the effects of estrogen and TAM on gastric cancer did not control for *H. pylori* infection, a major confounding factor(1, 4).

Transgenic INS-GAS mice over-express human gastrin, a phenotype associated with increased risk for gastric glandular atrophy and cancer in humans(7). INS-GAS mice infected with *H. pylori* or *H. felis* develop gastric carcinomas 28 weeks post-infection (WPI) in a male-predominant fashion(8), paralleling the development of human gastric cancer after decades of chronic *H. pylori* gastritis(7–9). Similar to *H. pylori*-infected humans, INS-GAS mice develop gastric atrophy, hypochlorhydria, intestinal metaplasia, dysplasia and gastric cancer(2, 8, 10). Eradication of *H. pylori* in male INS-GAS mice reduced gastric cancer risk, with earlier antibiotic therapy being more efficacious(11), implying that the progression of *H. pylori* carcinogenesis may be reversible up to a point. We previously demonstrated that ovariectomized female INS-GAS mice developed *H. pylori*-induced gastric cancer whereas E2 supplementation of ovariectomized female mice was protective(9). We also reported that E2 treatment, but not castration, prior to *H. pylori* infection attenuated gastric lesions by increasing *Foxp3+* and interleukin 10 (*IL-10*) expression and decreasing *IFN-γ* and *IL-1β* expression(12).

In this study, we determined the effects of E2 and TAM treatment on chronic *H. pylori* infection and examined the mechanisms by which E2 and/or TAM affect gastric lesions in INS-GAS male and female mice. Following 16 weeks of sham or *H. pylori* infection, pellets containing placebo, E2, TAM, or both E2 and TAM were subcutaneously implanted in male INS-GAS mice, while placebo or TAM pellets were implanted in female mice. We sought to clarify E2 and TAM's effects on gastric cancer incidence, expecting gastric cancer prevention (E2 alone), exacerbation of precancerous gastric lesions (TAM alone) or the

blocking of E2's protective effects (E2 and TAM treatment). At 28 WPI, the effects of E2 and TAM on histopathology, gene expression and immune cell infiltration in the stomach, as well as serum cytokine levels, were characterized.

Materials and Methods

Bacteria and Mice

Helicobacter pylori SS1 was cultured using Brucella broth with 5% fetal bovine serum under microaerobic conditions (10% H2, 10% CO2, 80% N2)(9). Fifty-two female and 99 male specific pathogen-free (including *Helicobacter* spp.) INS-GAS FVB mice were maintained in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International, fed standard mouse chow (RMH3000, Purina Mills, Inc.) *ad libitum*, and using husbandry practices and protocols approved by the MIT Committee on Animal Care, as previously described(9).

Experimental design

Eight-week-old, male and female mice were either infected with 1×10^8 colony forming units of *H. pylori* or dosed with broth only on alternate days for a total of 3 doses(8–9). At 4 and 16 weeks post-infection (WPI), 4 and 20 mice, respectively, were euthanized to confirm *H. pylori* infection. At 16 WPI, pathology was assessed to establish severity of gastritis prior to hormone treatment. At 28 WPI, the mice were euthanized and the presence of pellets was confirmed. Mice that lost the pellet during the course of the study were excluded. Based on hormone treatment and infection status, the following twelve groups of mice were used in subsequent analyses (a) uninfected males treated with placebo (UMP, n=12), (b) infected males treated with placebo (IMP, n=10), (c) uninfected males treated with E2 (UME, n=12), (d) infected males treated with E2 (IME, n=9), (e) uninfected males treated with TAM (UMT, n=6), (f) infected males treated with TAM (IMT, n=7), (g) uninfected males treated with E2 and TAM (UMET, n=7), (h) infected males treated with E2 and TAM (IMET, n=4), (i) uninfected females treated with placebo (UFP, n=9), (j) infected females treated with placebo (IFP, n=10), (k) uninfected females treated with TAM (UFT, n=1), and (l) infected females treated with TAM (IFT, n=9). Three of nine IME were euthanized before 28 WPI due to poor body condition and were excluded from serum cytokine and immunohistochemistry analyses.

Subcutaneous implantation of placebo, 17β-estradiol and Tamoxifen pellets

At 16 WPI, surgical placement of subcutaneous time-release pellets containing placebo (5mg, NC-111, Innovative Research of America), E2 (0.25mg, NE-121) or TAM (15mg, NE-361) was performed under anesthesia as described previously(9). Mice received 0.5 ml of lactated Ringer's solution intraperitoneally prior to surgery. E2 timed-release pellet results in a serum E2 level in the range from proestrus levels to slightly supraphysiological(9). Expected concentration of TAM was the range of 2–3 ng/ml (Personal communication from Innovative Research of America).

Sample collection and histological analysis

Immediately following CO_2 euthanasia, blood was collected by cardiac puncture, serum was separated and stored at −80°C. Testes and epididymis or uterus were removed and wet weight was measured. The stomach and proximal duodenum were removed and incised along the line of the greater curvature. Luminal contents were removed and the mucosa was rinsed with sterile PBS.

Individual linear gastric strips from the lesser curvature were sectioned, frozen in liquid nitrogen and stored at −80°C for DNA and RNA extraction. For histopathologic evaluation,

linear strips extending from the squamocolumnar junction to the proximal duodenum were taken along the lesser curvature, fixed overnight in 10% neutral-buffered formalin, embedded in paraffin and cut into four μm-thick sections for hematoxylin and eosin (H&E) staining. A board-certified comparative pathologist (N.M.P.), blinded to treatment groups, scored gastric lesions in the corpus on an ascending scale of 0 to 4 for inflammation, epithelial defects, atrophy, hyperplasia, mucous metaplasia, hyalinosis, intestinal metaplasia, and dysplasia according to previously published criteria(13). Gastric lesions in the antrum were also scored on an ascending scale of 0 to 4 for inflammation, epithelial defects, hyperplasia and dysplasia. A dysplasia score of 3 was considered carcinoma *in situ* or low-grade gastrointestinal intraepithelial neoplasia (GIN), while a dysplasia score of 3.5–4 represented intramucosal carcinoma or high-grade GIN(13–14). Both low grade and high-grade GIN were classified as gastric cancer. A gastric histologic activity index (GHAI) was calculated as the sum of scores for inflammation, epithelial defects, atrophy, hyperplasia, intestinal metaplasia, and dysplasia for the corpus and inflammation, epithelial defects, hyperplasia and dysplasia for the antrum. Hyalinosis and mucous metaplasia were excluded from the GHAI as they develop spontaneously in mice(13).

Immunohistochemistry for neutrophils and macrophages

Neutrophils and macrophages were quantified by immunohistochemistry as previously described(15) using antibodies for myeloperoxidase [1:75] (MPO) (RB-373-A; Thermo Scientific), a neutrophil-specific marker, and F4/80 [1:150] (MF48015; Caltag Laboratories), a macrophage-specific marker, in five mice per group. Five fields of MPO+ or F4/80+ cells were counted in the corpus at 40× magnification per mouse.

Confirmation of *H. pylori* infection by PCR

At 4 and 16 WPI, prokaryotic and eukaryotic DNA was extracted from gastric tissue using the High Pure PCR Template purification kit (Roche). *H. pylori* infection was confirmed by PCR of the *ureC* gene as described previously(16).

17β-estradiol serum levels

Serum E2 levels were measured using an estradiol EIA kit (Cayman Chemical Company) with a 1:4 serum dilution according to manufacturer's instruction.

mRNA analysis

Total RNA was extracted using Trizol (Invitrogen) and the RNeasy kit with DNAse treatment (Qiagen). RNA quality was determined using a RNA 6000 Nano total RNA Kit (Agilent Technologies). RNA was hybridized to Agilent 4x44K Whole Mouse Gene Expression Microarrays following the One-Color Microarray-Based Gene expression Analysis, Low Input Quick Amp Labeling protocol. Data was collected using an Agilent Microarray Scanner and Feature Extraction 9.1.

Processing of the raw gene expression data was performed using Partek® Genomics Suite™ software, where microarray data were first normalized by quantile using Robust Multi-Chip Average (RMA)(17). Data were then filtered for expression levels above background noise (>abs[180]) which resulted in a reduction of probesets from 41,174 to 21,549. Differential gene expression was defined as a significant difference in mRNA levels between the groups compared where the following statistical requirements were set: (1) fold change of ≥ 1.5 or ≤ -1.5; (2) p-value < 0.05 (ANOVA); and (3) a false discovery rate corrected q-value < 0.05. To control the rate of false positives, q-values were calculated as the minimum positive false discovery rate that can occur when identifying significant hypotheses(18). Comparisons were performed between 1) infected males with placebo vs. infected males

with 17-β estradiol, and 2) infected males with placebo vs. infected males with TAM. Three microarrays were hybridized for each group except for infected males with TAM (n=2). Data have been deposited in NCBI's Gene Expression Omnibus (GSE29715). Molecular networks containing differentially expressed genes were algorithmically constructed based on connectivity and the known relationships among proteins using Ingenuity Pathways Analysis (IPA).

Serum cytokine and chemokine levels

Serum protein levels of chemokines and cytokines were measured using the Bio-Plex Mouse Cytokine 23-Plex panel (Bio-Rad Laboratories) following the manufacturer's instructions. Briefly, sera were diluted 1:4 in mouse-specific and bound to antibody-coated fluorescent beads, followed by biotinylated secondary and streptavidin-PE antibodies. Plates were read on the Bio-Plex array reader on the high photomultiplier tube setting with sample levels quantified using regression analysis of the kit standard curve.

Statistical analysis

Two-way analysis of variance (ANOVA) followed by Bonferroni post-tests were used to analyze the effect of sex and 17β-estradiol and Tamoxifen on gastric lesions. Student two-tailed *t*-tests were used to analyze differences in serum E2 and gastric inflammatory cells. Mann-Whitney tests were used for serum cytokine responses. Analyses were done with GraphPad Prism 5.0, or Microsoft Excel 2007. *P*-values less than 0.05 were considered significant.

RESULTS

E2 and Tamoxifen reduce reproductive tissue size and serum E2 concentrations through different mechanisms

Efficacy of the placebo, E2, TAM and dual (E2 and TAM) treatments was confirmed by gross pathology and by serum E2 levels. In male mice, E2 and dual treatment reduced the size of testes and seminal vesicles regardless of infection status. Placebo and TAM treatment had no effect on reproductive tissues. E2 and dual treatment caused interstitial cystitis in male mice, which has been associated with E2(19), and TAM treatment in males caused inguinal hernias. The ratio of testes and epididymis to body weight was computed (Fig. 1). Both E2 and dual-treated mice had a significant reduction in the ratio compared to either untreated or TAM mice regardless of infection status (all $P<0.001$, except infected TAM males vs. infected dual males $P<0.01$). Serum E2 levels were significantly higher in infected E2 males (137.9 ± 29.9 pg/ml) and dual-treated males (154.0 ± 15.5 pg/ml) compared to untreated males (88.9 ± 24.7 pg/ml) ($P<0.05$ and 0.001, respectively) (Fig. 2).

In female mice, TAM reduced the ratio of uterus to body weight compared to untreated mice ($P<0.001$) in infected mice, but due to pellet loss, this comparison was not performed in uninfected mice (Fig. 1). Infection status had no effect on the ratio of uterus to body weight. E2 was significantly reduced in infected TAM females compared to uninfected untreated females ($P<0.001$) (Fig. 2).

E2, Tamoxifen and dual treatment prevent gastric cancer in infected males

After confirming *H. pylori* infection by PCR at 4 and 16 WPI (data not shown), gastric lesions were quantified using the gastric histologic activity index (GHAI) at 16 WPI revealing robust, *H. pylori*-induced gastric pathology in male and female mice (M=12.2 ± 1.9, F=11.4 ± 1.2) compared to uninfected controls (M=6.5 ± 1.8, F=5.1 ± 0.5) (both $P<0.001$). At this point, uninfected and infected male mice were divided into four groups: a) untreated/placebo (UMP and IMP), b) E2 (UME and IME), c) TAM (UMT and IMT) and d)

E2 and TAM (UMET and IMET); and uninfected and infected female mice were divided into two groups: a) untreated/placebo (UFP and IFP) and b) TAM (UFT and IFT).

After 12 weeks of hormone treatment, GHAI was significantly reduced in IME (12.6 ± 3.7), IMT (11.9 ± 0.7) and IMET (13.9 ± 1.9) compared to IMP (17.1 ± 1.2) (P<0.001, 0.001 and 0.01, respectively) (Fig. 3A and 3C). The reduction in overall gastric pathology demonstrated that therapeutic treatment with E2 reduced gastric lesions caused by chronic *H. pylori* infection. Unexpectedly, TAM was protective, indicating that in this model, TAM may act agonistically, activating estrogen signaling locally or systemically. IMET mice did not experience increased protection, suggesting that both E2 and TAM may act through overlapping and/or nonadditive pathways. Examining the individual histologic parameters in infected males, foveolar hyperplasia and dysplasia, two precancerous lesions, were found significantly decreased in all three treated groups. A significant decrease in inflammation was seen in relation to IME and IMT mice, while epithelial defects were only significantly decreased in relation to E2 treatment (Fig. 4 and Supp. Table 1). Intestinal metaplasia was significantly decreased in response to E2 or dual treatments. Forty percent of IMP mice had high-grade gastrointestinal intraepithelial neoplasia (GIN), or intramucosal carcinoma, while IME, IMT and IMET mice had no incidence of GIN (Fig. 4 and Supp. Table 1). No significant differences were observed with regard to mucous metaplasia, hyalinosis and oxyntic atrophy between any treated group and the untreated group. IME, IMT and IMET mice (0.4 ± 0.2, 0.6 ± 0.4, 1.1 ± 0.5, respectively) had significantly reduced antral pathology compared to IMP (2.5 ± 1.1) (all P<0.001).

UME and UMET mice also had less corpus pathology (3.0 ± 1.1 and 1.8 ± 1.1, respectively) compared to UMP (5.8 ± 1.8) (P<0.01 and 0.001, respectively), but UMT mice were not protected (4.5 ± 0.9) (Fig. 3A, 3C). In uninfected males, epithelial defects, oxyntic atrophy, and dysplasia were all significantly decreased in UME, as well as in UMET, mice compared to UMP mice, while UMT mice experienced no significant changes in any of the lesion categories. Foveolar hyperplasia was decreased only as a result of dual treatment. There was no significant difference in any of the uninfected male groups with regard to inflammation, mucous metaplasia or intestinal metaplasia (characterized by columnar elongation of foveolar epithelium with or without interspersed goblet cells) (Fig. 3C). In contrast, TAM treatment in females did not alter any of the lesion categories in infected or uninfected females (Fig. 3B and Supp. Table 1).

E2 and Tamoxifen decreased MPO+ neutrophils and F4/80+ macrophages in the stomach

Neutrophils expressing myeloperoxidase (MPO) were present in lower numbers in the stomach of IME and IMT mice (10 ± 2 cells/40× field and 13 ± 7 cells/40× field, respectively) compared to IMP mice (21 ± 4 cells/40× field, P<0.01 and P=0.072, respectively) (Fig. 5A). *H. pylori* infection increased neutrophil numbers in the stomach in IMP compared to UMP mice (3 ± 2 cells/40× field, respectively, P <0.001), and in IFP compared to UFP mice (21 ± 6 cells/40× field vs.1 ± 2 cells/40× field, respectively, P<0.001) (Fig. 5A). Despite decreased neutrophil counts, IME and IMT mice had increased neutrophilic infiltration compared to UMP mice (P< 0.001 and P<0.05, respectively). However, significant decreases in neutrophil numbers were observed in IME mice, while decreasing trends were observed in IMT mice, indicating deregulation of neutrophil recruitment by estrogen signaling.

A minor decrease in macrophages expressing F4/80 was observed in IME and IMT mice (28 ± 7 cells/40× field and 27 ± 8 cells/40× field, respectively) compared to IMP mice (34 ± 9 cells/40× field, P=0.276 and 0.229, respectively) (Fig. 5B). *H. pylori* infection increased macrophage infiltration, regardless of treatment group, compared to UMP mice (9 ± 5 cells/40× field; compared to infected males: untreated, P<0.001; E2, P<0.01; TAM P<0.01) and

in IFP compared to UFP mice (25 ± 9 cells/40× field vs. 5 ± 2 cells/40× field, $P<0.01$) (Fig. 5B).

E2 and Tamoxifen deregulate genes associated with cellular movement and cancer in infected mice

Gene expression analysis was performed on samples from IMP, IME and IMT mice to determine if a characteristic expression profile was observed in E2 and TAM reduction of gastric cancer. IME and IMT mice differentially expressed 363 and 144 genes ($Q<0.05$), respectively, compared to IMP mice (Complete list of genes in Supp. Table 2A & 2B). Comparison of both data sets yielded 61 commonly deregulated genes in IME and IMT compared to IMP mice (Supp. Table 2C).

Using IPA, genes deregulated by E2 and TAM resulted in the generation of 25 and 12 networks respectively (For the top 5 networks, see Supp. Table 3A & 3B). Analysis of the 61common genes between both E2 and TAM treated mice generated 7 networks (Supp. Table 3C). The most significant network from each comparison is highlighted for further evaluation (Supp. Figs. 1–3).

Within the "molecular and cellular functions" category, "cellular movement" was the function most highly associated with IME and IMT (See Supp. Table 4B, 5B & 6B for a complete list). Among the genes associated with cellular movement, *CXCL1*, a murine IL-8 homolog, as well as *IL-1α* and *Fosl1*, modulators of IL-8, were downregulated by E2 or TAM. IME upregulated gastric expression of *CCL19* (*MIP3β*), *CCL21a*, *CXCL15* and *IL-17b* while downregulating *IL-33*, a IL-1 family cytokine. IMT downregulated *CCL2*, *CCL7*, and *CCL12* (Supp. Table 2). *CXCL1* was associated with the most significant networks and top biological functions in both groups.

Cancer was the "disease or disorder" most associated with E2 and TAM, as well as in the dataset of overlapping genes (Supp. Table 4A, 5A & 6A). Genes differentially expressed affected oncogenic processes such as tumorigenesis, hyperproliferation, metastasis and neoplasia. Furthermore, both E2 and TAM treatments significantly affected genes linked to diseases mediated by immune and inflammatory responses, e.g. the hypersensitivity response or cardiovascular functions. Among cancer-associated genes, stress response genes (*DNAJA1* and *HSPA1A*), extracellular remodeling genes (*MMP10*, *PLAUR*, *SERPINE1*, and *GDF15*) and a component of the AP-1 transcription complex (*FOSL1*) were downregulated in IME and IMT mice, indicative of the reduced severity of gastric lesions. IME and IMT mice had upregulated genes associated with the Wnt/β-catenin pathway including a phosphatase (*PPP2R2B*) and two receptors (*SFRP2* and *FZD6*). IME mice also had downregulated gastric expression of additional metalloproteinases (*MMP3* and *MMP13*) while upregulation was noted in *SFRP4*, a Wnt receptor. IMT mice had downregulated growth factor, *IGF1*, which mediates insulin like effects (Supp. Table 2).

E2 modulates serum CXCL1 protein levels

Serum cytokine protein levels were measured in both uninfected and infected mice of the following groups: untreated males, E2 males and untreated females (Fig. 6). Basal differences in immune responses were observed between genders; UFP mice had elevated Th2 cytokines, interleukin 5 (IL-5) and IL-13, compared to UMP ($P<0.05$ and 0.01, respectively).

E2 treatment modulated serum cytokine responses in IME compared to IMP mice. CXCL1, a neutrophil chemokine, serum levels were decreased in both IME and IFP compared to IMP mice ($P<0.05$ and 0.001, respectively). Macrophage inflammatory protein 1-alpha (MIP1α or CCL3) and MIP1β (or CCL4) were significantly higher in IFP compared to IMP mice

(P<0.01 and 0.05, respectively) but were not different between IME and IFP mice, indicating a slight E2-mediated increase in MIP1α/β levels. IL-6 was increased significantly in IME compared to IMP mice (P<0.05).

A subset of cytokines were significantly different between genders and were unaffected by E2. IL-1β, IL-5 and monocyte chemoattractant protein-1 (MCP-1 or CCL2) were significantly decreased in IMP and IME compared to IFP mice (P<0.05, 0.01, 0.05 for IMP vs. IFP and P<0.05, 0.05 and 0.05 for IME vs. IFP). IL-12p70 was increased in IFP compared to IME mice (P<0.05) but not significantly compared to IMP mice (P=0.08).

Discussion

Although estrogen is hypothesized to reduce gastric cancer in women(1) due to its immunomodulatory effects(20), few studies involving male and female animals with a recognized sexual dimorphism in gastric cancer incidence have analyzed the role of 17β-estradiol and *H. pylori* in gastric carcinogenesis. In a six week study, E2 treatment of ovariectomized *H. pylori* infected gerbils increased acute inflammation and epithelial cell proliferation in the stomach(21). In contrast, using ovariectomized INS-GAS female mice infected with *H. pylori* for 28 weeks, we demonstrated that E2 treatment reduced epithelial cell proliferation, attenuated gastritis and reduced the development of GIN(9). We hypothesized that E2 was protective due to decreases in pro-inflammatory mediators like *IL-1β* and *iNOS* and increases in anti-inflammatory mediators like *IL-10*(9). Prophylactic E2 administration, but not castration, also reduced *H. pylori* induced gastric lesions in male INS-GAS mice while increasing gastric FoxP3+ regulatory T cells(12).

In the present study, we investigated the role of E2 and TAM in reducing gastric cancer in male and female *H. pylori* infected INS-GAS mice; E2, TAM or dual treatment prevented the formation of gastric cancer in infected male mice. The attenuation of gastric lesions by E2 and TAM treatments was accompanied by a downregulation of pro-inflammatory cues, a known effect of E2 treatment(20).

Previous studies suggest that in humans, E2 decreases gastric cancer risk, while TAM promotes gastric cancer(1, 5). However, our findings demonstrated that TAM prevented gastric cancer in IMT, and did not exacerbate disease in IFT mice. Given TAM's classification as a SERM, our results suggest that TAM may act agonistically in the stomach of INS-GAS mice. Two possible explanations for the differences between our findings and the findings in the epidemiological studies summarized by Chandanos et al.(1) are subject age and the cumulative TAM dose. Studies associating TAM with increased gastrointestinal cancer risk are composed of, or include a high percentage of, postmenopausal women(4–5), while our study used breeding age female mice. The effect of age and estrus cycles on TAM modulation of estrogen signaling requires further investigation. In the context of male mice, estrogen signaling induced by E2, and possibly TAM, were protective, suggesting that increased estrogen signaling may be beneficial in males, as seen in humans(1). Human studies indicate that the cumulative dose of TAM is crucial for increasing cancer risk(5). Breast cancer patients are commonly prescribed 20mg/day (0.3 mg/kg for a 70 kg person) for 5 years(22). Using TAM concentrations that inhibit breast cancer in mice(23), mice were treated with a higher dose of 0.18mg/day (6.8 mg/kg for a 25g mouse) for 12 weeks. Given differences in drug metabolism(24) and the short lifespan of mice, the murine model may not be ideal to assess longer term effects of chronic TAM treatment.

A systems biology approach was used to understand the biological implications of gene expression changes induced by E2 and TAM in male mice. Compared to IMP mice, both treatments reduced gastric pathology via common mechanisms, such as affecting cellular

movement or decreasing inflammatory signals (Supp. Table 4 & 5). Our analysis highlighted the importance of decreased *CXCL1* (also keratinocyte chemoattractant (KC) or growth-related oncogene-α (GROα)) expression in the stomach of IME and IMT. Moreover, lower serum CXCL1 protein levels in IFP and IME mice, and significantly decreased neutrophilic infiltration in IME mice confirmed the importance of CXCL1.

CXCL1 is a pro-inflammatory chemokine that recruits neutrophils and is upregulated in many diseases including cancer and cardiovascular disease(25–26). CXCL1 mediates changes in the microenvironment that promote tumor formation(25, 27). Additionally, its receptor, CXCR2, which is highly expressed on neutrophils and macrophages, is important in immune cell recruitment. Absence of CXCL1 or CXCR2 decreases macrophage infiltration and related lesions in a model of atherosclerosis(26). Furthermore, E2 decreases monocyte adhesion by reducing CXCL1 and CXCR2 expression in vitro(28–29). As E2 attenuates nuclear factor kappa beta (NF-κB) translocation(30), E2 could reduce CXCL1 levels by decreasing NF-κB binding upstream of CXCL1 (UCSC Genome bioinformatics).

Recent clinical studies link high CXCL1 expression and serum levels to gastric cancer(31–32). A single nucleotide polymorphism in the IL-8 promoter region (IL-8-251 T to A) that increases IL-8 levels is also associated with increased gastric cancer risk(33). K-ras overexpressing mice had increased *CXCL1* mRNA levels, which correlated with increased dysplasia scores(27).

A positive feedback loop couples CXCL1 secretion and neutrophil recruitment. In response to *H. pylori*-mediated CXCL1 gradients, neutrophils infiltrate the infection site and aid in the recruitment of macrophages. In addition to secreting more CXCL1, neutrophils induce *CXCL1* expression in macrophages and gastric epithelia(34). As CXCL1 promotes a tumorigenic microenvironment, a decrease in CXCL1 would inhibit cancer formation by decreasing neutrophil numbers, reducing *H. pylori*-related lesions, and dampening pro-inflammatory signals. Further studies are required to determine how E2 or TAM initially disrupt this positive feedback loop. Although local *CXCR2* expression was unchanged by treatment, decreases in *CXCL1* or *CXCR2* expression in circulating neutrophils, or a decrease in epithelial *CXCL1* expression, would disturb the feedback mechanism and this possibility merits further research.

Additionally, E2 and TAM affected expression of other cytokines regulating inflammatory responses. *IL-1α* was decreased in IME and IMT mice, likely reducing levels of chronic inflammation(35). CCR7 ligands (*CCL19* and *CCL21*), which are involved in tolerogenic responses(36), were increased in IME mice. IMT mice had decreased expression of *CCL2*, *CCL7* and *CCL12*, three ligands of CCR2, a receptor linked to inflammation-mediated diseases(37).

Gender and E2 systemic effects were explored by measuring serum cytokine levels. Cytokines associated with Th2 immune responses (IL-5 and IL-13) and monocytes were elevated in female mice. IL-13, which promotes alternative macrophage activation(38), was elevated in UFP compared to UMP mice. Alternatively activated, or M2, macrophages promote Th2 immunity during the resolution phase of inflammation. Compared to IMP, IFP mice also produced more monocyte chemokines (MIP1α and MIP1β) and cytokines secreted by monocyte-derived cells (IL-1β, IL-12p70 and MCP-1)(35, 37). While many of these are pro-inflammatory cytokines, the attenuated gastritis in females might be mediated by a greater number of M2 macrophages, as E2 mediates, possibly through increased Th2 cytokines, the alternative activation of macrophages and the reduction of classically activated macrophages and neutrophils(39). The role of M2 macrophages in stabilizing the progression of chronic inflammation in female INS-GAS mice requires further studies.

Surprisingly, serum IL-6 levels did not correlate with gastric cancer as noted in male-predominant liver cancer(40), as IMP had lower IL-6 levels than IME mice. Since IL-6 has both pro- and anti-inflammatory properties(41) and can be both downregulated and upregulated by E2(20), its role in gastric cancer and the INS-GAS model requires further investigation.

The disruption of the tumorigenic microenvironment by E2 and TAM may promote protection by decreasing oncogenic pathway activity. E2 and TAM downregulated the urokinase-type plasminogen activator receptor (*PLAUR*) and two of its regulators, *GDF15* and *SERPINE1* (also *PAI-1*)(42). *PLAUR* anchors urokinase, serving as a focal point for proteolytic activity during wound healing(43). Increased PLAUR and PAI-1 levels are associated with cell invasion, metastasis, and angiogenesis, and have been associated with *H. pylori* infection as well as poor prognosis in gastric cancer patients(43–44). As *PLAUR* is regulated by β-catenin(45) and *H. pylori* increases β-catenin translocation(46), E2 and TAM may decrease progression of gastritis by increasing expression of two Wnt repressors, Frizzled-6 (*FZD6*)(47) and secreted frizzle-related protein 2 (*SFRP2*)(48). E2 induction of sFRP-2 induction has been noted(49), and its silencing is associated with increased gastric cancer risk(48).

Sex differences in gastric cancer incidence, the protective effect of prolonged fertility in females and the reduced risk among women taking postmenopausal hormones, are elements suggesting that sex hormones play a protective role in *H. pylori* associated gastric cancer. Our findings suggest that both E2 and TAM decrease gastric cancer by decreasing neutrophilic infiltration, attenuating the chronic inflammatory response, and decreasing oncogenic signaling. These highly interrelated mechanisms result in the reduction of neutrophilic infiltrate by CXCL1, which reduces the exposure of the stomach to oxidative stress, a cause of DNA mutagenesis, which in turn decreases pro-inflammatory cellular infiltrates and delays the progression of gastric cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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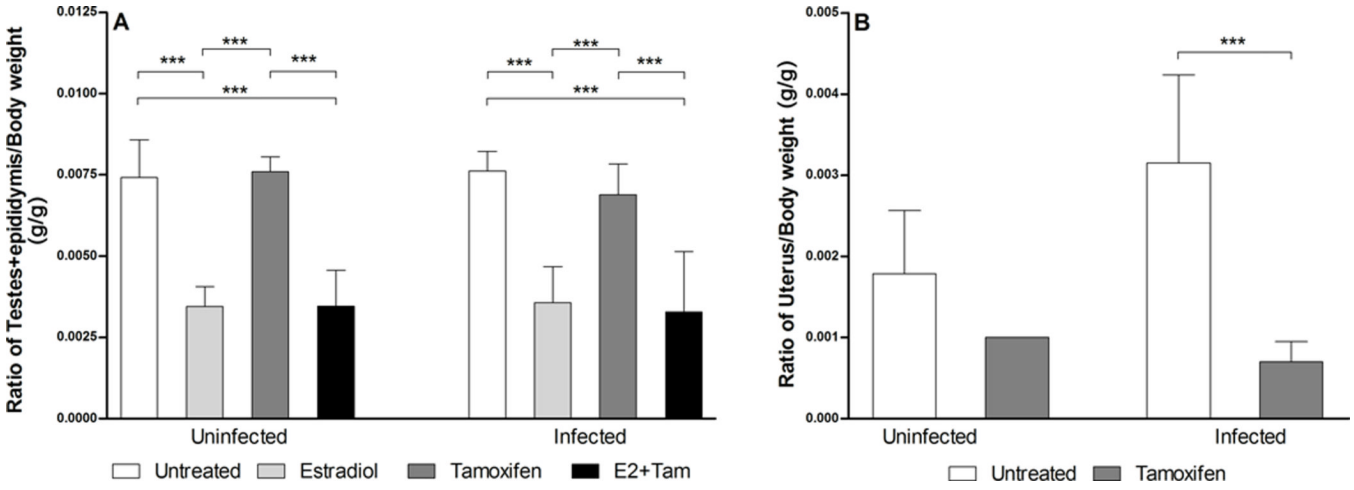

Figure 1.

Ratios of reproductive tissues/body weight of uninfected and *H. pylori*-infected mice with hormone treatment. A) Testes and epididymis/body weight ratios (g/g) in male mice. B) Uterus/body weight ratios (g/g) in female mice (UFT n=1 due to pellet loss). ***P<0.001. Error bars represent SD.

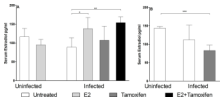

Figure 2.
Serum E2 levels in A) males and B) females after E2, TAM or dual treatment. **P*<0.05, ***P*<0.01, ****P*<0.001. Error bars represent SD.

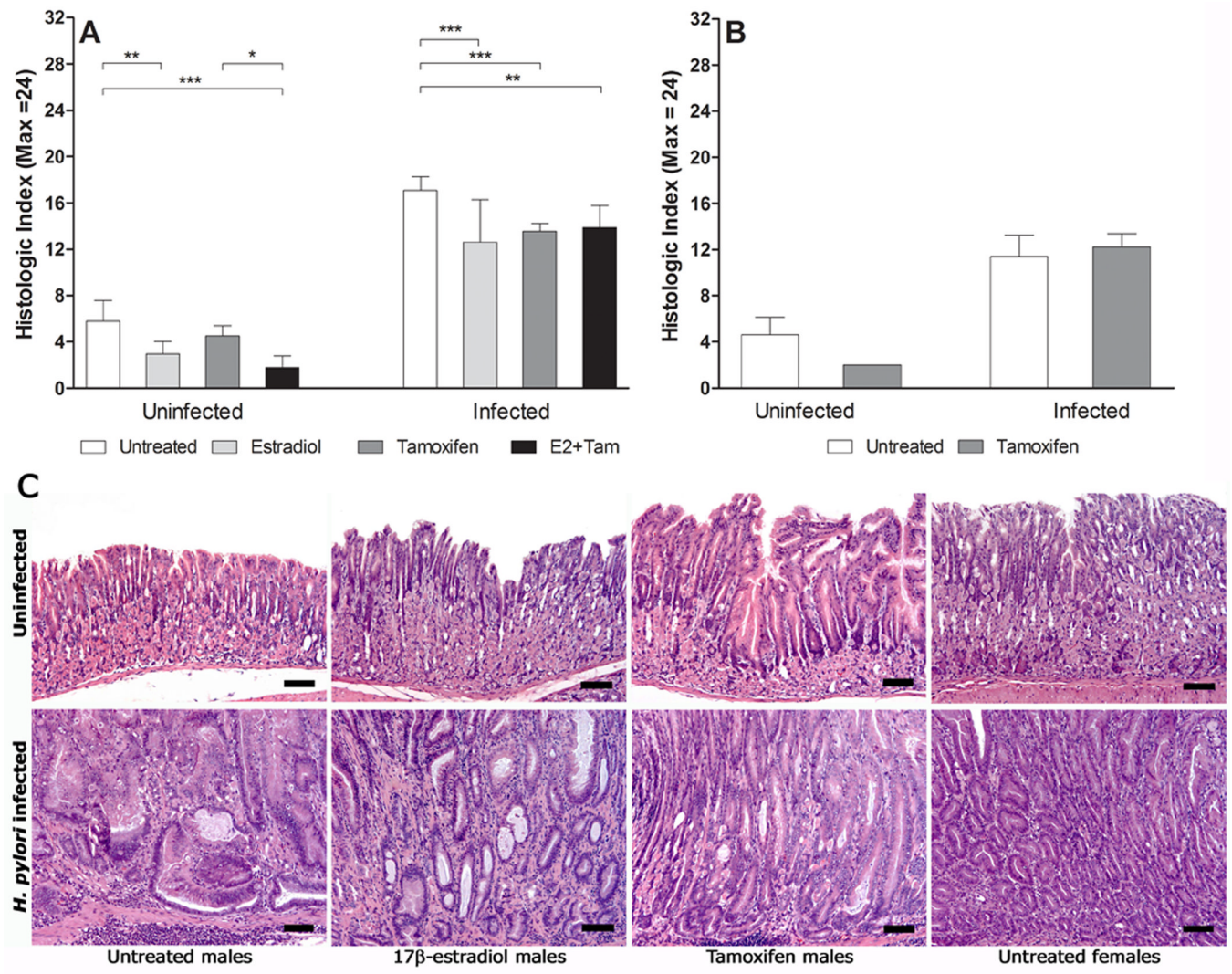

Figure 3.

Corpus pathology after 28 weeks of *H. pylori* infection and 12 weeks of hormone treatment. A) Cumulative histopathology score of male mice and B) female mice. C) Representative H&E-stained sections (Original magnification = 10×; bar = 200μm). **P*<0.05, ***P*<0.01, ****P*<0.001. Error bars represent SD.

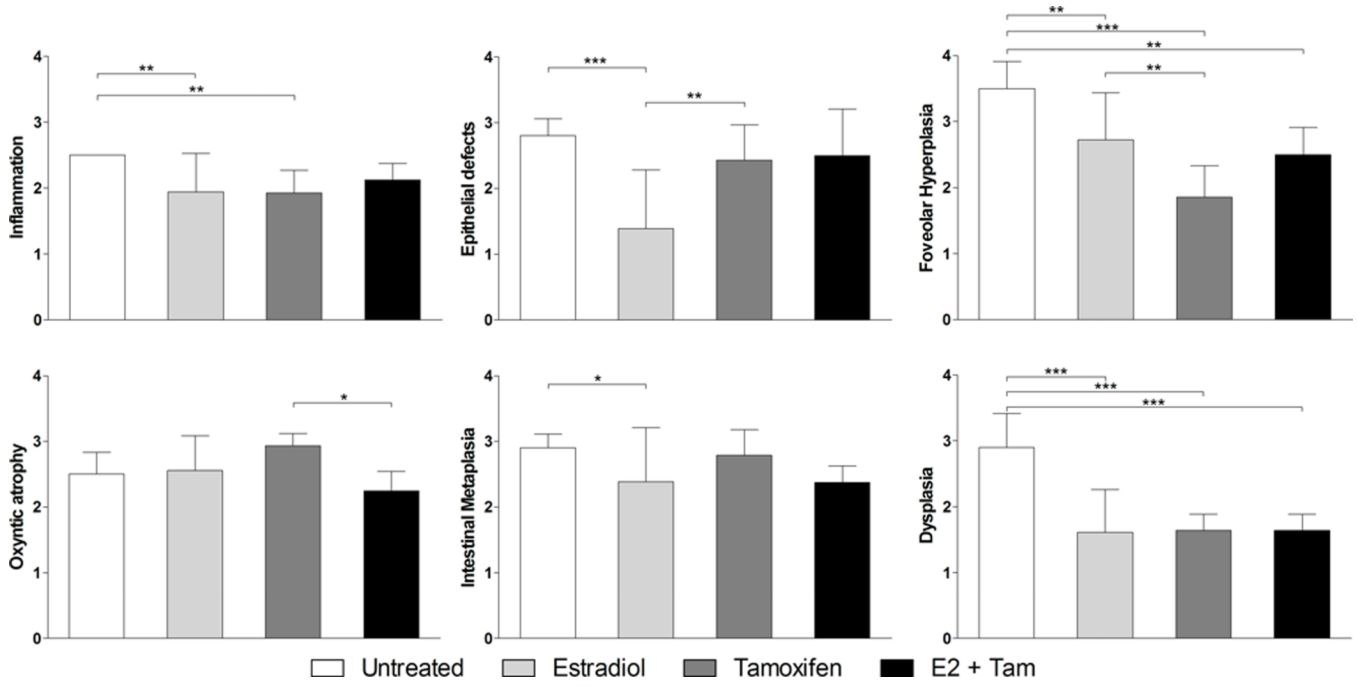

Figure 4.

Individual histopathology scores for gastric lesions of the corpus after 28 weeks of *H. pylori* infection and 12 weeks of hormone treatment in infected male mice. **P*<0.05, ***P*<0.01, ****P*<0.001. Error bars represent SD.

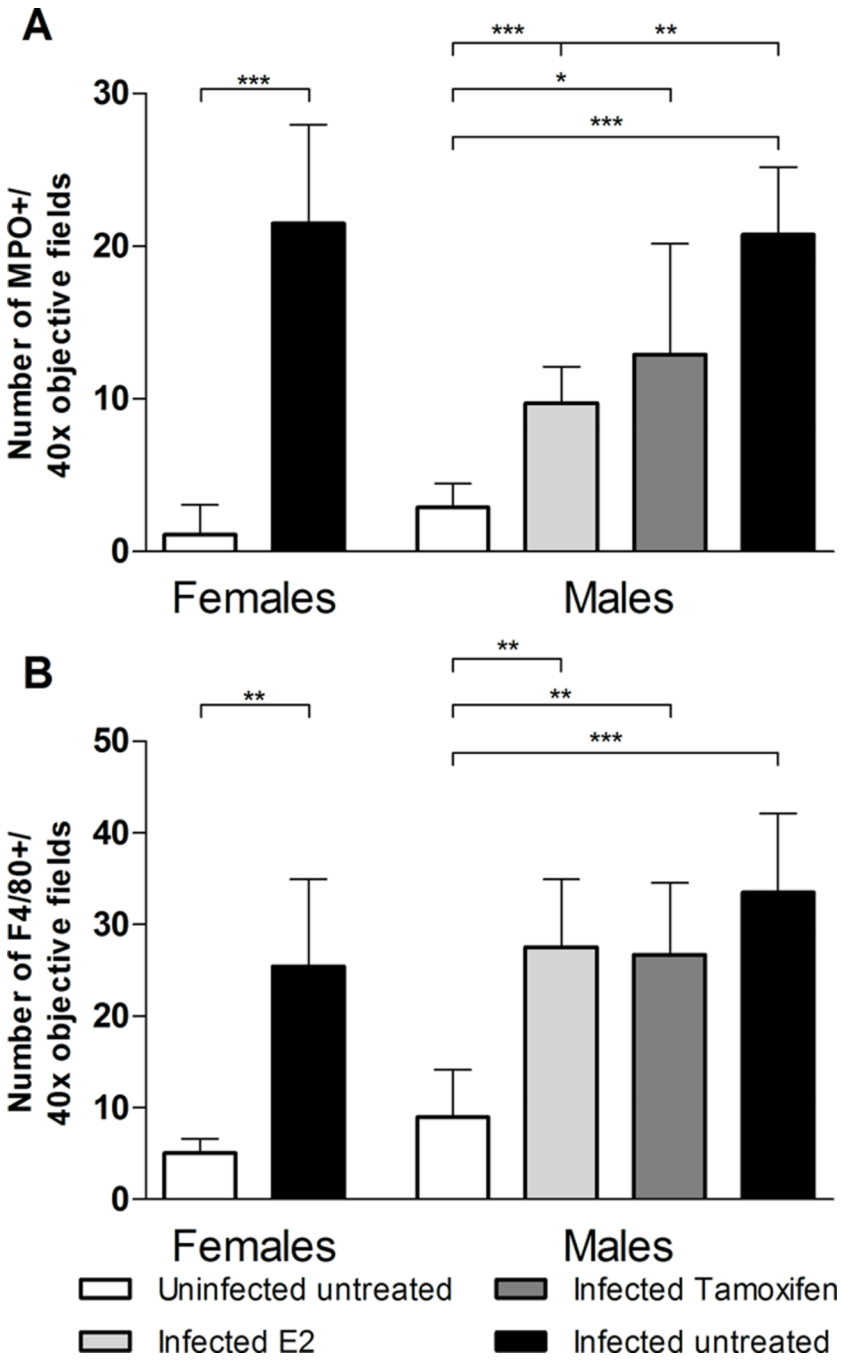

Figure 5.

Immune cell infiltration of A) MPO+ neutrophils and B) F4/80+ macrophages in uninfected and *H. pylori*-infected mice after E2 or TAM treatment. **P*<0.05, ***P*<0.01, ****P*<0.001. Error bars represent SD.

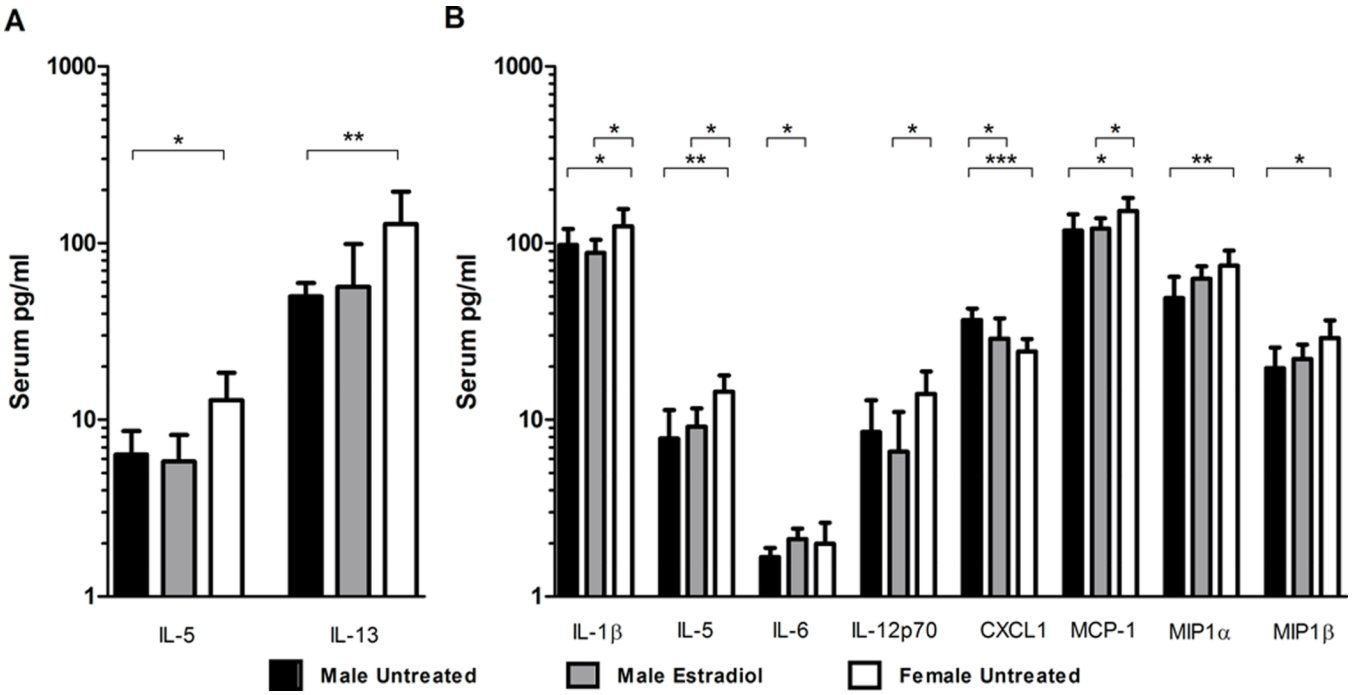

Figure 6.

Serum levels of cytokines and chemokines in A) uninfected and B) *H. pylori*-infected untreated males, E2 males and untreated females. **P*<0.05, ***P*<0.01, ****P*<0.001. Error bars represent SD.