17β-Estradiol Protects Females against Influenza by Recruiting Neutrophils and Increasing Virus-Specific CD8 T Cell Responses in the Lungs

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ABSTRACT
17β-Estradiol (E2) treatment limits the pathology associated with pulmonary diseases caused by pathogens, allergens, and asthma, partly by reducing the production of proinflammatory cytokines and chemokines. To test the hypothesis that E2 protects against influenza A virus (IAV) infection by altering the recruitment and activity of innate immune cells and T cells, chemokine concentrations were measured and innate and adaptive immune cells were enumerated from the lungs of E2- and placebo-treated ovariectomized female C57BL/6 mice following infection. Females treated with E2 experienced less morbidity but had similar lung virus titers to placebo-treated females. Females treated with E2 had lower induction of CCL2 but higher CCL3 and CXCL1 responses in their lungs than placebo-treated females. Pulmonary recruitment of neutrophils, NK cells, macrophages, and dendritic cells was increased following infection, but only neutrophil numbers were greater in E2-treated than placebo-treated females. Neutrophils enhance the responses of influenza virus-specific CD8 T cells to promote virus clearance and improve the outcome of infection. Total numbers of virus-specific CD8 T cells were not altered by treatment with E2, but the proportion of gamma interferon (IFN-γ)- and tumor necrosis factor alpha (TNF-α)-producing, virus-specific CD8 T cells was increased. Neutrophil depletion in E2-treated females increased morbidity, reduced pulmonary production of chemoattractants for neutrophils, and reduced IFN-γ production by virus-specific CD8 T cells. Neutrophils mediate both inflammation and tissue repair during IAV infection and are regulated by E2 to improve the outcome of influenza in females.

IMPORTANCE
Severe influenza is associated with excessive inflammation that leads to tissue damage. We demonstrate that estradiol (E2) is a potent anti-inflammatory hormone that reduces the severity of influenza A virus infection in females. Treatment of female C57BL/6 mice with E2 does not affect virus replication but rather alters the production of chemokines, pulmonary recruitment of neutrophils, and the cytokine responses of virus-specific CD8 T cells to protect females against severe influenza.

Despite the availability of safe and effective vaccines and antivirals, annual influenza epidemics still result in 3 to 5 million cases of severe infection and between 250,000 and 500,000 deaths worldwide (1). Influenza pandemics and avian influenza outbreaks can result in severe disease that is associated with increased production of proinflammatory factors and the development of immunopathology (2). Often overlooked is the fact that the sex and hormonal status of an individual can regulate inflammatory responses and the development of immunopathology during influenza A virus (IAV) infection (3, 4).

Changes in hormone concentrations caused by natural fluctuations during the menstrual cycle, pregnancy, and menopause or following use of oral contraception or hormone replacement therapy affect pulmonary disease outcome (5, 6). Many inflammatory-mediated pulmonary diseases, including allergy and asthma, are more severe in women than men, with disease severity often changing at puberty, during the menstrual cycle, and after menopause (6). Women are 2 to 6 times more likely to be hospitalized and/or die following infection with respiratory pathogens, including Mycobacterium tuberculosis, pandemic IAVs (e.g., 2009 H1N1), and avian H5N1 virus (3, 7, 8). Mouse models of asthma, allergy, and respiratory infections collectively confirm that (i) females suffer a worse outcome from pulmonary diseases than males, (ii) lung function changes with circulating 17β-estradiol (E2) concentrations, and (iii) exogenous E2 treatment reduces pulmonary inflammation and symptoms of disease in females (9–15). In humans, administration of oral contraceptives that contain E2 reduces asthma exacerbations associated with premenstrual asthma by increasing regulatory T cell activity and reducing allergic inflammation in the respiratory tract (16). Sustained, as opposed to cyclical, concentrations of E2 are associated with improved outcome of respiratory diseases, including those caused by infections (11).

Estrogens, primarily E2, regulate cellular function in diverse cell types, including macrophages, dendritic cells (DCs), granulocytes, and lymphocytes (17). Estradiol has bipotential effects, with small or cyclical amounts enhancing proinflammatory cytokine responses and high or sustained concentrations reducing production of proinflammatory cytokines and chemokines (17). Exa-
diol can indirectly alter transcription of inflammatory genes by inhibiting NF-κB activity and recruiting steroid receptor cofactors that act as transcriptional repressors (18, 19).

The primary biological effects of estrogen are mediated through binding to one of two intracellular receptors, estrogen receptor alpha (ERα) or ERβ, that have cell-type-specific distributions (20, 21). These receptors serve as ligand-dependent transcriptional factors that regulate gene expression (22–24). Cell-specific responses to E2 depend on ER-mediated regulation of discrete genes and on the relative expression of ERα and ERβ (21). Both immune cells and nonimmune cells (e.g., epithelial cells, which are the principle cell type infected by IAVs), have intracellular ERs that act as transcriptional factors to regulate cellular function, including inflammatory responses (21). Signaling through ERα, but not ERβ, reduces pulmonary inflammatory cytokine responses and protects female mice during IAV infection (11).

Murine models of IAV pathogenesis demonstrate a protective role of exogenous treatment with E2 against infection-induced morbidity and mortality in females (11, 25, 26). Treatment of female mice with E2 protects against IAV by dampening inflammatory responses associated with tissue damage, including excessive production of gamma interferon (IFN-γ), tumor necrosis factor α (TNF-α), and CCL2, and by promoting higher antibody responses to influenza vaccination (11, 25, 26). Some (26), but not all (11), studies suggest that treatment of females with E2 affects type I IFN responses and virus replication in the lungs. Based on the current literature, sustained concentrations of E2 protect females against IAV infection, but whether this is by promoting antiviral defenses, reducing inflammatory responses, or both has not been conclusively determined. Despite the known effects of E2 on pulmonary inflammatory responses, little is known about the specific cell types affected by E2 signaling during IAV infection.

Conceptually, once infected, hosts can employ one of two defense strategies against viruses that are not mutually exclusive: they can engage responses that reduce virus replication, collectively referred to as "host resistance," or they can limit the damage caused by a given virus burden, referred to as "disease tolerance" (27). Whether E2 protects females by limiting the inflammation caused by host responses to IAV (i.e., by promoting disease tolerance), by reducing virus replication (i.e., enhancing resistance), or through both mechanisms is not clear and was evaluated in the present study. The present study also was designed to test the hypothesis that E2 alters the recruitment and activity of specific innate and adaptive immune cells to regulate the outcome of IAV infection in females.

MATERIALS AND METHODS
Animals and surgery. Adult (6- to 8-week-old) female C57BL/6 mice were purchased from NCI Frederick, housed in microisolater cages, and handled using biosafety level 2 (BSL2) practices. All animal procedures were approved by the Johns Hopkins University Animal Care and Use Committee. At 7 to 9 weeks of age, mice were anesthetized with an intraperitoneal injection of ketamine (i.p.) and xylazine subcutaneously. Mice were euthanized by i.m. injection with a ketamine-xylazine cocktail and then intranasally (i.n.) inoculated with 30 μl of Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) or PR8 diluted in DMEM, corresponding to 1.78 MLD50 of PR8 results in significant mortality 8 to 11 days postinoculation (p.i.); therefore, in this study, sample collection was limited to the first week p.i. For protein and virus quantification, mock- and virus-infected females were euthanized at 7 days p.i., at which time, plasma, uterine horns, and whole lungs were collected. For protein and virus quantitation, excised lungs were snap-frozen and stored at −80°C until homogenized in DMEM supplemented with 1% penicillin–streptomycin (Pen/Strep) and 1% l-glutamine (Invitrogen) and centrifuged to remove cellular debris. For enumeration of immune cells, mock- and influenza virus-infected females were euthanized at 1, 3, 5, or 7 days p.i., at which time plasma, uterine horns, and whole lungs were collected. Prior to excision, lungs were perfused with 5 ml of 1 × phosphate-buffered saline (PBS) through the right ventricle of the heart. Single-cell suspensions were generated from lungs and draining lymph nodes that were minced and incubated for 45 min at 37°C in the presence of collagenase II (1 mg/ml; Invitrogen) and then pressed through a 100-μm-pore nylon cell strainer (BD Biosciences, San Jose, CA) and treated with ACK lysis buffer (Quality Biological) to deplete red blood cells. Total viable cells within each single-cell suspension were calculated by trypan blue exclusion (Invitrogen) using a hemocytometer.

Cytokine and chemokine quantification. Supernatants from lung homogenates were used to measure interleukin-1β (IL-1β), IL-4, IL-5, IL-13, IL-33, CCL3, CXCL1, CCL5, and transforming growth factor β (TGF-β) by enzyme-linked immunosorbent assay (ELISA) (R&D Systems and BD Biosciences) and CCL2, IL-12(p70), tumor necrosis factor alpha (TNF-α), gamma interferon (IFN-γ), IL-10, and IL-6 with the mouse inflammation cytometric bead array (BD Biosciences).

Flow cytometry analysis of innate immune cells. For quantification of TGN-α-inducible nitric oxide synthase (iNOS)-producing dendritic cells (tipDCs) (CD11b+ Ly6c+MHC-II+ iNOS+), a subset of lungs were resuspended at 1 × 106 cells/ml in complete DMEM and stimulated with medium or lipopolysaccharide (LPS) (100 ng/ml; Sigma) on 12-well plates in the presence of GolgiPlug for 4 h at 37°C in 5% CO2 prior to antibody staining. The viability of all cells was determined by fixable LIVE/DEAD violet viability dye (Invitrogen). Following Fc receptor blocking with anti-CD32/CD16, innate immune cell populations were identified as follows: alveolar macrophages (CD11b+CD11c+MHC-II+Ly6g-), conventional dendritic cells (cDCs) (CD11b+CD11c+MHC-II+Ly6g-),
inflammatory monocytes (CD11b⁺ CD11c⁻ MHC-II- Ly6g⁻), monocyte-derived dendritic cells (moDCs) (CD11b⁺ CD11c⁺ MHC-II⁺ Ly6g⁺), neutrophils (CD11b⁺ CD11c⁺ MHC-II⁻ Ly6g⁻), natural killer (NK) cells (CD11b⁺ CD11c⁻ NK1.1⁺), and plasmacytoid dendritic cells (pDCs) (CD11b⁻ CD11c⁺ MHC-II⁺ Ly6g⁻). Cells were stained with appropriate concentrations of the following antibodies: Alexa Fluor 647-conjugated anti-Ly6c (ER-MP20; AbD Serotech), fluoroscein isothiocyanate (FITC)-conjugated anti-Ly6g (1A8), allophycocyanin (APC)-conjugated anti-CD11c (HL3), phycoerythrin (PE)-conjugated anti-MHC II (AF6-120.1), APC-Cy7-conjugated anti-CD11b (M1/70), and FITC-conjugated anti-NK1.1 (PK136; ebiscience). Intracellular staining with PE-conjugated anti-TNF-α (MP6-XT22), rabbit anti-iNOS (M19; Santa-Cruz, Dallas, TX), and FITC-conjugated goat anti-rabbit IgG (Santa-Cruz) was performed following fixation and permeabilization with Cytofix/Cytoperm and Perm/Wash buffer. Data were acquired using a LSRII (FACS Diva software; BD Biosciences) and analyzed using FlowJo (Tree Star, Inc.). Total cell counts were calculated by multiplying the percentage of each cell population by the total cell counts acquired by trypan blue exclusion using a hemocytometer. All staining procedures were performed at 4°C unless stated otherwise. All reagents were purchased from BD Biosciences unless stated otherwise.

**Flow cytometry analysis of T cells.** For quantification of intracellular cytokine production in CD4 and CD8 T cells, cells were resuspended at 2 × 10⁶ cells/ml in RPMI 1640 (Cellgro) supplemented with 10% fetal bovine serum (FBS) (Fisher Scientific) and 1% Pen/Strep, and 100 µl of cells was plated in 96-well round-bottom plates and incubated at 37°C in 5% CO₂ for 4 to 5 h with no stimulation, phorbol myristate acetate (PMA) (50 ng/ml)-ionomycin (750 ng/ml) (Sigma-Aldrich) or 1 µM peptide antigen (CD8, NP₃₆₆−₃₇₄; CD4, hemagglutinin [HA₂₁₁−₂₅₅] or NP₃₁₁−₃₂₅) (ProImmune) in the presence of GolgiPlug as described previously (31, 32). Following stimulation, cells were stained with appropriate concentrations of the following antibodies: peridinin-chlorophyll protein (PerCP)-Cy5.5-conjugated anti-CD4 (RM4-5), FITC-conjugated anti-CD25 (7D4), PerCP-Cy5.5-conjugated anti-CD8a (53–67), PE-conjugated DNP₆₆₆–₃₇₄ tetramer (NIH Tetramer Core Facility) after staining with the LIVE/DEAD far-red viability dye (Invitrogen) and blocking Fc receptors with anti-CD32/CD16. Intracellular staining with PE-conjugated anti-IFN-γ (MP6-XT22), FITC-conjugated anti-IL-4 (MP6-XT22), and APC-conjugated anti-CD11c (HL3) was performed following fixation and permeabilization with Cytofix/Cytoperm and Perm/Wash buffer (BD Biosciences). Intracellular staining with PE-conjugated anti-IL-17A (TC11-18H10) was performed following fixation and permeabilization with Cytofix/Cytoperm and Perm/Wash buffer (BD Biosciences). Cells were acquired using a FACS Calibur (Cellquest Software) and analyzed using FlowJo (Tree Star, Inc.). Total cell counts were calculated by multiplying the percentage of each cell population by the total viable cells acquired by trypan blue exclusion using a hemocytometer. All staining procedures were performed at 4°C unless stated otherwise. All reagents were purchased from BD Biosciences unless stated otherwise.

**Neutrophil depletion.** For neutrophil depletion, mice were administered rat anti-mouse anti-Ly6g antibody (1A8; BioXCell, West Lebanon, NH) or rat IgG2a isotype (2A3; BioXCell) diluted in sterile filtered PBS. On days 3 and 5 p.i., neutrophil depletion antibody or isotype was administered intraperitoneal (i.p.) (0.5 mg in 80 µl) injection and i.n. (0.2 mg in 30 µl) instillation as described previously (33).

**Statistical analyses.** Morbidity data were analyzed with a multivariate analysis of variance (MANOVA) with one within-subjects variable (days) and one between-subjects variable (treatment), and significant interactions were further analyzed using planned comparisons. Virus titers, protein concentrations, and T cell influx were analyzed with r tests. Innate immune cell quantities were analyzed with 2-way ANOVAs with day p.i. and treatment as the independent variables, and significant interactions were further analyzed using the Tukey method for pairwise multiple comparisons. Pearson product moment correlational analyses were used to measure dependence between variables, and the Fisher r-to-z transforma-
placebo-treated females (Fig. 1B) ($P < 0.0001$). Consistent with previous studies (11), titers of infectious virus were not different between E2- and placebo-treated females, suggesting that E2 treatment does not promote resistance against IAV infection (i.e., reduced virus replication) (Fig. 1C). Conceptually, another host strategy that can protect against the detrimental effects of infection is “disease tolerance,” which is defined as a reduction of the negative impact of infection on host fitness or survival (34). By comparing the fitness consequences (i.e., body mass loss in the present study) and virus titers of females treated with E2 or placebo, we determined that females treated with E2 experienced a significantly lower fitness cost (i.e., less weight loss) at any given virus titer than females not treated with E2 (Fig. 1D) ($P < 0.005$). Taken together, these data suggest that E2 protects females not by making them more resistant to infection but by making them more tolerant to the costs associated with infection.

**Treatment with E2 suppresses proinflammatory responses and increases pulmonary production of chemokattractants for neutrophils.** The pathogenesis of IAV infection is associated with an excessive inflammatory response to infection, rather than uncontrolled virus replication (35–37). Consistent with previous studies (11), inflammatory responses commonly associated with severe IAV outcome, including IFN-γ and CCL2, were reduced in E2-treated compared with placebo-treated females (Fig. 2A and data not shown) ($P < 0.05$ in each case). To ascertain whether the protective effects of E2 were mediated by a skewing of the immune response toward an anti-inflammatory profile, the induction of anti-inflammatory cytokines was assessed during IAV infection. Pulmonary concentrations of IL-4, IL-10, IL-13, IL-33, and TGF-β did not differ between E2-treated and placebo-treated females (data not shown).

To further examine whether E2 alters the induction of chemotactic factors following IAV infection, an additional panel of chemokines were measured in the lungs. The induction of CCL5, a chemokine predominantly involved in the recruitment of lymphocytes, was not affected by E2 treatment (Fig. 2C). The induction of chemokines associated with monocyte and neutrophil recruitment, including CCL3 and CXCL1, however, was higher in E2-treated females than in placebo-treated females (Fig. 2B and D) ($P < 0.05$ in each case). In summary, E2 may regulate the pulmonary environment during IAV infection by reducing responses associated with immunopathology and enhancing responses associated with the recruitment of innate immune cells, in particular neutrophils, into the lungs.

**Recruitment of neutrophils into the lungs during IAV infection is E2 dependent.** Because E2 altered the chemokine profile, we next analyzed the kinetics of recruitment of innate immune cells into the lungs during IAV infection (see Fig. S1 in the supplemental material). Consistent with previous studies (38–41), over the course of IAV infection (i.e., 3 to 7 days p.i.), there was a significant influx of natural killer (NK) cells, monocytes, plasmacytoid dendritic cells (pDCs), conventional DCs (cDCs), monocyte-derived DCs (moDCs), and tipDCs into the lungs compared with in mock-infected females (Table 1) ($P < 0.05$). There were, however, no differences in the kinetics of recruitment of these innate immune cell populations between E2-treated and placebo-treated females (Table 1). Only the influx of neutrophils into the lungs was increased both over the course of infection and to a greater extent in E2-treated females than placebo-treated females (Fig. 3A to C) ($P < 0.05$).

**17β-Estradiol treatment increases the cytokine responses of virus-specific CD8 T cells during IAV infection.** Neutrophils can contribute to the maintenance of CD8 T cell effector function in the lungs during IAV infection (42, 43). To establish whether E2 treatment alters T cell recruitment or function during IAV infection, we assessed the number and cytokine production from CD4 and CD8 T cells in the lungs and draining lymph nodes during IAV infection (see Fig. S2 in the supplemental material). The total number of CD4 T cells in the lungs and draining lymph nodes was significantly increased in IAV-infected compared with mock-infected females (data not shown) ($P < 0.05$); there were, however, no differences in the total numbers of CD4 T cells or the proportions of IFN-γ+, IL-4, or IL-17-producing CD4 T cells or Foxp3+ regulatory T cells in the lungs (Fig. 4A to C) or draining lymph nodes (data not shown) of E2- and placebo-treated females during IAV infection.

The total number of CD8 T cells and the number of virus-specific CD8 T cells in the lungs and draining lymph nodes significantly increased after IAV infection (data not shown) ($P < 0.05$). There were, however, no differences in the total number of CD8 T

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**FIG 2** Exogenous 17β-estradiol (E2) treatment alters chemokine responses in the lungs during IAV infection. Adult C57BL/6 female mice were ovariec-...
cells or the number of virus-specific CD8 T cells in the draining lymph node (data not shown) or the lungs (Fig. 5A and B) between E2- and placebo-treated females.

We next assessed whether cytokine production by influenza virus-specific CD8 T cells was affected by E2 treatment. There was no effect of E2 treatment on the median fluorescence intensity (MFI) for IFN-γ or TNF-α in CD8 T cells from either the lungs (Fig. 5C) or lymph nodes (data not shown) during IAV infection. In contrast, the proportions of IFN-γ- and TNF-α-producing CD8 T cells in the lungs, but not the lymph nodes (data not shown), following ex vivo influenza antigen stimulation were significantly increased in cells from E2-treated mice (Fig. 5D) (P < 0.05 in each case). The proportions of polyfunctional CD8 T cells that produced both IFN-γ and TNF-α following ex vivo antigen stimulation did not differ between placebo- and E2-treated females (Fig. 5D). In sum, E2 selectively increases the proportion of virus-specific CD8 T cells producing either IFN-γ or TNF-α during IAV infection rather than the quantity of cytokine production/cell, suggesting that the effects of E2 on cytokine production might be indirect rather than directly on CD8 T cell transcription of cytokines.

The protective effects of E2 during IAV infection are neutrophil dependent. Neutrophils present antigen via MHC class I and II complexes, express the costimulatory molecules CD80 and CD86, and activate T cells during infection (44, 45). To test the hypothesis that neutrophils are required for E2-mediated protection against IAV, neutrophils were depleted from both placebo- and E2-treated, IAV-infected mice by treatment with anti-Ly6g, and the efficacy of the depletion, virus titers, cytokine, and chemokine concentrations, and the recruitment and cytokine responses of CD8 T cells were analyzed. Following neutrophil depletion, there were significantly fewer neutrophils in the lungs and draining lymph nodes of both placebo- and E2-treated mice administered anti-Ly6g antibody compared with those treated with the isotype control (Fig. 6A and data not shown) (P < 0.005 in each case). Among the placebo-treated females, who already had significantly lower numbers of neutrophils in their lungs than E2-treated females during IAV infection (Fig. 6A) (P < 0.005), depletion of neutrophils did not significantly alter morbidity during IAV infection (Fig. 6B). E2-treated females depleted of neutrophils, however, exhibited a greater loss of body mass over the 7 days of infection compared with nondepleted E2-treated females (Fig. 6D) (P < 0.05). Depletion of neutrophils from either placebo- or E2-treated females did not impair their ability to control virus replication (Fig. 6D). Compared with nondepleted E2-treated females, depletion of neutrophils from E2-treated females reduced the induction of CCL3 and CXCL1 in the lungs during IAV infection (Fig. 6E and F) (P < 0.05 in each case). There was no effect of neutrophil depletion on the already low induction of CCL3 and CXCL1 in placebo-treated females during IAV infection (Fig. 6E and F).

Neutrophil depletion in E2-treated females did not alter the total number of CD8 T cells (Fig. 6G) or the number of influenza virus-specific CD8 T cells in the lungs (Fig. 6H) in the lungs. Depletion of neutrophils in placebo-treated females appeared to reduce total numbers of CD8 T cells (Fig. 6G) (P < 0.05), but the elevated numbers of CD8 T cells in the isotype control, placebo-treated females has not been observed consistently (Fig. 5A). Neutrophil depletion diminished the proportion of IFN-γ- but not TNF-α (data not shown)-producing virus-specific CD8 T cells in the lungs of E2-treated mice (Fig. 6I) (P < 0.05). In the draining lymph node, depletion of neutrophils in E2-treated females did not affect the expansion of CD8 T cells, numbers of virus-specific CD8 T cells, or production of cytokines by CD8 T cells (data not shown).

### Table 1

<table>
<thead>
<tr>
<th>Cell subset</th>
<th>Treatment</th>
<th>Mock treated</th>
<th>1</th>
<th>3</th>
<th>5</th>
<th>7</th>
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<tbody>
<tr>
<td>Alveolar macrophages (CD11b+ CD11c+)</td>
<td>Ovx</td>
<td>0.53 ± 0.27</td>
<td>0.337 ± 0.159</td>
<td>0.166 ± 0.034</td>
<td>0.371 ± 0.238</td>
<td>0.090 ± 0.059</td>
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<td>MHC-IIint Ly6g-</td>
<td>Ovx + E2</td>
<td>0.358 ± 0.231</td>
<td>0.410 ± 0.195</td>
<td>0.112 ± 0.015</td>
<td>0.070 ± 0.027</td>
<td>0.055 ± 0.024</td>
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<tr>
<td>cDCs (CD11b+ CD11c+ MHC-II+ Ly6g+)</td>
<td>Ovx</td>
<td>1.105 ± 0.269</td>
<td>1.118 ± 0.231</td>
<td>3.798 ± 0.432</td>
<td>10.109 ± 2.329</td>
<td>30.612 ± 8.028⁸</td>
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<tr>
<td>MHC-II+ Ly6g-</td>
<td>Ovx + E2</td>
<td>0.570 ± 0.171</td>
<td>1.371 ± 0.149</td>
<td>3.428 ± 0.437</td>
<td>6.269 ± 0.407</td>
<td>21.945 ± 3.333⁸</td>
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<td>Inflammatory monocytes (CD11b+ CD11c+ MHC-II+ Ly6g+)</td>
<td>Ovx</td>
<td>5.552 ± 1.105</td>
<td>2.516 ± 0.255</td>
<td>4.241 ± 0.449</td>
<td>14.817 ± 1.256⁸</td>
<td>15.815 ± 1.533⁸</td>
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<tr>
<td>MHC-II- Ly6g-</td>
<td>Ovx + E2</td>
<td>3.639 ± 0.639</td>
<td>2.865 ± 0.268</td>
<td>4.224 ± 0.326</td>
<td>7.737 ± 0.199⁸</td>
<td>16.378 ± 0.789⁸</td>
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<td>moDCs (CD11b+ CD11c+ MHC-II+ Ly6ghi)</td>
<td>Ovx</td>
<td>0.175 ± 0.077</td>
<td>0.137 ± 0.018</td>
<td>1.021 ± 0.178</td>
<td>3.120 ± 0.876</td>
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<td>MHC-II- Ly6g-</td>
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<td>0.175 ± 0.120</td>
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<td>NK cells (CD11b+ CD11c- NK1.1+)</td>
<td>Ovx</td>
<td>8.576 ± 1.527</td>
<td>3.689 ± 0.705</td>
<td>13.114 ± 0.617</td>
<td>53.061 ± 8.029⁸</td>
<td>154.513 ± 35.635⁸</td>
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<td>MHC-II- Ly6g-</td>
<td>Ovx + E2</td>
<td>6.827 ± 1.403</td>
<td>4.239 ± 0.528</td>
<td>16.519 ± 6.93</td>
<td>47.881 ± 86.38</td>
<td>150.155 ± 16.993⁸</td>
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<td>pDCs (CD11b+ CD11c+ MHC-II- Ly6g+)</td>
<td>Ovx</td>
<td>0.459 ± 0.138</td>
<td>0.516 ± 0.191</td>
<td>0.716 ± 0.089</td>
<td>2.747 ± 0.459</td>
<td>5.292 ± 1.529⁸</td>
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<td>MHC-II- Ly6g-</td>
<td>Ovx + E2</td>
<td>0.510 ± 0.292</td>
<td>0.485 ± 0.120</td>
<td>0.634 ± 0.014</td>
<td>1.921 ± 0.215</td>
<td>3.690 ± 1.269⁸</td>
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<td>tipDCs (CD11b+ Ly6c+hi TNF-α+ iNOS+)</td>
<td>Ovx</td>
<td>0.692 ± 0.257</td>
<td>1.079 ± 0.449</td>
<td>2.062 ± 1.089</td>
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<td>31.856 ± 4.164⁸</td>
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<tr>
<td>MHC-II- Ly6g-</td>
<td>Ovx + E2</td>
<td>0.636 ± 0.268</td>
<td>1.511 ± 0.622</td>
<td>3.533 ± 0.965</td>
<td>6.526 ± 0.977</td>
<td>41.065 ± 2.440⁸</td>
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a Ovx, placebo-treated ovariectomized; Ovx + E2, 17β-estradiol-treated ovariectomized.

b Data are means ± SEM. Data were analyzed with 2-way ANOVAs followed by Tukey’s tests, with significant differences between E2-treated and placebo-treated females at an individual time point represented by * (P < 0.05) and significant differences between samples from mock-infected and IAV-infected females represented by # (P < 0.05).
FIG 3 Treatment with 17β-estradiol (E2) increases the influx of neutrophils into the lungs during IAV infection. Adult C57BL/6 female mice were ovariectomized and treated with placebo (Ovx) or exogenous E2 (Ovx+E2) followed by intranasal inoculation with medium alone (mock) or influenza A virus (IAV). Neutrophil populations were defined as CD11b<sup>+</sup> CD11c<sup>-</sup> MHCIIC<sup>+</sup> Ly6g<sup>+</sup> (A and B). The total number of neutrophils was enumerated from whole lungs of placebo- and E2-treated females on several days p.i. (C). Bars represent means ± SEM. Significant differences between placebo- and E2-treated females, as determined by post hoc analyses, are represented by an asterisk (P < 0.05; n = 7 or 8/treatment).

DISCUSSION

Sex steroid hormones are significant regulators of the immunological balance between virus clearance and inflammatory disease during IAV infection. Collectively, data from this article and previous studies (11, 26) illustrate that adult female mice administered sustained concentrations of E2 or an ERα agonist show greater survival of IAV infection, reduced morbidity, reduced production of inflammatory proteins, increased production of chemokactrants for neutrophils, increased pulmonary infiltration of neutrophils, and increased percentages of cytokine-producing, virus-specific CD8 T cells compared with placebo-treated females. Depletion of neutrophils in E2-treated females reversed the protective effects of E2 on the outcome of IAV, indicating that neutrophils play a fundamental role in the protective effects of E2 against IAV infection. These data have direct relevance to our understanding of why the outcome of influenza differs between the sexes, during pregnancy, and after reproductive senescence (7, 46).

The causes of severe influenza are multifaceted and involve viral and host-mediated factors. While the initial recognition of IAV and responses of innate immune cells are necessary for activation of lymphocytes that control and eliminate virus, if these host inflammatory responses become excessive, then this can lead to the development of immunopathology. Neutrophils can both limit and contribute to inflammation during IAV infection in mice (38, 47). Neutrophils can phagocytize IAV-infected cells, control virus replication and inflammation, and activate IAV-specific CD8 T cells in the lungs (33, 42, 43, 48, 49). Depletion of neutrophils prior to IAV infection results in greater morbidity and mortality as well as a lower magnitude of IAV-specific CD8 T cells in the lungs of infected mice (33, 38, 42, 49). The data from the present study support and expand the role of neutrophils in influenza pathogenesis by demonstrating that E2 regulates neutrophil recruitment and activity during lethal IAV infection in females. Studies (33, 42), including the present study, utilizing neutrophil depletion to evaluate the role of these cells in IAV pathogenesis have limited analyses to the first week post-lethal infection. The role of E2 regulation of neutrophil recruitment into the lungs during the resolution phase of infection, especially in response to sublethal infection, requires further investigation. Whether E2 enhances the expression of genes encoding negative regulators of inflammatory responses in neutrophils requires consideration and might explain why E2-regulated recruitment of neutrophils is beneficial, rather than detrimental (47), during IAV infection. In addition to the hormonal milieu, there could be sex-specific effects of neutrophils during IAV infection, with the beneficial effects occurring in female (38), rather than male (47), mice.

Neutrophils constitute a majority of circulating leukocytes and play a crucial role in the pathogenesis of many diseases. Treatment of either humans or mice with E2 increases numbers of neutrophils in the blood and lungs, respectively (50, 51). Neutrophils possess both ERα and ERβ (52, 53). Functionally, in vitro exposure of neutrophils to E2 (i) increases degranulation, elastase release, and production of reactive oxygen species, (ii) inhibits caspase 3/9 and delays apoptosis, and (iii) increases cell adhesion molecules and interactions with endothelial cells (54–56). Neutrophils produce and release cytokines and chemokines (e.g., CXCL1 and CXCL8), present antigen, and express surface receptors necessary for recruitment and activation of monocytes, DCs, and T cells, illustrating a central role for neutrophils in regulating innate and adaptive immune responses (57–59). Data from the present study demonstrate that in vivo treatment with E2 increases pulmonary concentrations of neutrophil chemoattractants, including CCL3 and CXCL1, and recruitment of neutrophils to the lungs during IAV infection. Whether E2 directly mediates the ef-
factor functions of neutrophils during IAV infection requires further consideration.

During IAV infection, CD8 T cells, which are critical for the control of IAV replication, kill infected cells and produce inflammatory cytokines (40, 60–63). Previous studies illustrate that treatment of female mice with supraphysiological (i.e., pregnancy) levels of E2 suppresses the number of IAV-specific CD8 T cells but does not affect IFN-γ production by IAV-specific CD8 T cells in the lungs (26). In the present study, treatment of females with physiological amounts of E2 did not alter the recruitment of CD8 T cells into the lungs but increased the percentages of IFN-γ-producing, virus-specific CD8 T cells. The production of IFN-γ by individual cells (i.e., MFI) in the lungs was not affected by E2. Only the proportion of cytokine-producing cells in the lungs was increased by the presence of E2. Although CD8 T cells possess both ERα and ERβ (21), the data from the present study suggest that E2 may not have a direct effect on CD8 T cell transcription of cytokines in response to IAV. Because neutrophil depletion significantly reduced the proportion of IFN-γ-producing CD8 T cells in the lungs, it is more likely that the effects of E2 on neutrophils result in differential responses (e.g., cytokine production) of IAV-specific CD8 T cells in the lungs during infection.

Influenza A viruses productively infect respiratory epithelial cells. While host cellular responses, including production of antiviral and inflammatory factors, have evolved to restrict IAV replication, IAVs have coevolved mechanisms (e.g., NS1) to inhibit cellular antiviral and inflammatory responses and promote virus replication (64). If treatment with E2 increased resistance against IAV, then E2-treated females would have lower virus titers than placebo-treated females. We have consistently observed that the kinetics of IAV replication in the lungs of E2-treated and placebo-treated females are similar (11). Furthermore, neutrophil depletion after IAV infection did not impair the ability of E2-treated females to control virus replication. We interpret these data to indicate that E2-treated females are no more resistant to IAV infection than placebo-treated female mice. If E2 is not functioning to eliminate IAV from the lungs, then this hormone might serve to limit the damage caused by infection. Reduction of the cytotoxicity directly caused by a pathogen or the immune-mediated damage caused by the host responses to a pathogen, without necessarily restricting virus replication, is referred to as “disease tolerance” (34). In the present study, treatment with E2 enabled females to maintain their overall health, as measured by changes in body mass, despite having virus titers that were equivalent to those of placebo-treated females. By downregulating the inflammatory responses that cause tissue damage (e.g., CCL2) and augmenting responses (e.g., CXCL1) associated with recruitment of cells (e.g.,

![FIG 4](Exogenous 17β-estradiol (E2) treatment does not affect CD4 T cell populations in the lungs. Adult C57BL/6 female mice were ovariectomized and treated with placebo (Ovx) or exogenous E2 (Ovx+E2) followed by intranasal inoculation with influenza A virus (IAV). At day 7 p.i., the total number of CD4 T cells (A), the proportion of cytokine-expressing CD4 T cells in response to influenza virus antigen (D₃₁H₂₁₃–₂₅₃) stimulation ex vivo (B), and the total number of FoxP3-expressing CD4 T cells (C) were analyzed in the lungs. Bars represent means ± SEM (n = 8/treatment).

![FIG 5](Exogenous 17β-estradiol (E2) treatment increases cytokine production from influenza virus-specific CD8 T cells in the lungs. Adult C57BL/6 female mice were ovariectomized and treated with placebo (Ovx) or exogenous E2 (Ovx+E2) followed by intranasal inoculation with influenza A virus (IAV). At day 7 p.i., the total number of CD8 T cells (A), the number of D₄₉NP₃₆₆–₃₇₄-specific CD8 T cells (B), the median fluorescence intensity (MFI) for cytokine production (C), and the proportion of cytokine-expressing CD8 T cells in response to influenza virus antigen (D₄₉NP₃₆₆–₃₇₄) stimulation ex vivo (D) were analyzed in the lungs. Bars represent means ± SEM. Significant differences between ovariectomized placebo and E2-treated females are represented by asterisks (P < 0.05; n = 8/treatment).
neutrophils and CD8 T cells) that are necessary for the control of IAV, we propose that E2 protects females from severe influenza by limiting the negative impact of infection on host fitness.

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