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Estradiol Resolves Pneumonia via ER-β in Regulatory T cells

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Abstract

Current treatments for pneumonia (PNA) are focused on the pathogens. Mortality from PNA-induced acute lung injury (PNA-ALI) remains high, underscoring the need for additional therapeutic targets. Clinical and experimental evidence exists for potential sex differences in PNA survival, with males having higher mortality. In a model of severe pneumococcal PNA, when compared to males, age-matched female mice exhibited enhanced resolution characterized with decreased alveolar and lung inflammation and increased numbers of Regulatory T cells (Tregs). Recognizing the critical role of Tregs in lung injury resolution, we evaluated if improved outcomes in females were due to estradiol (E2) effects on Treg biology. E2 promoted Treg suppressive phenotype in vitro and resolution of PNA in vivo. Systemic rescue administration of E2 promoted resolution of PNA in males independent of lung bacterial clearance. E2 augmented Treg expression of Foxp3, CD25 and GATA3, an effect that required ERβ, and not ERα signaling. Importantly, the in vivo therapeutic effects of E2 were lost in Treg depleted mice (Foxp3DTR). Adoptive transfer of ex vivo E2-treated Tregs rescued S. pneumoniae-induce PNA-ALI, a salutary effect that required Treg ERβ expression. E2-ERβ was required for Tregs to control macrophage pro-inflammatory responses. Our findings support the therapeutic role for E2 in promoting resolution of lung inflammation after PNA via ERβ Tregs.
Introduction

Pneumonia (PNA) is one of the leading causes of death worldwide, resulting in an estimated 2.74 million deaths (1). PNA can result in devastating acute inflammatory injury in the lung manifesting in acute lung injury (ALI) and clinically known as acute respiratory distress syndrome (ARDS). PNA can also worsen underlying co-morbid conditions and is associated with increased mortality months to years after the initial inciting event (2). Current treatments for ALI/ARDS caused by PNA are focused primarily on pathogen killing with antibiotics, but do not target excessive lung inflammation elicited by the host immune response (3). Given the morbidity and mortality of PNA even in the era of broadly available antibiosis and vaccinations, there is an urgent need for additional biological insight into the host responses to inflammation caused by incident PNA. Unfortunately, in part due to lack of mechanistic clarity, we currently have little understanding of how to modulate host factors in order to improve PNA outcomes.

Clinical and experimental evidence exists for sex differences in PNA outcomes, with males having higher severity(4) and mortality (4-6). Both humoral and cellular immune responses are known to be more active in females than in males (7, 8). The burden of infectious diseases is generally higher in men than in women (9-11). As a result, responses to vaccines and clearance of pathogens are enhanced in females compared to males, although this could lead to exuberant inflammatory responses in the female host (8, 12). Sex differences in immune responses are complex and can be explained by differential chromosomal organization, sex steroid levels, hormonal receptor distribution and expression in different cells, relative difference in immune cells composition and differential expression of pattern recognition receptors (PRRs) environmental exposures which can influence microbiome composition (8).
In these studies, we focused on estradiol (E2) which plays a significant role in regulating immunologic sex differences (13, 14). Estradiol (E2) has been implicated in the regulation of numerous inflammatory disorders (15), such as autoimmune encephalomyelitis (16) and severe acute respiratory syndrome coronavirus infection (17). E2 is protective in experimental systemic LPS and intestinal ischemic-induced ALI (18-20) when administered before the onset of the inflammatory insult. For experimental PNA, the role of E2 remains disparate. Protective effects have been described in a mouse pneumococcal pneumonia model (21, 22) while a pro-inflammatory role was observed in a pseudomonas aeruginosa pneumonia model (23). Studies of the protective effects of E2 have mostly focused on early phases of lung injury; to our knowledge, a potential role for estrogen in the resolution of PNA not been investigated.

Regulatory T cells (Tregs) comprise a small yet potent subpopulation of CD4+ lymphocytes (5-10%) in humans and mice (24, 25). Tregs maintain immunological self-tolerance and homeostasis by suppressing aberrant or excessive immune responses (26, 27). They express high levels of CD25 (the IL-2R α-subunit) and the forkhead box protein 3 (Foxp3) master transcription factor (28). We previously showed that CD4+CD25+Foxp3+ Tregs resolve experimental ALI by modulating the following critical pro-repair steps: 1) abrogation of macrophage pro-inflammatory responses, 2) augmentation of neutrophil efferocytosis (29), 3) limitation of fibroproliferation (30), and 4) augmentation of alveolar epithelial repair (31). In summary, Tregs are pivotal pro-repair cells after ALI (32, 33). Moreover, in a previous study we identified Tregs in the alveolar spaces of ALI patients (29, 34) and a recent study found increased Treg numbers in ALI survivors compared to non-survivors (35). Exogenous E2 has been shown to expand Tregs in mice (36). Understanding the links between E2 augmentation of Treg suppressive function could lead to development of Treg based therapeutics. (37, 38).

To investigate the role of E2 in the resolution phase of PNA, we established a model of resolution following pneumococcal-induced lung injury using intratracheal (i.t.) S. pneumoniae (PNA-induced ALI, PNA-ALI). We found that males and females have comparable early lung inflammatory responses to
S. pneumoniae. Despite similar bacterial burdens with the lung, male mice had sustained lung injury 6 days after initial infection and prolonged elevation of bronchoalveolar lavage (BAL) inflammatory cytokine expression observed including INF-γ, TNF-α, IL-6 and IL-12p70. Further, Treg numbers in both the BAL and lung were increased in female mice in the resolution phase of PNA. Exogenous systemic administration of E2 given as rescue treatment 48 hours after lung infection promoted resolution of PNA-ALI in male mice with decreased lung inflammation, decreased BAL inflammatory cytokines, and increased number of lung Tregs. This was also independent of effects on lung bacterial burden. The salutary effects of exogenous E2 were lost in Treg-depleted animals. Isolated Tregs stimulated in vitro with E2 showed an enhanced suppressive phenotype characterized by upregulation of their master transcription factor Foxp3, GATA3, surface expression of IL-2Rα (CD25) and the glucocorticoid-induced tumor necrosis factor receptor (GITR) expression. CD4+CD25- T conventional cell did not upregulate any of these markers in response to E2. ERα−/− but not ERβ−/− Tregs responded similarly to E2 as WT Tregs suggesting ERβ requirements in Treg estrogenic stimulation. To determine the functional necessity for the ERβ receptor on Treg, lymphocyte-deficient mice were treated with subtherapeutic doses of Tregs after S. pneumoniae injury. Animals were randomized to E2-stimulated Tregs derived from WT or ERβ−/−. Beneficial effects of E2-treated Treg were dependent upon ERβ−/− expression. In vitro co-culture studies demonstrate that the ability of Tregs to modify macrophage anti-inflammatory IL-10 production is augmented in E2-Treated Treg and the Treg ERβ contributes to suppression of macrophage generated pro-inflammatory cytokines. Our results provide support of estrogenic mechanisms involved in PNA-ALI resolution; identifying new targets regulating Treg function and therapeutics that enhance Treg pro-repair function.

**Results**

**Female sex displayed enhanced resolution of pneumonia**
Given prior reports showing increased Foxp3 expression and suppressive function effect by E2 on Tregs (36), a cell type we previously showed to be important for ALI resolution, we hypothesized that resolution of pneumonia would be enhanced in female mice in vivo. Age-matched WT male and female mice were challenged with S. pneumoniae. Although males displayed higher total BAL and lung cell counts 2 days after PNA, their early lung inflammatory response was similar than females as measured by similar increases in BAL protein and BAL neutrophil counts (Fig. 1B, 1C). However, while lung inflammation was largely cleared by 6 days after PNA-ALI in female mice, male mice experienced sustained weight loss (Fig. 1A), higher number of BAL total cells (Fig. 1C) with predominance of neutrophils (Fig. 1D). Moreover, the proportion of alveolar cells showed higher % neutrophils and lower % of macrophages in the male host by day 6 after PNA (Supplementary Fig. E1). The lung compartment profile of sustained inflammatory cells was similar to the alveolar compartment (Fig. 1G, H). The number of alveolar macrophages and lymphocytes was similar at all intervals after injury in males compared to females (Fig. 1E-F). Analysis of sex differences in BAL cytokines at over time during PNA demonstrated similar BAL pro-inflammatory cytokine profiles in both sexes at day 2 of PNA. In contrast, by day 6, female mice had significantly lower BAL INF-γ, IL-12, IL-6 and TNF-α than males (Supplementary Fig. E2). These data suggest that female mice, in contrast to males, displayed enhanced resolution of similar lung inflammation after S. pneumoniae.

**Alveolar and Lung Tregs increased in resolving females**

We next examined baseline Treg numbers and function in male and female mice in alveolar and lung compartments. At baseline, no differences in BAL and lung cell counts were observed. Lung Treg numbers were similar in both sexes at baseline (Supplementary Fig. E3). Baseline expression of Treg glucocorticoid-induced TNFR-related protein (GITR) in the lung of female mice were higher than that of male mice, while both sexes had similar Treg expression for Foxp3, CD25 and GATA3
(Supplementary Fig. E3). During resolution, *S. pneumoniae*-injured female mice displayed a higher absolute fold increase in the number of alveolar (Fig. 2A) and lung Tregs (Fig. 2B) compared to their male counterparts. The proportion of Tregs in alveolar and lung compartments was significantly increased compared to males at day 6 (Fig. 2C, 2D). Notably, Foxp3 (Fig. 2E) and Ki-67 (Fig. 2F) expression was higher in females than males during resolution, indicating enhanced proliferative state. Treg GATA3 expression was similar in males and females (Fig. 2G). Consistent with enhanced lung injury resolution, females demonstrated increased Treg numbers both in the lung and alveolar compartment. Further, markers of suppressive phenotype were enhanced in female Tregs relative to males.

**Exogenous Estrogen enhanced Regulatory T cell (Tregs) suppressive phenotype**

To determine the effect of exogenous E2 on Tregs, we cultured the CD4+CD25+ (Tregs, >85% Foxp3+) cells with anti-CD3/CD28 beads, and stimulated them with either E2 or vehicle for 72 hrs, prior to measuring markers associated with Treg suppressive phenotype. E2 treatment increased expression of the Treg master transcription factor Foxp3 expression (Fig. 3A). Similarly, E2 increased CD25 (IL-2R alpha) expression in Treg cells (Fig. 3B). Moreover, expression of GATA-3 and GITR proteins was increased in E2-stimulated Treg cells (Fig. 3C-D). GATA-3 is a transcription factor known for its role on the migration of Treg cells to inflamed sites, while GITR enhances proliferation of functionally competent Treg cells. Other Treg markers known to play important roles in Treg biology but not altered by E2 stimulation included Ki-67, CD62L, CD69, CD39, PD-1, CTLA-4, CD44 and CD40L (Supplementary Fig. E4). In order to determine if the effects of E2 are specific for Tregs, we evaluated the effect of E2 treatment on cultured conventional CD4+ T cells (CD4+CD25--; < 1% Foxp3+). In contrast to Tregs, E2 had no effects on Foxp3, GATA3 (Supplementary Fig. E5), CD25 or GITR expression (not shown). It is worth noting that the effect by E2 on Treg cells were independent of the presence of exogenous IL-
These results showed that exogenous E2 robustly enhanced Treg suppressive phenotype \textit{in vitro}.

**Therapeutic estradiol accelerated resolution of lung injury in males**

We hypothesized that exogenous E2 could promote the resolution of acute lung injury in males given the favorable phenotype observed in females (Fig. 1) and the enhanced Treg phenotype seen \textit{in vitro} (Fig. 3). To avoid potentially blunting the initial inflammatory response to \textit{S. pneumoniae}, we started rescue treatment with E2 at day 2 after lung injury. Male mice treated with vehicle group had sustained weight loss, whereas E2-treated male mice regained weight (Fig. 4A). At day 6 after lung injury, E2-treated male mice, but not vehicle-treated mice, displayed a resolving phenotype similar with female mice, with reduced BAL protein (Fig. 4B), decreased BAL neutrophils counts (Fig. 4D) and increased BAL Treg numbers (Fig. 4E). Total BAL cell count was not statistically different following E2 therapy (Fig. 4C), while lung cell counts were diminished (Fig. 4F). Lung profile parallel alveolar compartment with decreased lung inflammatory cells, inflammatory cytokines (Supplemental Fig. E7) and increased lung Treg (Figs. 4 G-H). Similar to female mice, the proportion of Tregs in alveolar and lung compartments of E2-treated mice was increased (Supplementary Fig. E6). Moreover, E2-treated males displayed higher Ki-67 expression in their Tregs (Supplementary Fig. E6), indicating a higher proliferative state. Analysis of BAL cytokines demonstrated that systemic exogenous E2 in males reduced BAL pro-inflammatory cytokines including \textit{INF-\gamma}, IL-12, IL-6, TNF-\alpha and IL-1\beta without influencing KC, IL-10 or IL-4 levels (Supplementary Fig. E7). Representative lung H-E sections showed clearance of lung inflammation in the male E2 treated group (Fig. 4I). In summary, rescue therapeutic administration of E2 promoted resolution of acute lung injury in males associated with decreased inflammatory cytokine production and increased number and proliferation of lung Treg cells.
Rescue Estradiol did not impact lung bacterial clearance

A potential explanation for improved resolution as a function of sex or mediated by exogenous estrogen is enhanced lung bacterial clearance. In order to investigate both sex differences and whether exogenous E2 had an effect on *S. pneumoniae* clearance during resolution. We injected exogenous E2 or vehicle on day 2, 3 and 4 after lung injury. On day 6 after injury, lungs were harvested and homogenized for determination of colony forming units (CFU) for *S. pneumoniae*. Male and female mice had no demonstrable difference in bacterial loads and E2 treatment of male mice further showed no difference in bacterial load (Fig. 5A). Moreover, to determine whether E2 exhibited direct bactericidal activity, we cultured *S. pneumoniae* in the presence of increasing concentrations of E2 (1-1000 µM) or vehicle. After culturing for 24 hours, CFU were counted. We observed no difference of CFU between E2 and vehicle-treated *S. pneumoniae*, suggesting a lack of direct bactericidal activity by E2 (Fig. 5B). These studies suggest that sex differences in PNA outcomes and the E2 therapeutic effects were unlikely to be due to modulation of lung bacterial burden.

Tregs are required for E2 enhanced resolution

In order to determine if the salutary effects of E2 required Tregs *in vivo*, we used and treated Foxp3DTR mice with exogenous diphtheria, efficiently depleting Tregs. Male Foxp3DTR mice and age-matched counterparts WT mice received diphtheria toxin beginning 2 days before *S. pneumoniae* injury and every other day thereafter. E2 was given intraperitoneal daily starting on days 2, 3 and 4 (Fig. 6A). We confirmed lung Treg depletion in diphtheria toxin-treated Foxp3DTR mice compared with WT mice 5 days after *S. pneumoniae* injury (Supplementary Fig. E8). In contrast to the beneficial effects of E2 in injured WT mice, E2 treatment in Treg-depleted Foxp3DTR mice did not accelerate lung injury resolution as measured by persistent elevated BAL total cell counts (Fig. 6C), BAL neutrophils (Figure 6D), lung neutrophils (Fig. 6F), and histological changes (Fig. 6G). Tregs measured in the BAL were significantly
higher in Treg sufficient WT mice treated with E2 (Fig. 6E). Lung H-E sections showed persistent injury in the Treg-depleted hosts, despite E2 therapy. In contrast, WT Treg sufficient animals that received E2 displayed enhanced resolution (Fig. 6G). Interestingly, compared to vehicle-treated Foxp3DTR mice, E2-treated Foxp3DTR mice showed lower BAL protein (Fig. 6B), suggesting potential additional Treg-independent mechanisms in E2-mediated lung inflammation recovery. These data confirmed that E2-enhanced resolution of lung inflammation required Tregs.

**E2 enhanced Treg suppressive phenotype required ERβ**

The predominant effects of E2 are mediated through two distinct estrogen receptors (ER), ER alpha (ERα) and ER beta (ERβ). ERα and ERβ signaling can display redundant effects in vitro; however, distinct expression patterns in cells and tissues have shown diverse biological effects in vivo. In order to evaluate the receptor requirement for Treg suppressive phenotype, Tregs isolated from WT, ERα−/− and ERβ−/− mice were cultured with anti-CD3/CD28 beads with or without E2 in vitro. Similar to the in vivo findings, E2 induced Foxp3 expression in both WT and ERα−/− Treg. In contrast, E2 did not induce Foxp3 expression in ERβ−/− Tregs (Fig. 7A). ERα−/− Tregs responded to E2 similar to WT with increased Foxp3, GATA3, CD25 and GITR while ERβ−/− Tregs were unresponsive. This hyporesponsive phenotype was not generalized as ERβ−/− Tregs responded to exogenous IL-2 in vitro (Supplementary Fig. E10). In summary, ERβ was required for E2 to exert its effect on Tregs in vitro.

**E2 pro-repair function required ERβ to resolve pneumonia**

To address the contribution of ERβ to E2-mediated effects on Treg biology, we evaluated ex vivo E2-treated Tregs in S. pneumoniae-induced ALI. Previously, we demonstrated that the therapeutic adoptive transfer of Tregs requires 1 X10⁶ cells/mouse and that transferring lower numbers would be
insufficient to mediate robust resolution\textsuperscript{34}. \textit{S. pneumoniae}-injured lymphocyte-deficient \textit{Rag-1}\textsuperscript{-/-} mice received 0.25 X 10\textsuperscript{6} WT vehicle treated Tregs or E2-treated WT or \textit{ER\beta}-/- Tregs via retro-orbital injection 1 hour after i.t. \textit{S. pneumoniae}. Five days after injury, mice that received a subtherapeutic dose of E2-treated \textit{ER\beta}-/- Tregs, displayed unremitting lung inflammation characterized by elevated BAL protein (Fig. 8A), high BAL total cell counts (Fig. 8B), high BAL neutrophil counts (Fig. 8C and Supplementary Fig. E11) and total lung neutrophil counts (Fig. 8E and Supplementary Fig. E11) as well as total lung cell counts (Fig. 8D). Thus, a non-resolving lung inflammatory phenotype was observed in \textit{Rag-1}\textsuperscript{-/-} mice that received E2-treated \textit{ER\beta}-/- Tregs. In contrast, lung injury resolution occurred in mice that received E2-treated WT Tregs (Fig. 8A-8F). Flow cytometry confirmed successful adoptive transfer and homing to the alveoli and lungs independent of ER\textbeta expression (Fig. 8F-8G and Supplemental Fig. E10).

Interestingly, the absolute numbers of recovered adoptively transfer Tregs did not differ among groups (Supplementary Fig. E10), although E2-treated WT Tregs displayed higher % BAL Treg and Foxp3 expression (Fig. 8G-8H). In contrast, transfer of E2-treated \textit{ER\beta}-/- Tregs resulted in higher BAL and total lung cellularity, BAL neutrophil counts, loss of E2-mediated increases in the proportion of BAL Tregs. Analysis of Treg phenotypic markers indicates that \textit{ER\beta}-/- Tregs treated with E2 had similar expression of proliferative markers Ki67 but diminished expression of CD39 (Supplemental Fig. E12.) This, coupled with diminished expression of Foxp3 and insufficient rescue in \textit{Rag-1}\textsuperscript{-/-} mice indicate that \textit{ER\beta}-/- Tregs have reduced suppressive phenotype and function. In summary, ex vivo treatment of an otherwise ineffective Treg dose facilitated lung injury resolution and Treg expression of ER\textbeta was necessary for Treg-mediated resolution of \textit{S. pneumoniae}-lung injury.

**Tregs modulate macrophages responses via E2-ER\beta**

To begin to understand the cellular effector targets mediating the salutary effects of E2-ER\textbeta in Treg, we co-culture macrophages with Tregs. Tregs were isolated from WT or \textit{ER\beta}-/- spleens and cultured
for 48 hours in the presence or absence of E2 (10^{-5} M). Pre-treated Tregs were then transferred into
direct contact with LPS (100 ng/ml) stimulated bone–marrow derived macrophages (BMDM) at a ratio
of 1:2 (lymphocyte: macrophage). After 24 hours in co-cultures, cells were harvested for intracellular
cytokine staining for macrophage–derived cytokines. Non-primed WT Tregs abrogated macrophage
TNF-\( \alpha \) while increasing anti-inflammatory IL-10 production (Fig. 9A-9C). E2 primed WT Tregs
surprisingly did not abrogate macrophage TNF-\( \alpha \) responses, but rather further increased their IL-10
production compared to non-primed WT Tregs (Fig. 9A). In contrast, E2 primed ER\( \beta^-/- \) Tregs
exacerbated macrophage pro-inflammatory responses with higher BMDM IL-6 and TNF-\( \alpha \) production
while IL-10 production was still enhanced compared to co-culture with non-primed WT Tregs (Fig. 9A-
C). These findings support an E2-ER\( \beta^- \) role for Tregs to modulate macrophages inflammatory
responses.

Discussion

We reported therapeutic efficacy for exogenous E2 in promoting resolution of experimental pneumonia-
induced ALI. Our findings supported that E2-mediated beneficial effects were dependent on
CD4+CD25+Foxp3+ (Tregs) cells. E2 enhanced Treg pro-repair phenotype and function to mediate
and accelerate resolution of lung inflammation induced by pneumonia. The E2 modulation was
dependent on Tregs and expression of estrogen receptor \( \beta \) (ER\( \beta^- \)), while independent on antibacterial
properties.

Although there is no ideal model that recapitulates the complex underlying mechanisms of human acute
lung injury (39), we chose a direct model of pneumonia with *Streptococcus pneumoniae*. *S.
pneumoniae* is one of the leading causes of pneumonia worldwide (40), it produces a robust initial lung
inflammatory response that is reproducible and a resolution phase that can be evaluated over time. We
treated animals with E2 starting at day 2 after initial injury for several reasons. First, we focused on the
resolution phase, a distinct stage with active immunological mechanisms (41, 42) that could offer new
therapeutic targets. Second, pre-treatment or early delivery of E2 could blunt the peak inflammatory injury and thus hasten resolution of inflammation. Lastly and most importantly, patients often present days after their onset of pneumonia, and thus, assessing delayed treatment (i.e., rescue) effects provides a more clinically relevant therapeutic model. Systemic fluids and antibiotics, cornerstone treatments for pneumonia, were not used in our studies to avoid confounding variables, although they could be employed in future studies using more severe models of lung infections, conditions which could necessitate multiple interventions strategies.

The burden of infectious diseases is generally higher in men than in women (6, 10). Pre-clinical models of lung inflammation have demonstrated a protective effect of females over males (17, 43-46), however there has been a lack of cellular/molecular mechanisms that provides an explanation for the female salutary effects. Our studies support the role of sex as a major determinant in resolution of pneumococcal PNA. Compared to their male counterparts, female mice exhibited enhanced resolution of severe pneumococcal PNA with decreased lung inflammation and enhanced clearance of alveolar and lung neutrophils. This was independent of effects on bacterial clearance. In contrast to reports using pretreatment with E2 (18, 20), resolution can be accelerated by E2 administered 2 days after established lung injury. The therapeutic treatment of males with E2 had no effect on lung bacterial load clearance, indicating E2 was not bactericidal and did not affect bacterial burden, but rather targets enhanced pro-repair mechanisms.

Therapeutic effects of E2 have reported in models of carrageenan-induced lung injury (47) and models of sepsis induced by cecal ligation and puncture (48). These studies employed either preventive or early treatment strategies, limiting their clinical translation. We chose rescue therapeutic administration of E2 in order to minimize the potential impact on early beneficial inflammatory responses in the lung early in bacterial lung infection.
E2 also modulates macrophage responses and reprogram them to an alternative activated and anti-inflammatory states (49-51). Most bacterial infections induce classically activated macrophage, which are important for initially clearance of infections and subsequent skewing to a pro-repair state that promotes healing. Our experiments using Treg-depleted animals suggest that E2-mediated pro-repair effects were independent on direct E2 effects on macrophages but support a model in which E2 treated Treg modify macrophage responses. In the normal host, E2 could contribute via modulation of macrophages to an alternative activated state and thus promote Treg numbers and their suppressive phenotype (52, 53). We also observed decreased BAL protein in Treg-depleted animals that received E2. This effect suggest non-immune cells such as endothelial cells could also be relevant targets of E2 in vivo. E2 regulates vascular inflammation with anti-inflammatory effects through direct antioxidant effect, generation of nitric oxide, reduction of endothelial cell apoptosis and suppression of cytokines (54). Although E2 administration has potent effects in multiple cells types, our studies support a requirement for Tregs to mediate E2 salutary effects in resolution of pneumococcal-induced ALI.

Resolution of lung injury is an active process. Tregs maintain immunological self-tolerance and homeostasis by suppressing aberrant or excessive immune responses harmful to the host (27). We showed that CD4+CD25+Foxp3+ Tregs resolve experimental ALI by modulating critical pro-repair steps: 1) abrogation of macrophage pro-inflammatory responses, 2) augmentation of neutrophil efferocytosis (29), 3) limitation of fibroproliferation (30), and 4) augmentation of alveolar epithelial repair (31). In this report, we determined the feasibility of using E2 to promote Treg function in vivo and ex vivo to improve PNA-ALI outcomes.

We have observed that females had higher BAL and lung Treg numbers with higher Foxp3 and Ki-67 expression (marker of proliferation) after injury, indicating that sex hormones could enhance the suppressive function and proliferative rate of Tregs during resolution. Although there are several sex hormonal differences in females compared to males, we focused on E2 given reports of its effect on Treg cells (36, 55-57). Arruvito described a positive correlation of E2 levels with Treg numbers in
women (58). Polanczyk demonstrated that E2 treatment of isolated CD4+ splenocytes increased their CD25 protein expression and induced FOXP3 mRNA (55). They showed enhanced suppressive activity of Tregs isolated from E2-treated mice when co-culture with T effector cells.

In order to evaluate the E2 effects on Treg suppressive phenotype, we performed an extensive survey of Treg proteins using multicolor flow cytometry. Our immunophenotyping evaluation of Tregs suggested several mechanisms involved in E2-enhanced lung repair. First, E2 augmented the expression of the Treg master transcription factor, Foxp3. Enhanced Foxp3 correlates with higher immunoregulatory and suppressive function (59). Interestingly, E2 induced Foxp3 expression in Tregs but not in CD4+ CD25- cells. This is in contrast to a previous report showing E2 promoted the conversion of CD4+CD25- T cells to CD4+CD25+ T cells. Tai showed a subtle increase in Foxp3 expression in CD4+CD25- T cells treated with E2, from 1% of to 3% in total CD4+ cells in a representative sample (57). Second, we also found another critical Treg transcription factor, GATA3, regulated by E2. GATA3 is essential for the homeostasis and stability of Tregs. GATA3 is required to maintain high levels of Foxp3 and CD25 expression in inflammatory sites (60, 61). We observed different effects of E2 on Treg GATA3 expression, with increased expression in vitro, but unchanged in vivo. The expression of GATA3 in Tregs can be negatively regulated by a number of inflammatory cytokines including IL-6, IL-27, and IL-12. These inflammatory cytokines may contribute to the observed decreased GATA3 expression observed in the inflamed hosts (62-65), while in vitro Treg stimulation with E2 significantly increased GATA3 expression. To our knowledge, the modulation of E2 on Treg GATA3 expression has not previously been reported. Third, glucocorticoid-induced tumor necrosis factor receptor-related protein (GITR) was induced by E2. GITR is expressed at high levels in activated T cells and Tregs. Although GITR is not essential for Treg suppressive function in vitro (66); GITR has an important role in Treg expansion (67), with lower number of Tregs seen in GITR-knockout mice (68, 69). GITR activation on Tregs can exert distinct roles based on which signaling pathways are activated. The role of Treg GITR is also dependent on the experimental context (homeostatic vs inflammatory).
For instance, activation of the NF-κB pathway may result in Treg expansion (67), while Siva protein activation may result in their apoptosis (70). The specific role of E2 induction of Treg GITR expression will need to be further investigated in context of lung inflammation.

In summary, E2 increased several critical components of Treg function, and the synergistic effect of these upregulation in Treg markers likely led to an enhanced Treg function. Collectively, both quantitative factors (augmentation of Treg suppressive function) and qualitative factors (increased Treg proliferation and numbers) may underlie the pro-resolution effect of E2. E2 functions primarily through two intracellular estrogen receptors (ERα and ERβ) (71). These receptors are ubiquitously expressed (72) with a wide range of estrogen receptors (ER) expression across tissues, remarkably with few sex differences (73). Other reports indicate that ERα is necessary for E2-mediated upregulation of intracellular PD-1 expression in Treg contributing to its suppressive function (74). In contrast, we found that ERα was not required for therapeutic E2-mediated augmentation of Treg suppressive phenotype and function. In contrast, Treg expression of ERβ was necessary for E2-mediated resolution of pneumococcal ALI and phenotypic changes of Tregs in vitro.

The contribution of ERα and ERβ on E2-mediated effect may vary based on the source of the Tregs. E2-mediated effect on induced Tregs (iTregs) have been reported. These studies showed that specific depletion of ERα on total CD4+ cells attenuated the E2 response of iTreg (52). We focused on thymic-derived Tregs (tTregs), and not iTregs. Although we cannot exclude a relative contribution of iTregs, our adoptive transfer experiments used exclusively Tregs. ERα−/− Tregs stimulated with E2 upregulated Foxp3, GATA-3, CD25 and GITR expression in a similar manner to WT. In contrast, ERβ−/− Tregs failed to upregulate this “estrogenic signature”. This was not a sign of global hyporesponsiveness in vitro. ERβ−/− Tregs treated with IL-2 showed a robust increase in Foxp3 and CD25 expression confirming that the hyporesponsive state was specific for E2. Individuals with multiple sclerosis have lower expression
ERβ in Tregs compared to controls (75) underscoring the potential importance of ERβ expression in Tregs in humans.

Regulatory T cells orchestrate resolution of lung inflammation and promote lung repair through cellular interactions with macrophages (29), alveolar epithelial cells (31) and likely endothelial cells. Our findings suggest that E2-treated Tregs enhance the production of macrophage IL-10 while had no additive effects in downregulating macrophage pro-inflammatory responses. These findings were ER-β independent. However, although E2-treated ER-β/- Tregs were able to augment macrophage IL-10 production, their effect on macrophage pro-inflammatory responses was significantly augmented, elucidating the enhanced alveolar inflammatory milieu observed in our adoptive transfer experiments with E2-treated ER-β/- Tregs (Fig. 8). E2-ERβ signaling in Tregs could be responsible to Treg cell lineage commitment and maintenance of Foxp3 expression (76) and a lack of it could render these cells into ex-Foxp3 Treg cells with a promiscuous and pro-inflammatory effect (e.g. Th1 or Th17). Treg lineage tracing experiments will be needed to evaluate this hypothesis. We did not observe Tregs enhancing macrophage TGF-β in our co-culture experiments, a finding we had previously described (29). These co-culture experiments were different from previous, as Tregs were cultured for 48 hours and maximally stimulated before their co-culture with stimulated macrophages. E2-ERβ signaling in Tregs and their pro-repair effects on other immune and non-immune injured cells will need the focus of future studies. Moreover, the transcriptional and pro-resolution signatures induced by E2-ERβ signaling in Tregs will yield valuable information and provide other targets for resolution of pneumonia.

The present study has limitations and raises several questions. What other cell types are modulated in response to E2 in the setting of lung injury resolution? A recent investigation showed that E2 inhibited the LPS-induced IL-6 inflammatory response, resulting in inhibition of NF-kB transcriptional activity via GPR30/GPER1 in monocytes (77). Yang reported that estrogen-mediated activation of lung
macrophage nitric oxide synthase-3 (NOS3) was involved in female resistance to pneumonia (22). Our studies do not directly evaluate if physiological levels of estradiol were sufficient to mediate its pro-repair effects. E2 can display different effects on human monocytes/macrophages, with low doses enhancing the production of pro-inflammatory cytokines while high doses reducing their production (15). We also did not address if androgens or other sex hormones modulate Tregs during PNA resolution. Androgens and progesterone have been reported to increase Treg population and Foxp3 expression (78-80). Our studies focused on the therapeutic implication of exogenous E2 and did not systematically define alternative determinants for sex differences in the resolution of PNA-lung injury.

We believe our findings have translational relevance to PNA-ALI. Although systemic administration of E2 represents a potential therapeutic strategy, \textit{ex vivo} treatment of Tregs with E2 followed by cell transfer could improve E2’s therapeutic index. Tregs could be sorted from individuals with severe PNA and \textit{ex vivo} primed and stimulated with E2 (24-48 hours stimulation) with subsequent transfer back to the host (37). We have shown the feasibility of this approach (81) and others have suggested as a potential therapeutic strategy for Treg immunotherapy (38, 82, 83).

In conclusion, we reported a role for rescue treatment with E2 in the resolution of PNA. Tregs were indispensable for the resolution of PNA. Moreover, E2 pro-repair effects required Tregs and specifically ER\(\beta\) expression. We hope to provide the foundation for non-antibiotic therapeutic targets for PNA-induced lung injury and potential consideration of cellular therapy with “conditioned” Tregs.

**Methods**

**Animals**

C57BL/6 Wild-type (WT), \(\text{Rag-1}^{-/-}\), \(\text{ER}^{\alpha/-}\) and \(\text{ER}^{\beta/-}\) mice were purchased from Jackson Laboratory (Bar Harbor, ME). \(\text{Foxp3}^{DTR}\) mice were a gift from Dr. Alexander Rudensky (Sloan-Kettering Institute, New York, NY). Animals were bred and housed in a pathogen-free facility. All animal protocols were approved by the Johns Hopkins Animal Care and Use Committee. Mice aged 8 to 12 weeks were used.
Male mice were age matched with counterpart female mice. Both females and males were harvested simultaneously at specified time intervals after infection.

**Streptococcus pneumoniae preparation**

*Streptococcus pneumoniae* (*S. pneumoniae*; serotype 19, ATCC 49619) was purchased from American Type Culture Collection (Manassas, VA). Bacteria were grown overnight at 37°C in a 5% CO₂ incubator on blood agar plates, 5% sheep blood in tryptic soy agar (ThermoFisher, Waltham, MA). About 10 colonies were then suspended in Todd-Hewitt Broth (Becton Dickinson, Sparks, MD) supplemented with 17% (v/v) Fetal Bovine Serum (ThermoFisher) and incubated at 37°C with shaking at 225 rpm for 4-6 hours until an OD₆₀₀ 0.3 was reached. The media was distributed into 1 mL aliquots and flash-frozen in liquid nitrogen before storage at -80°C. Freshly thawed aliquot was used to challenge mice and subsequently plated to confirm the colony forming units (CFU) instilled.

**Preparation of Mice**

Mice were anesthetized with intraperitoneal (i.p.) ketamine/acetylpromazine (100 and 2.5 mg/kg, respectively) prior to intubation with a 20-gauge catheter. After anesthesia and tracheal intubation, *Streptococcus pneumoniae* (ATCC, 49619) or Todd Hewitt broth (RPI, T47500, Mt.Prospect, IL) was injected into the trachea. 25 µg β-Estradiol (TOCRIS Biosciences, UK) or vehicle was given on days 2, 3 and 4 through intraperitoneal injection after inoculation. For Treg depletion, diphtheria toxin (DT; List Biologicals, Campbell, CA) was administered intraperitoneally 2 days and 1 day before intratracheal instillation of bacteria and then on days 1, 3 after inoculation. At day 5 or day 6 after *S. pneumoniae* instillation, mice were anesthetized and killed by isoflurane (FlurisoTM, MWI, ID).

**Analysis of BAL Fluid**

BAL was obtained by cannulating the trachea with a 18-gauge catheter. The bilateral lung was lavaged twice (each aliquot 1 ml; calcium-free PBS); total returns averaged 1.6-1.8 ml/mouse. BAL was
centrifuged at 500 g for 5 minutes at 4°C. The cell-free supernatants were stored at –80°C for later analysis. The cell pellet was diluted in PBS, and total cell number was counted with a hemocytometer after staining with trypan blue. Differential counts were done on cytocentrifuge preparations by counting 300 cells per sample (Cytospin 3; Shandon Scientific), stained with Hema 3TM Stat Pack (Fisher HealthCare, PA, USA) according to instructions. Total protein was measured in the cell-free supernatant using Pierce BCA protein assay kit (Thermo Scientific, Rockford, IL).

**Preparation of lung single cell suspension**

Lungs were gently minced using a MACS Dissociator (Miltenyi Biotec Inc.CA) and incubated at 37°C in an enzyme cocktail of RPMI containing 5 mg/ml collagenase I (Worthington, NJ) and 1mg/ml DNase (Sigma, MO), then mashed through a 70-μm nylon cell strainer (BD Falcon). Red cells were lysed using ACK lysing buffer (Quality Biological, MD) and then single cell suspension was obtained.

**Lung morphology**

Lungs from animals were inflated to 25 cm H2O with formalin solution (Sigma) for histologic evaluation by H&E staining as previously described (29).

**Flow Cytometry**

BAL cells and single lung suspension were prepared for FACS analysis with a live-dead discriminator and fluorochrome-conjugated antibodies. Cells were incubated with Fc Block (BD Biosciences) Ab to block Fcγ III/II receptors before staining with a specific Ab. The following Abs (BD Biosciences — Pharmingen) were used for surface staining: BUV395-conjugated anti-CD4 (clone GK1.5) and BV650-conjugated anti-CD25 (clone PC61), PE-Cy7-conjugated anti-CD39 (clone 24DMS1), APC-Cy7-conjugated anti-CD62L (clone MEL-14),BV510-conjugated anti-CD44 (clone 1M7), BV605-conjugated anti-PD-1 (clone 29F.1A12), BV711-conjugated anti-GITR (clone DTA-1), BUV737-conjugated anti-CD69(clone H1.2F3), BV786-conjugated anti-CD40L(clone MR1),. For intracellular staining, cells were
fixed and permeabilized with Foxp3 staining buffer (eBioscience), then stained with APC-conjugated anti-Foxp3 (clone FJK-16s), PE-CF594-conjugated anti-GATA3 (clone L50-823), PerCP-eFlour710-conjugated anti-Ki-67 (clone SoLA15), BV421-conjugated anti-CTLA-4 (clone UC10-4B9). Lymphocytes were gated with characteristic low forward scatter/side scatter, using a FACSARia instrument and FACSDiva for data acquisition (Becton Dickinson) and Flowjo for analysis (Tree Star Inc.). Mean fluorescence intensity was calculated as the mean of the positive population fluorescence.

**BAL cytokines measurements**

BAL supernatant was collected following centrifugation of the cellular components and stored at −80° until further processing. Cytokine measurements were performed using the Mesoscale Discovery platform.

**Lymphocyte Culture**

Splenic CD4+CD25+ cells (about 85-90% Foxp3+) and CD4+CD25- (<1% Foxp3+) cells were isolated using magnetic bead separation (Miltenyi Biotec, Auburn, CA). Cells were plated in media with Mouse T-cell activator CD3/CD28 Dynabeads (ratio 1:1; Gibco, Waltham, MA). Cells were incubated for 72 hours with vehicle, E2 at 10\(\mu\)M. Cells were then stained for flow cytometry.

**Adoptive Transfer**

CD4+CD25+ isolated from male WT or ER\(\beta\)-/ splenocytes were cultured with vehicle or 10 \(\mu\)M E2 for 48 hrs as above. Cells were spun down and resuspended in 100 \(\mu\)l PBS. 0.25 X10\(^6\) live Tregs were administered via retro-orbital injection into male Rag-1-/ mice 1 hour after intratracheal *S. pneumoniae*.

**Bone marrow-derived macrophages**

BMDM were isolated from WT C57BL/6 femurs by flushing with 10 ml of RPMI medium using a 27-gauge needle into 6 well plates. Single cell suspensions were centrifuged (300 g) and resuspended in RPMI medium containing M-CSF (40 ng/ml) and plated for a total of 7 days before co-cultures.
**Macrophage-Treg co-cultures**

Tregs isolated were cultured for 48 hours with CD23/CD28 dynabeads in the presence or absence of estradiol 10 µM. Cells were harvested and counted and transferred to wells containing LPS-stimulated BMDM at a lymphocyte to macrophage ratio of 1:2 for 24 hours. At 20 hours, brefeldin A was added for intracellular cytokine staining.

**Statistical Analysis**

Groups of 3 to 7 mice were used for all experiments and repeated at least twice. *In vitro* experiments were performed in triplicate and repeated at least three times. Values are reported as mean and SEM. Differences between groups were compared using Mann–Whitney U test. Multiple group comparisons were performed using one-way ANOVA with Tukey’s multiple comparisons test. Two-factor comparisons were performed using two-way ANOVA Tukey’s multiple comparisons test. Significance was determined at P values less than 0.05. Statistics were performed using GraphPad Prism or R (84).

**Study approval**

The Institutional Animal Care and Use Committee of the Johns Hopkins University School of Medicine approved the animal procedures performed in this study.

**Figure Legends**

**Figure 1. Female mice display enhanced resolution of pneumonia.** Age-matched WT male and female mice were challenged with intratracheal *S. pneumoniae* (4 x 10^6 CFU/mouse) and followed over time. Lung injury parameters were harvested on days 0, 2 and 6. (A) Body weight over time relatively to baseline at day 0. (B-D) BAL total protein (B), BAL total cell count (C), and BAL differential cell counts (D-F) were determined over time in female and male WT mice after intratracheal *S. pneumoniae*. (G-H) Total lung total cell counts and lung neutrophil counts were determined in female and male mice.
after intratracheal *S. pneumoniae*. Two-way ANOVA was used. Mice, n=6-7 per group per time point. *P <0.05. Values reported as mean ± SEM.

**Figure 2. Alveolar and Lung Tregs increased in resolving females.** BAL and lung regulatory T cells (Treg) numbers, suppressive phenotype and proliferative capacity were measured by flow cytometry in males and female WT animals on days 2 and 6 after intratracheal *S. pneumoniae*-induced lung injury. (A-B) Fold change for BAL (A), lung (B) Treg numbers and BAL(C), lung (D) Treg percentage compared to male levels at day 2 after *S. pneumoniae*. BAL Treg expression of its master transcription Foxp3 (E), proliferative state by intracellular Ki-67(F) and transcription factor GATA3(G) expression were determined by mean fluorescence intensity and compared over time. Normalization followed by two-way ANOVA. Mice, n=6-7 per group per time point. *P <0.05. Values reported as mean ± SEM.

**Figure 3. Estrogen enhances Regulatory T cell (Tregs) suppressive phenotype in vitro.** CD4+CD25+ Treg were isolated from WT mice splenocytes and cultured in the presence of anti CD3/CD28 beads and stimulated with either vehicle or estradiol (E2; 10 µM) for 72 hours. Multicolor flow cytometry was performed to assess E2 dependent changes in Treg suppressive phenotype. Treg expression for Foxp3 (A), CD25 (B), GATA3 (C) and GITR (D) were measured and expressed as mean fluorescence intensity (MFI +/- SEM). The Mann-Whitney test was used for all MFI.*P <0.01 n =25-30 per group.

**Figure 4. Therapeutic estradiol accelerates resolution of lung injury in male WT animals.** WT male mice were challenged with intratracheal *S. pneumoniae* (4 x 10^6 CFU/mouse). On day 2 after injury, rescue treatment with intraperitoneal estradiol (25 µg/mouse/dose) was administered daily on days 2, 3 and 4. Lung injury markers were measured on day 6 after lung injury. (A) Body weight over time relatively to baseline at day 0. Fold changes compared to males that received intraperitoneal vehicle after *S. pneumoniae* were measured for BAL total protein (B), BAL total cell counts (C), BAL neutrophil counts (D) BAL Treg numbers (E) Lung total cell counts (G), lung neutrophils (H) and lung Tregs (I) are
shown. Representative lung H-E sections 6 days after injury were stained with hematoxylin and eosin. Original magnification: X100. Two-way ANOVA was used for weight. Normalization to fold change followed by Mann-Whitney test was used for protein and cell counts. *P <0.05, n = 7 per group.

**Figure 5** Estradiol does not alter lung bacterial counts after *S. pneumoniae*. Lung homogenates (A) were obtained from WT male and female mice after intratracheal *S. pneumoniae* (4 x 10^6 CFU/mouse) on day 6 and plated in blood agar plates overnight at 37°C and counted for colony forming units (CFU). To determine if estradiol has direct antibacterial effects, *S. pneumoniae* was plated with vehicle or 1, 100 or 1000 µM E2 overnight in blood agar plates, CFU were counted. (Mann-Whitney test was used. n = 7 per group). Values reported are mean ± SEM.

**Figure 6.** Salutary effects of E2 require Tregs. Male WT and *Foxp3^DTR^* mice were challenged with intratracheal *S. pneumoniae* (3 x 10^6 CFU/mouse). All groups received diphtheria toxin (day-2 at 50 µg/kg, subsequent doses at 10 µg/kg) and estradiol (E2, 25 µg/mouse/dose) treatment was given intraperitoneal daily on days 2, 3 and 4 as shown in schematic (A). Lung injury markers were measured on day after injury. BAL total protein (B), BAL total cell counts (C), BAL neutrophil counts (D), BAL Tregs (E) and lung neutrophils (F) were measured 6 days after *S. pneumoniae* injury. (E). Representative lung sections were stained with hematoxylin and eosin. Original magnification: X100 (G). One-way ANOVA was used for statistics. *P <0.05, (n = 3-8 per group). Values reported are mean ± SEM.

**Figure 7.** Estradiol augmentation of Treg suppressive phenotype is ERβ-dependent. CD4+CD25+ Tregs were isolated from males WT, *ERα^-/-^* and *ERβ^-/-^* splenocytes and cultured in the presence of anti CD3/CD28 beads and stimulated with either vehicle or estradiol (E2; 10 µM) for 72 hours. Multicolor flow cytometry was performed for the expression of Foxp3(A), CD25(B), GATA-3(C) and GITR(D) and measured by mean fluorescence intensity (MFI) Two-way ANOVA was used for statistics. *P <0.05 for
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Values reported are mean

**Figure 8. Estradiol augments Treg function in an ERβ-dependent manner.** Male WT and ERβ⁻/⁻ Tregs were cultured in the presence of anti CD3/CD28 beads and stimulated with either vehicle or estradiol (E2; 10 μM) for 48 hours. Cells were collected and 0.25 X 10⁶ Tregs adoptively transferred (AT) (retro-orbital) 1 hour after intratracheal S. pneumoniae (3 X 10⁶ CFU/mouse) in lymphocyte-deficient Rag-1⁻/⁻ mice. Lung injury markers were measured at day 5 and expressed as fold change compared to group of Rag-1⁻/⁻ mice AT with WT Tregs cultured ex vivo with vehicle (ethanol). BAL protein (A), BAL total cell counts (B), BAL neutrophil counts (C) lung total cell counts (D), lung neutrophil counts (E), lung %Tregs of total lung cells (F), BAL %Tregs of total BAL cells (G) and their relative Treg Foxp3 expression were measured. Normalization followed by Kruskal-Wallis test was used for statistics. *P <0.05, n=5-8, values reported are mean ± SEM.

**Figure 9. Tregs modulate macrophages responses via E2-ERβ.** WT and ERβ⁻/⁻ Tregs were isolated and culture for 48 hours with CD23/CD28 dynabeads in the presence or absence of estradiol (10 μM). Tregs were harvested, counted and transferred to wells containing LPS-stimulated bone marrow-derived macrophages. BMDM at a lymphocyte to macrophage ratio of 1:2 for 24 hours. Last 4 hours of co-cultures, brefeldin A was added. Co-culture cells were harvested for intracellular flow cytometry. Macrophage IL-10 (Fig. 9A), IL-6 (Fig. 9B and TNF-α (Fig. 9C) were measured as mean fluorescence intensity (MFI) and as a % positivity from total macrophage subpopulation (CD4 negative population). One-way ANOVA was used for statistics. *P <0.05, (n = 3-8 per group). Values reported are mean ± SEM.

**Author contributions**
YX and TP carried out the experiments. QZ provided technical expertise. AB, LW acquired data. KS analyzed data. YX and FD wrote the manuscript. RD and KS provided intellectual input. FD, RD and YX designed and supervised the study. All authors read and approved the final manuscript.

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The authors have declared that no conflict of interest exists.

References

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