Supraphysiologic Vaginal Estrogen Therapy in Aged Mice Mitigates Age-Associated Bladder Inflammatory Response to Urinary Tract Infections

Importance Bladder diseases characterized by chronic inflammation are highly prevalent in older women, as are recurrent urinary tract infections (rUTIs). Recurrent urinary tract infections lead to chronic inflammation of the bladder mucosa and cause lower urinary tract symptoms that persist even after the infection is cleared. Vaginal estrogen therapy (VET) has long been used for the treatment of rUTIs; however, its mechanism of action remains unclear.

Objectives The objective of this study was to examine the mechanism(s) by which VET affects bladder inflammation and response to rUTIs.

Study Design Here, we induced surgical menopause in aged (18 months old) mice followed by VET. Mice were then infected with uropathogenic *Escherichia coli*, and course of infection was investigated. Inflammatory cytokine response was assessed before and during infection using enzyme-linked immunosorbent assay. RNA sequencing analysis was used to compare the inflammatory status of the young versus aged bladder and principal changes confirmed via quantitative reverse transcriptase–polymerase chain reaction to determine the effects of VET on bladder inflammation. Impact on age-associated bladder tertiary lymphoid tissue formation was evaluated histologically.

Results In the ovariectomized aged model, VET not only mitigated uterine atrophy but was also associated with reduced rUTIs, number of bacterial reservoirs, dampened immune response, and promotion of terminal differentiation of urothelial cells. Bladder tertiary lymphoid tissue lesions were also reduced with VET, with an associated decrease in signals important for bladder tertiary lymphoid tissue formation. Finally, we determined that VET reverses age-associated upregulation of inflammatory genes and pathways.

Conclusions Our data suggest that VET is effective by reducing age-associated hyperinflammatory conditions in bladder mucosa and in enhancing the host response to infection.

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WHY THIS MATTERS

Supplemental estrogen therapy has long been used for the treatment of recurring urinary tract infections (rUTIs) in postmenopausal women. Targeted vaginal estrogen therapy (VET) has previously been shown to be effective in improving common lower urinary tract symptoms in women. However, how VET leads to improvement is still poorly understood. In our study, we show VET is associated with reduction in the inflammatory signals associated with rUTIs and general inflammatory milieu of the aged bladder mucosa. Vaginal estrogen therapy also is positively associated with limiting the number of bacterial reservoirs that have the potential to reseed new infections. In addition, VET is associated with increased terminally differentiated umbrella cells that line the bladder lumen. Finally, VET is associated with reduction in signals that lead to formation of age-associated bladder lymphoid lesions, which are tertiary lymphoid tissue also found in bladders of postmenopausal women and tightly linked with increased rUTI frequency. Together, our work provides supporting mechanistic data for the efficacy of VET in limiting inflammatory lesions and rUTIs.

As the world population continues to grow, so does the aging population. By 2050, the world's population of individuals older than 60 years is projected to make up 22% of the population.[1] The aging body undergoes a number of changes, which is further compounded in women because of the reduction of the sex hormone, estrogen,

postmenopause. Up to 50% of postmenopausal women will experience urinary tract infections (UTIs), the second most common infection in the elderly, accounting for approximately 25% of all infections.[2] Urinary tract infections result in an estimated 13 million outpatient visits and cost $2 billion per year in the United States alone.[3] Urinary tract infections have an unusual propensity to recur, and more than 50% of UTI cases in women older than 55 years recur within 1 year despite appropriate antibiotic treatment.[4,5] Moreover, recurrent UTIs (rUTIs) lead to chronic inflammation of the bladder mucosa and cause lower urinary tract symptoms that persist even after the infection is cleared.[6–8] Thus, there is great need to understand the mechanisms driving chronic inflammation and to determine therapeutic regimens to treat and prevent UTIs in the elderly.

Hormone therapy with estradiol (E2) has long been used to treat a number of urinary symptoms in postmenopausal women.[9–17] Estrogen treatment supplementation has been associated with increased production of antimicrobial peptides in humans and strengthening of urothelial barrier function.[18] Targeted vaginal estrogen therapy (VET) has been shown to decrease the rate of rUTIs in postmenopausal women and reduce the levels of proinflammatory cytokine, interleukin 6 (IL-6).[10,19–23] Although the effectiveness of VET has been shown, the mechanism(s) by which it could work remain to be elucidated.

Prior studies have used mouse models to address the interplay between aging and immunity and the impact of estrogen therapy on UTIs.[18,24,25] Our group and others have utilized the ovariectomized (OVX) young mouse as a model of induced hormone depletion and demonstrated that OVX mice harbored greater bacterial burden, increased formation of bacterial reservoirs that can seed rUTIs, and elevated circulating IL-6. Estradiol supplementation limited the infection and inflammatory response in part by rapidly promoting urothelial regeneration via BMP4 signaling.[25] Lüthje et al[18] confirmed that ovariectomy results in higher bacterial burdens in the bladder and urine. In addition, they reported that urines of postmenopausal women (who have low estrogen) exhibited higher urothelial exfoliation during UTI than did urines of premenopausal women. These studies indicate a clear role of estrogen in modulating mucosal defenses in mice and women. Recent work has begun to uncover how aging itself fundamentally alters the bladder immune landscape. Ligon et al[26] demonstrated that unperturbed aged bladders harbor tertiary lymphoid tissue (bladder tertiary lymphoid tissue [bTLT]), which serves as centers for B-cell recruitment, activation, and differentiation into plasma cells. Bladder tertiary lymphoid tissues appear as a feature of aging concomitant with reproductive senescence (~9 months of age) and appear to be unique to female bladders, indicating that "menopause/reproductive senescence" is a key transition for age-associated changes in the urinary tract. In addition, bTLT formation was shown to be dependent on tumor necrosis factor α (TNF-α) and Chemokine (C-X-C motif) ligand 13.[26]

Here, we utilize a murine model of aging to improve our understanding of the impact of aging and postmenopausal state on bladder inflammatory status and uropathogenic *Escherichia coli* (UPEC) infection pathogenesis and determine the mechanism underlying VET efficacy in ameliorating these conditions. Using aged mice (18 months old), we show that aging is associated with a baseline increase in inflammatory status, increased rUTIs, and elevated proinflammatory response to infection. We find that VET restores the age-associated uterine tissue atrophy with the mice re-entering estropause, a significant decrease in rUTI concomitant with increased terminal differentiation. Further, we found that VET reverses the age-associated upregulation of inflammatory-related genes and pathways in aged mice and reduces the number and size of bTLTs via downregulation of TNF-α and CXCL13. Together, our findings elucidate a mechanism of action of VET and support use of VET in postmenopausal women with or without a history of UTIs.

MATERIALS AND METHODS

Mice

All aged mice were 18 months old and were obtained from the National Institute on Aging. Young mice, 6–10 weeks old, were obtained from The Jackson Laboratory. Mice were held in a pathogen-free and temperature controlled facility, with a 12-hour light-dark photocycle. All animal protocols were approved and in accordance with Washington University School of Medicine Institutional Animal Care and Use Committee standards.

Measurement of Estrus

Rodent estrous cycling occurs every 4–5 days, consisting of 3 stages: estrus, metestrus, and diestrus. It

has been shown that at approximately 9–12 months of age, rodents begin to experience irregular estrous cycles called estropause and eventually stop cycling all together (termed *anestrous state*).[27–29] These stages were visualized using the vaginal smear technique described by Byers et al.[30]

Ovariectomies

Ovaries of female 18-month-old C57BL/6 female mice were removed as previously described.[25] In short, 2 small dorsal skin incisions were made around the ovarian fat pad. The ovaries and the attached uterine horns were located and pulled through the incision. The ovaries were then removed using a single cut, applying pressure to the uterine horn to stave off bleeding. Preoperative care included the administration Buprenorphine Sustained Release (0.5–1.0 mg/kg). All mice were allowed at least 2 weeks to recover.

E2 Supplementation

Vaginal E2 cream (ESTRACE [Warner Chilcott, Mississauga, Ontario, Canada] cream 0.01%) was applied to mice immediately following ovariectomy. Each mouse received 0.1 mg/kg of E2 directly into the vagina, daily for 2 weeks. Graduated pipette tips were cut above the first notch to create a larger opening. The tip was then filled with the cream until it reached the following notch. After 2 weeks, the mice received the same dose of E2 twice a week, throughout the duration of the infection.

Infection

UT189[31] from a frozen glycerol stock was grown statically at 37°C for 24 hours in 20 mL of Luria-Bertani (LB) broth. The overnight culture was then subcultured into fresh 20 mL of LB broth and grown for 16-18 hours. Mice were anesthetized and transurethrally catheterized with 50 µL of 10^7 CFU (colony-forming units) bacterial suspension diluted in phosphate-buffered saline (PBS). Mice were euthanized day 1 through day 14 after infection, in accordance with the experimental design.

Urine Analysis

Throughout infection, urine samples were obtained from each mouse. Urine samples were then serially diluted in sterile PBS; 5 µL of each dilution was then seeded on LB plates for a total of 6 replicates. Plates were counted and calculated as CFU/mL. Urine cytology was obtained by spinning down 10 µL of urine and 40 µL sterile PBS, onto microscope slides. These slides were then fixed, using an open flame, and stained using the Papanicolaou protocol.

Tissue Histology

After the mice were euthanized, bladders were removed and immediately fixed in methacarn (60% methanol, 30% chloroform, 10% acetic acid). Fixed bladders were then embedded in paraffin, and 5-µm-thick sections were stained with hematoxylin and eosin.

Immunofluorescence Analysis

Bladder tissues were sectioned as described previously and then stained using the following antibodies: mouse monoclonal antibody to Uroplakin III (Fitzgerald 10R-U103A; Fitzgerald Industries Intl, Concord, Massachusetts), chicken polyclonal antibody to cytokeratin-14 (1:200; BioLegend 906004, San Diego, CA), mouse monoclonal antibody to E-cadherin (1:250; BD Biosciences, Franklin Lakes, NJ 610181), rabbit polyclonal antibody to *E coli* (1:250; US Biological e3500-26, Swampscott, MA), rabbit monoclonal antibody to p27KIP1 (1:250; Abcam 190851, Cambridge, United Kingdom), and rabbit monoclonal antibody to cytokeratin 5 (1:250; Abcam 53121). Following three 5-minute washes with PBS, secondary antibodies to Alexa 488, Alexa 594, and Alexa 647 (1:500; Invitrogen, Waltham, MA) were applied. Superficial cell nuclei positive for p27kip1 were quantified and analyzed 14 days postinfection (dpi).

Quiescent Intracellular Reservoir Quantification

Four separate 5-µm serial sections over a thickness of 200 µm per bladder were stained for *E coli* (to visualize bacteria) and E-cadherin (to visualize the outline of epithelial cells). The total number of quiescent intracellular reservoirs (QIRs) was quantified and reported as number of QIRs per bladder.

Bladder Tertiary Lymphoid Tissue Quantification

Hematoxylin-eosin–stained sections were imaged using Hamamatsu NanoZoomer 2.0-HT System (Hamamatsu Photonics, Hamamatsu City, Japan). The numbers of bTLT per bladder were quantified, and the total area of bTLT was analyzed using NDP.veiw2 software as described by Ligon et al.[26]

RNA Sequencing

Preparation of bladders for RNA isolation, sequencing, and data analysis was previously described.[26] In short, bladders were snap frozen and homogenized for RNA isolation using RNeasy Mini Kit (Qiagen, 74101, San Diego, CA). Preparation of libraries was done with Ribo-Zero rRNA depletion kit (Illumina, San Diego, CA) and then sequenced on HiSeq3000 (Illumina, San Diego, CA). Reads were then aligned to the Ensemble top-level assembly with STAR version 2.0.4b. Gene counts were produced from the uniquely aligned unambiguous reads by Subread:feature Count version 1.4.5, and transcript counts were derived by Sailfish version 0.6.3 (Kingsford Group Software, Pittsburgh, PA). Using RSeQC version 2.3 (Liguo Wang, Houston, TX), sequencing performance was determined for the total number of aligned reads; total number of uniquely aligned reads, genes, and transcripts detected; ribosomal fraction; known junction saturation; and read distribution over known gene models. All gene counts were then imported into R/Bioconductor package EdgeR (University of Melbourne, Melbourne, Australia), and Trimmed mean of M values (TMM) normalization size factors were calculated to adjust samples for differences in library size. Some features, such as ribosomal and any feature not expressed in at least 3 samples, were excluded from further analysis, and TMM size factors were recalculated to create effective TMM size factors. These factors and matrix counts were then imported into R/Bioconductor package Limma, and the voomWithQualityWeights function was used to calculate the observed mean-variance relationship of every gene/transcript and sample. Generalized linear models were then used to test for gene/transcript level differential expression, which were filtered for false discovery rate–adjusted P values less than or equal to 0.05 (adapted from Ligon et al[26]).

Real-Time Reverse Transcriptase–Quantitative Polymerase Chain Reaction)

RNA was isolated from flash-frozen bladders from non-infected mice, using TRIzol (Invitrogen 15596018). Samples were then treated with DNase I (Invitrogen 18068015) to remove any contaminating DNA. cDNA was synthesized using SuperScript II Reverse Transcriptase (Invitrogen 18064014). Expression of genes of interest was detected via real-time reverse transcriptase–quantitative polymerase chain reaction using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad 1725275, Hercules, CA) on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad). List of primers used can be found in Table 1. Relative expressions were calculated using the Double Delta Ct value and normalized to 18S.

Enzyme-Linked Immunosorbent Assay

Serum and urine samples collected from mice were analyzed according to the manufacturer's protocol. The following kits were used: mouse IL-6 (R&D Systems DY406, Minneapolis, MN) and mouse CXCL13/BLC/BCA-1 (R&D Systems DY470).

Statistical Analysis

For time-course experiments, 2-way analyses of variance with matching were used with Bonferroni post hoc tests for individual time points. To determine significance between 2 samples, unpaired t test, paired t test, or nonparametric Mann-Whitney U test was performed by using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA). $P < 0.05$ was used as the cutoff for statistical significance. To determine significance between treatment groups using real-time reverse transcriptase–quantitative polymerase chain reaction, unpaired t test was performed using GraphPad Prism software.

TABLE 1. Primer Sequences Used in qPCR Analysis

Gene Name	Forward Primer (5′-3′)	Reverse Primer (5′-3′)
IL-18r1	TCACCGATCACAAATTCATGTGG	TGGTGGCTGTTTCATTCCTGT
Cd163l1	TGGCCTCTGAGTTTAGGGTCT	CCCTTGGTGTCGAACCAGC
Cd68	TGTCTGATCTTGCTAGGACCG	GAGAGTAACGGCCTTTTTGTGA
Clec4e	AGTGCTCTCCTGGACGATAG	CCTGATGCCTCACTGTAGCAG
TNFAIP8l2	TCAGCTCAAAGAGTCTGGCAC	CTGGTCTCGTCGATAAAGAGATG
IL-4	GTCTGCATCAAGACGCCATG	CGTTGCTGTGAGGACGTTTG
IL-1β	GCAACTGTTCCTGAACTCAACT	ATCTTTTGGGGTCCGTCAACT

qPCR, quantitative polymerase chain reaction.

RESULTS

VET Restores Estrous Cycling in Aged Mice

Unlike humans, estrogen levels in aged mice do not typically display a significant decrease,[27–29] and thus, "aged" may not directly be reflective of "postmenopausal" mice. We determined whether OVX-aged mice constitute a bona fide "postmenopausal aged mouse model" to better understand the potential mechanism(s) of VET action in the aged mouse bladder. Ovariectomized aged mice were surgically generated and treated with VET (0.1 mg/d) daily for 2 weeks. Mice were then infected with UPEC for up to 14 days, and VET was continued twice a week for the duration of the study (Fig. 1A).

We found that serum E2 levels remained unchanged in aged mice before and after ovariectomy; however, levels significantly increased in aged mice treated with VET (Fig. 1B). Rodents exhibit age-associated uterine horn atrophy, which could be reversed with VET (Fig. 1C). We observed that both aged and OVX-aged mice undergo anestrous evident with the presence of leukocytes and cornified epithelial cells throughout the cycle, whereas VET-treated OVX-aged mice appear to reenter estropause and display changes in amount of leukocytes and cornified and nucleated epithelial cells in the mucous smears (Fig. 1D). Hereafter, we continued subsequent analyses with "aged" to represent OVX-aged animals and "aged + VET" to represent OVX-aged mouse cohorts given VET.

VET Reduces Bacterial Reservoirs and rUTI in Aged Mice

We previously assessed the role of systemic E2 instillation on UPEC pathogenesis in a young OVX mouse model.[25] To determine the effect of VET on the UPEC pathogenic cycle, we infected aged (18 months old) female mice with and without VET and monitored the progression of infection over 14 dpi. Although there was no impact of VET in the initial states of infection in aged mice similar to what was previously observed in young mice,[25] we found VET treatment was associated with significantly fewer instances of spontaneous bacteriuria indicative of rUTIs (Fig. 2A). Following UPEC infection, we see the formation of QIRs within Lamp-1–positive vesicles, which can seed rUTIs.[31,32] Given the significant decrease in rUTIs in the aged + VET

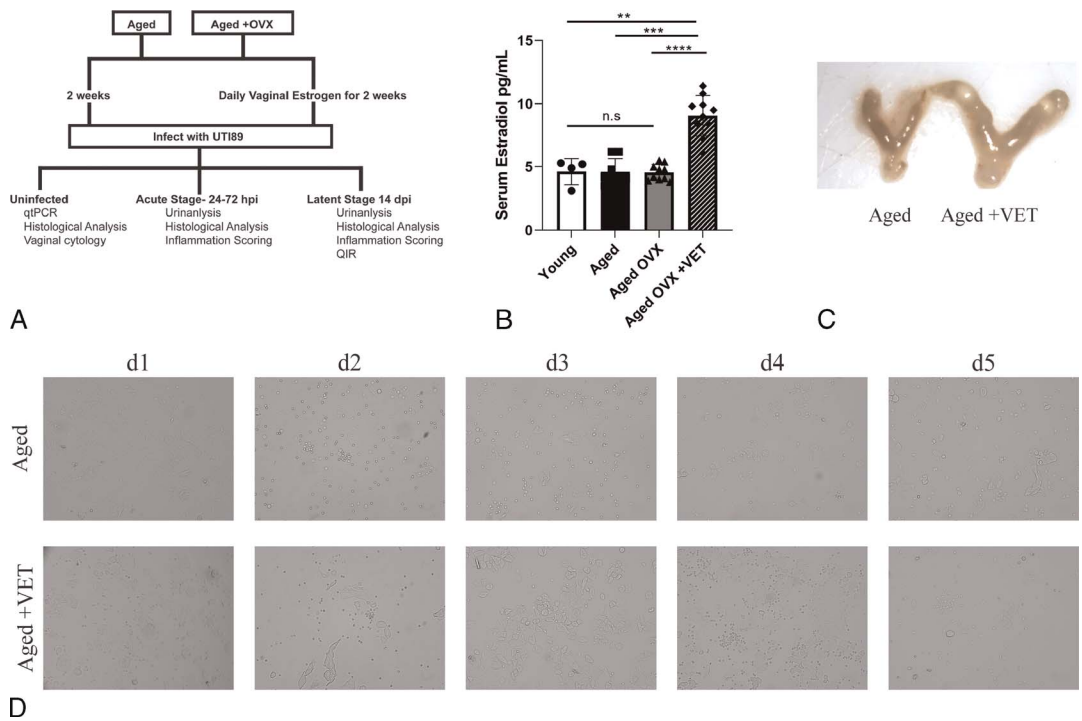

FIGURE 1. Vaginal estrogen therapy restores estrous cycling in aged mice. (A), Experimental design depicting experimental strategy. (B), Serum estradiol levels in young (n = 4), aged (n = 8), aged ovariectomized (n = 10), and aged ovariectomized mice treated with VET (n = 11). (C), Uterine horns of aged and aged VET mice, 4 weeks after ovariectomy. (D), Images of unstained wet smears from aged and aged VET mice depicting stages of estrous cycle, 3 weeks following the start of VET. OVX, ovariectomized; QIR, quiescent intracellular reservoir; VET, vaginal estrogen therapy.

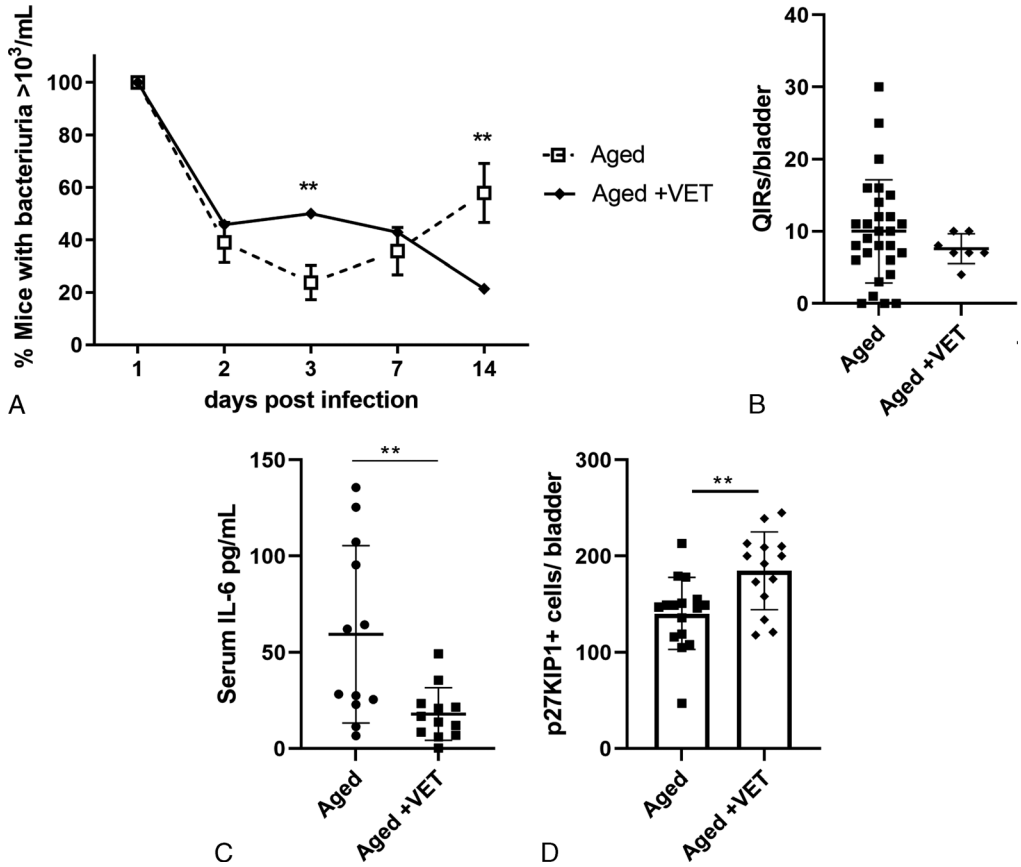

FIGURE 2. Vaginal estrogen therapy is associated with reduced rUTI and circulating IL-6 levels and increased differentiation status. (A), Percentage of mice with positive UPEC urine titers >10^3 CFU/mL at given time points (aged n = 19-41, aged + VET n = 14-24). (B), Quantification of QIRs in aged (n = 28) versus aged + VET (n = 7) mouse bladders at 14 dpi. (C), Serum IL-6 levels in aged versus aged + VET mice, 14 dpi (n = 24). (D), Quantification of p27kip1-positive nuclei in aged (n = 30) and aged + VET mice, at 14 dpi. CFU, colony-forming units; dpi, days postinfection; IL-6, interleukin 6; QIRs, quiescent intracellular reservoirs; rUTI, recurrent urinary tract infection; UPEC, uropathogenic *Escherichia coli*; VET, vaginal estrogen therapy.

group, we next sought to determine whether there was a decrease in QIRs. Histological analysis revealed fewer QIRs in the VET-treated mice (Fig. 2B).

VET Significantly Reduces Serum IL-6 Following UPEC Infection

Inflamm-aging is a process in which aged individuals develop chronic low-grade inflammation due to an increase in proinflammatory cytokines such as IL-6.[33] Studies have shown the importance of IL-6 production in response to UTI, in particular, controlling the host antimicrobial response and aiding in tissue regeneration.[32,34–36] Given the age-associated increase in IL-6 and its role as a proinflammatory cytokine during UTI, we next examined serum IL-6 levels following infection. We found that serum IL-6 was significantly reduced in the aged + VET group, 14 dpi (Fig. 2C).

VET Is Associated With Increase in Terminal Differentiation Markers in the Aged Urothelium

Superficial urothelial cell exfoliation due to infection has to be restored via terminal differentiation of underlying cells into new superficial cells with the activation of regenerative pathways to restore the urothelium, including BMP4 pathway leading to increased expression of p27kip1 indicating exit from the cell cycle and terminal differentiation.[37–41] Vaginal estrogen therapy–treated aged mice contained a significantly greater number of p27kip1-positive nuclei than aged mice suggesting that VET might promote terminal differentiation (Fig. 2D).

VET Reverses Age-Associated Upregulation of Inflammatory Genes and Cytokine Signals

To better understand the role of aging in the increased susceptibility to UPEC infection, we performed RNA sequencing analysis of bladders from young and aged

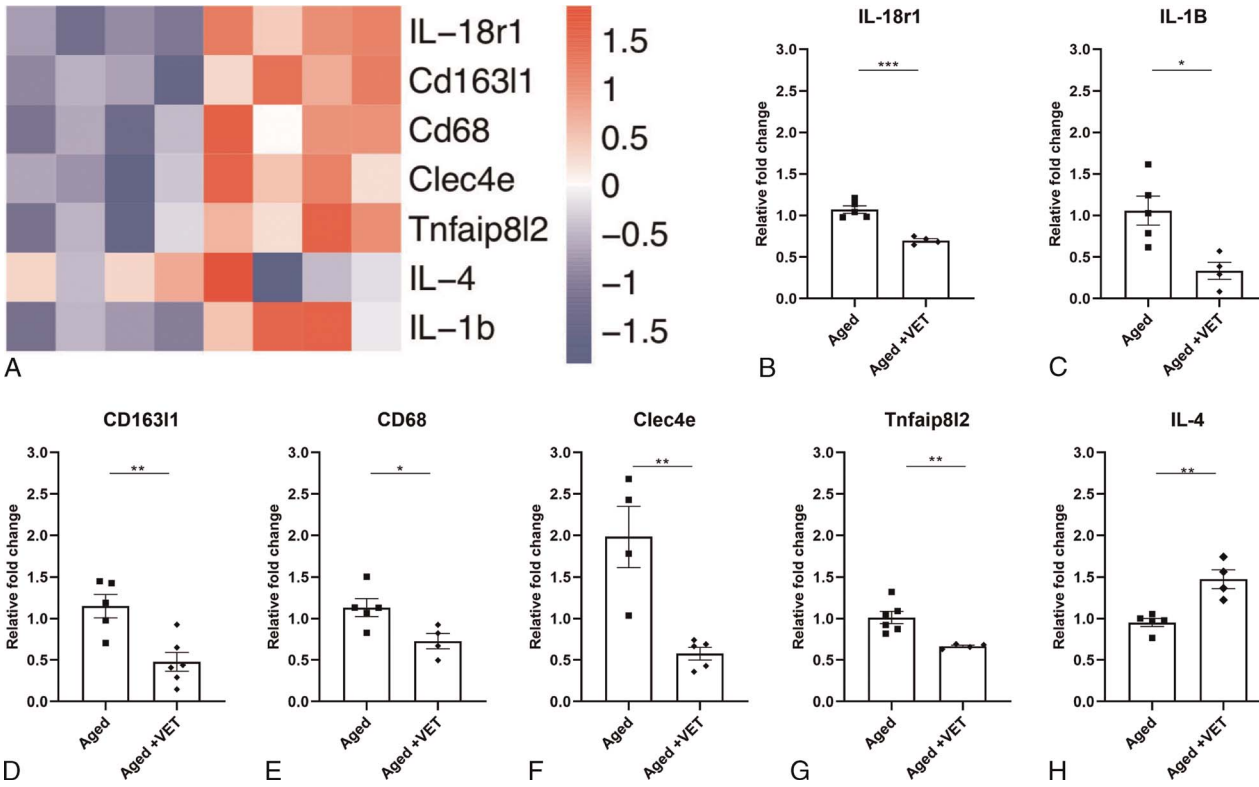

FIGURE 3. Vaginal estrogen therapy reverses age-associated upregulation of inflammatory genes and cytokine signals. (A), Heat map of inflammation-related genes from young (n = 4) and aged mice (n = 4) from RNA sequencing analysis. B-H, Real-time RT-qPCR analysis of inflammation-related genes: (B), IL-18r1, (C), IL-1β, (D), Cd163l1, (E), CD68, (F), Clec4e, (G), TNFAIP8l2, (H), IL-4 from uninfected bladders of aged (n = 3-6) and aged + VET (n = 3-6) mice. RT-qPCR, reverse transcriptase–quantitative polymerase chain reaction; VET, vaginal estrogen therapy.

mice. Pathway analysis of our uninfected controls revealed the upregulation of a number of inflammation-related genes in the transcriptome of aged mice compared with young mice (Fig. 3A). Using KEGG pathway analysis, we identified an upregulation of genes/pathways involved in inflammatory cell death, recruitment of macrophages (IL-18R1, IL-1β, and TNFAIP8L2), and response to tissue damage (CD68, CLEC4e). Increased expression of CD163L1, CD68, and Clec4e, as well as TNFAIP8I2, was evident in the aged uninfected bladders (Fig. 3A). Tissue repair requires macrophage polarization into proregenerative phenotype, which occurs via the secretion of anti-inflammatory cytokine IL-4 by T lymphocytes, which reduces production of proinflammatory cytokines.[42–44] We note that compared with young naive bladders, aged bladders express decreased expression of IL-4. Next, we sought to determine whether VET has an impact on these signals and pathways. We found that VET in aged mice resulted in a significant decrease in IL-18r1 (Fig. 3B) and IL-1β (Fig. 3C). We further showed that VET significantly reduces expression of CD163L1 (Fig. 3D), CD68 (Fig. 3E), CLEC4e (Fig. 3F), and TNFAIP8L2 (Fig. 3G). In contrast, we note an increase in expression of anti-inflammatory marker, IL-4 upon VET (Fig. 3H).

VET Induces Partial Regression of Bladder Tertiary Lymphoid Tissues in Aged Mice

We have recently shown that aged bladders are characterized by the presence of tertiary lymphoid structures (bTLTs) that begin to form at approximately 9 months of age in the aged female mouse.[26] Bladder tertiary lymphoid tissues comprise T and B cells with bona fide germinal centers and have increased expression of lymphoid chemokine, CXCL13,[45] and TNF-α.[26] We found that VET-treated aged mice had fewer bTLTs than aged mice alone (Figs. 4A, B), although this number was not significant. However, the size/volume of bTLT was reduced significantly in mice treated with VET (Fig. 4C). The formation of bTLT in the aging bladder is dependent on the inflammatory cytokine TNF-α and the expression of chemokines such as CXCL13.[26] We found that VET-treated aged mice express significantly reduced tissue TNF-α compared

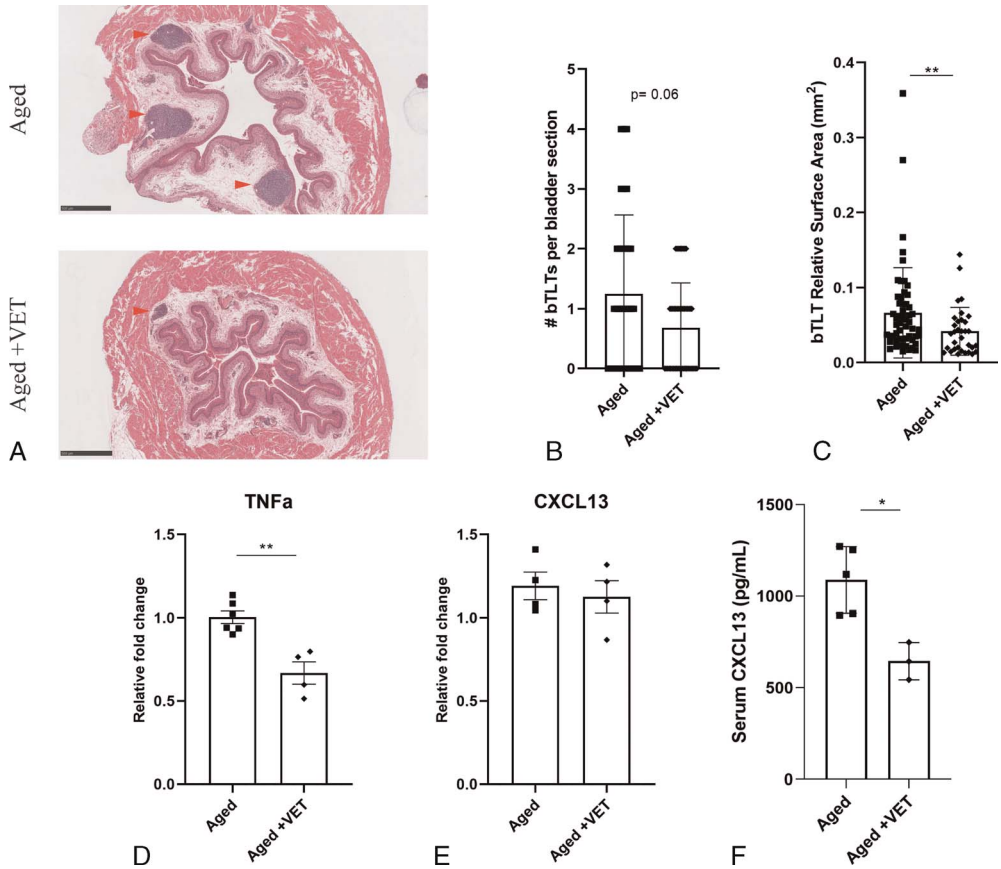

FIGURE 4. Vaginal estrogen therapy induces partial regression of bladder tertiary lymphoid tissues in aged mice. (A), Tissue histology of aged and aged + VET mice, 3 weeks following VET treatment. Red arrowheads denote bTLTs. Quantification of the number (B), and relative surface area (C), of each bladder tertiary lymphoid tissue in aged (n = 44) and aged + VET (n = 47) mice. Bars denote average number of bTLTs and average relative surface area. Real-time RT-qPCR analysis of uninfected bladder tissue TNF-α (D), and Cxcl13 (E), from aged (n = 4-6) and aged + VET (n = 4) mice. (F), Serum Cxcl13 levels of aged (n = 4) and aged + VET (n = 3) mice. bTLT, bladder tertiary lymphoid tissues; RT-qPCR, reverse transcriptase–quantitative polymerase chain reaction; TNF-α, tumor necrosis factor α; VET, vaginal estrogen therapy.

with aged bladders (Fig. 4D). Although tissue CCXL13 remained unchanged (Fig. 4E), serum CCXL13 levels were significantly reduced in VET-treated animals (Fig. 4F).

DISCUSSION

The menopausal transition in a woman is commonly associated with symptoms and changes in the urogenital tract, including vaginal atrophy, urinary urgency, frequency, and increased susceptibility to UTIs. Vaginal estrogen therapy has been shown to have utility in the management of urologic health.[46,47] The mechanisms driving the use of VET remain to be fully elucidated. In the current study, we used a mouse model of OVX-aged mice to examine the impact of VET on age-associated inflammatory status of the bladder before and after a UTI. We found that VET was sufficient to reduce baseline inflammation in the aged bladder as well as limit the inflammatory response after infection. Further, we show that VET reduced the prevalence of bacterial reservoirs, serum IL-6 following infection, and subsequent incidence of rUTIs in addition to upregulating the expression of urothelial regenerative markers. Finally, we demonstrate that VET significantly reduced the size of tertiary lymphoid tissue (TLT) lesions associated with the aging bladder. These lesions have been associated with an increased incidence of rUTIs. Our findings provide a foundation for exploring mechanisms of VET action and further underscore the importance of considering VET for women as they undergo menopause.

Removal of ovaries in aged mice does not exacerbate age-associated changes in estrogen levels and reproductive tissue. Unlike humans, mice do not undergo traditional menopause[48] and do not experience a notable decrease in estrogen levels, although they undergo estropause and loss of fertility by 9 months of age. Nevertheless, we note a significant impact of age on bladder physiology, function, immunity, and more. The lack of change in E2 levels in aged mice following

ovariectomy may be due to an increase in adipocyte tissue mass associated with aged mice, which can significantly contribute to circulating estrogen levels in postmenopausal women.[49,50] Adipose tissue has been shown to be a source of estrogen synthesis.[49,50] Other studies have shown that steroid levels following gonadectomy do not necessarily reflect steroid levels found in other regions of the body.[51] Thus, local levels of estrogen could well be affected even if systemic levels may not be altered. Indeed, ovariectomy does have an effect on reproductive tissue with atrophied appearance of the uterine horns in OVX mice that did not receive estrogen. In contrast, the uterine horns of the mice that received estrogen therapy had a normal appearance. One possibility is that the local effect could be due to alteration in estrogen receptors, ERα and Erβ, expressed in the urothelium. Whether the levels of estrogen receptors change with age is not clearly determined and warrants further investigation. Aging is also associated with an altered immune landscape of the urinary tract,[26] pelvic floor dynamics,[52] increased oxidative stress,[53] and altered neural control.[54] These aspects could also indirectly influence estrogen action.[52]

Normal estrogen levels have long been associated with anti-inflammatory functions such as downregulation of proinflammatory cytokines and resolution of inflammation by macrophages and other cells of the innate and adaptive immune system.[55–57] Loss of estrogen coincides with the increased susceptibility to a number of autoimmune diseases such as rheumatoid arthritis and multiple sclerosis[58–60] and enhanced immune responses.[56,61] Tissue resident macrophages play an instrumental role in the homeostatic maintenance, sensing, and responding to tissue damage. As an important effector cell of the innate immune system, macrophages use a number of receptors to sense and respond to danger signals efficiently.[62–64] CD163L1 is a group B scavenger receptor family and is highly expressed on macrophages where it colocalizes with CD163.[65] Similarly, CD68, which is expressed primarily by mononuclear phagocytes and tissue macrophages, is a scavenger receptor.[66–68] Macrophage inducible Ca^{2+}–dependent lectin receptor (Mincle), which is encoded by the gene CLEC4E, is expressed by myeloid cells, including monocytes and dendritic cells and functions as a tissue sensor.[69–71] TNFAIP8l2 functions as a myeloid cell–derived immune checkpoint regulator.[72] Increased expression of TNFAIP8l2 is associated with autoimmune diseases.[73,74] TNFAIP8l2 expression was increased in aged bladders. We noted an increase in these markers related to resident macrophages and inflammation in our aged bladders. Thus, together, our findings in naive bladders indicate that age is associated with a higher proinflammatory phenotype.

We suggest that VET is effective as a therapy because of dampening of proinflammatory pathways in aged bladders while promoting a tissue regenerative phenotype. The antimicrobial properties of E2 in the bladder promote regeneration and barrier function of the urothelium following UPEC infection, and our data support the use of VET to reduce rUTIs in the aged "postmenopausal" state.[18,19,25] Our data show that, at baseline, uninfected aged bladders express higher levels of IL-18 and receptor IL-18r1, which is necessary for IL-18 signaling, along with proinflammatory cytokine IL-1β. These pathways are indicative of capase-1–dependent inflammatory response,[75] wherein proforms of IL-1β and IL-18 are cleaved into mature proinflammatory cytokines and released upon cell lysis, thereby promoting a robust inflammatory host response.[76–78] We also note the decreased expression of proinflammatory cytokine IL-1B and an increase in the expression of anti-inflammatory cytokine, IL-4. Reducing the proinflammatory state of the aged bladder before UPEC infection may be key in reducing the overall enhanced inflammatory response and subsequent damage to the urothelium. Vaginal estrogen therapy induces downregulation of age-associated proinflammatory responses, including IL-6 levels, which is consistent with our previous studies described in both mice and menopausal women.[19,25]

Excessive inflammation associated with age may result in increased damage to the urothelium and delay in urothelial regeneration. Vaginal estrogen therapy might be particularly useful in dampening inflammation and promoting terminal differentiation of urothelial cells following UPEC infection. This is consistent with an increase in terminal differentiation markers in aged bladders treated with VET. We note an increase in p27Kip1-positive cells (depicting differentiated superficial urothelial cells) in mouse bladders that received VET and speculate that the uptick in differentiated cells could help reduce the number of niches for QIR formation. It has previously shown that OVX young mice had increased QIR formation[18,25] and that this is reversed following estrogen therapy.[25] Although exfoliation of infected superficial urothelial cells during a UTI serves as a defense mechanism to remove UPEC from the bladder, the excessive exfoliation we note in aged mouse urines coupled with the increased inflammatory signals could facilitate invasion and establishment of reservoirs in the underlying cell layers. Previous work has shown that loss of superficial urothelial cells

Simply Stated

Urinary tract infections (UTIs) are one of the most common infections worldwide, and they recur frequently. Postmenopausal women experience a significant number of recurrent UTIs (rUTIs). Hormone therapy with estradiol has long been used to treat a number of urinary tract symptoms in postmenopausal women. In particular, vaginal estrogen therapy (VET) has been shown to be effective in treating rUTIs in postmenopausal women; however, the mode of action is not well understood. Here, we used an aged female animal model (18-month-old mice) representative of postmenopausal women who are 65+ years old, to study how VET works. We found that VET mitigated uterine atrophy associated with "menopause" and reduced the number of rUTIs. Vaginal estrogen therapy dampened the increased immune response associated with rUTIs and promoted epithelial differentiation. Lastly, we found that lymphoid lesions found in both postmenopausal women's bladder tissue and in aged mouse bladders and associated with rUTIs in mice and women were reduced following VET, and the signals important for their formation were also decreased. Our work provides further support for a beneficial role of VET in restoration of genitourinary tract homeostasis, reduction of rUTIs, and promotion of anti-inflammatory response.

exposed the underlying cells, and UPEC could establish additional QIRs within those cells.[38]

Recent work has uncovered the age-dependent development of TLT in mouse bladders commencing upon reproductive senescence.[26] Aging was also associated with substantial changes to the naive bladder immune system revealing novel subsets of macrophages and dendritic cells, as well as an enrichment of B and T cells.[26] These tertiary lymphoid structures are organized with bona fide germinal centers with distinct B and T cells zones and are dependent on TNF-α and CXCL13 for their formation/maintenance. Somewhat unexpectedly, we determined that VET appears to induce regression of TLT. Our data suggest VET may reduce the size and volume of TLT in part by dampening the expression of TNF-α and CXCL13. The presence of these structures in aged mice and their subsequent regression following VET needs to be further addressed.

Our study is not without some limitations. Testing only 1 dose of E2 may have limited our ability to the find optimal dosage required for effectiveness. Similarly, we used only 1 VET regimen (modeled after the average clinical patient's prescription and scaled to body weight of an 18-month-old female mouse), as well as a limited treatment course (maximum of 4 weeks). This limits our understanding of the long-term effects of VET on rUTIs. Further work needs to be done to examine the long-term beneficial usage of VET for the treatment of rUTIs. Given that mice typically do not experience reduction in serum E2 levels even though they undergo reproductive senescence and alterations in estrous cycles, it is possible that the addition of VET could have resulted in supraphysiologic systemic levels of E2 in the mouse. Finally, whether and how estrogen signaling may directly or indirectly block the formation of bladder TLT is not elucidated, although our results suggesting that it is via regulation of CXCL13 warrant further investigation. However, if true in postmenopausal women, this understanding has the potential for being transformative in terms of therapy to limit rUTIs and dampen the inflammatory milieu in the aged bladder mucosa.

During infection, a subset of UPEC subverts host-mediated immune defenses to form QIRs that can persist indefinitely and lead to subsequent cases of reinfection.[31] Conventional UTI treatments such as antibiotics are ineffective in the elimination of QIRs.[79] Understanding the role of E2 in the formation of these reservoirs has the potential to unveil potential therapeutic targets for the treatment of rUTIs. It has been shown that ERα and ERβ agonist treatment induces protection against UPEC invasion in urothelial cells.[80] Reduction in estrogen receptors could occur with age and mediate local changes in the bladder. Estradiol has also been shown to affect UPEC virulence factors in a dose-dependent manner,[81] which could be another aspect to consider. Overall, our work provides further support for a beneficial role of VET in restoration of genitourinary tract homeostasis, reduction of rUTIs, and promotion of anti-inflammatory response.

The Women's Health Initiative sought to identify the risk and benefits of hormone (estrogen and progestin) therapy in postmenopausal women. The main risk factors reported by the study were that hormone therapy was associated with an increased risk of coronary heart disease and invasive breast cancer.[82] This led to widespread fear of the prescription of hormone therapy in postmenopausal women. However, recent studies are changing the conversation and acceptance and comfort with using VET to improve older women's genitourinary health.[59,60,83,84] Our work adds to and provides new evidence in support of VET as a therapy in the treatment of rUTIs in postmenopausal women.

ARTICLE INFORMATION

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