

Supplement

17 β -estradiol dysregulates host immune response to *Pseudomonas aeruginosa* respiratory infection and is modulated by estrogen receptor antagonism

¹Shadaan Abid, ¹ShangKui Xie, ¹Moumita Bose, ²Philip W. Shaul, ¹Lance S. Terada, ³Steven L. Brody, ⁴Philip J. Thomas, ⁵John A. Katzenellenbogen, ⁵Sung Hoon Kim, ⁶David E. Greenberg, ¹Raksha Jain

¹Division of Pulmonary and Critical Care Medicine, Department of Internal Medicine, University of Texas Southwestern Medical Center, Dallas, TX 75390

²Center for Pulmonary and Vascular Biology, Department of Pediatrics, University of Texas Southwestern Medical Center, Dallas, TX 75390

³Division of Pulmonary and Critical Care Medicine, Washington University School of Medicine, St. Louis, MO 63110

⁴Department of Physiology, University of Texas Southwestern Medical Center, Dallas, TX 75390

⁵Department of Chemistry, University of Illinois at Urbana-Champaign, Urbana, IL 61801

⁶Division of Infectious Disease, Department of Internal Medicine, University of Texas Southwestern Medical Center, Dallas, TX 75390

Supplementary methods

***Pseudomonas aeruginosa* growth assays**

For *in vitro* growth studies, overnight PAO1 cultures were washed and final pellets were re-suspended in 1 mL of LB broth. Concentrations were calculated using the optical density at 600 nm (OD600) and the previously determined number of colony-forming units (CFUs) for each strain to give CFUs/OD600. Cultures were diluted to a final concentration of 5×10^5 CFUs in LB broth. Assays were performed in non-pyrogenic polystyrene 96-well plates (Falcon tissue culture plates; BD Biosciences, San Jose, California). PAO1 was incubated with 1nM, 10nM and 100nM estrogen in a total volume of 300 ul that contained Roswell Park Memorial Institute 1640 (RPMI) medium with L-glutamine (Invitrogen, Carlsbad, California). The plates were incubated at 37°C and after 3, 6, 9, 12, 18 hours, the culture supernatants were diluted and spread on LB plates and then counted for CFUs.

Mucoid conversion of *P. aeruginosa*

P. aeruginosa isolate PAO1 was cultured and grown at 37°C in LB broth with subculture into fresh vehicle, E2 10 nM and P4 10 nM. The cultures were then plated onto Spirit blue agar (Fluka, St. Louis, Missouri) to detect the formation of mucoid colonies at 24, 48 and 72 hours.(1)

Table S1: Blood culture results at 24 hours

Strain and Dose	PAO1 3.5 x 10⁵ CFU	PAO1 8.5 x 10⁵ CFU	M57-15 1.2 x 10⁶ CFU
Male	Negative	Positive	Negative
Female	Negative	Positive	Negative

Table S2: Demographics of participants in neutrophil experiments (females with CF)

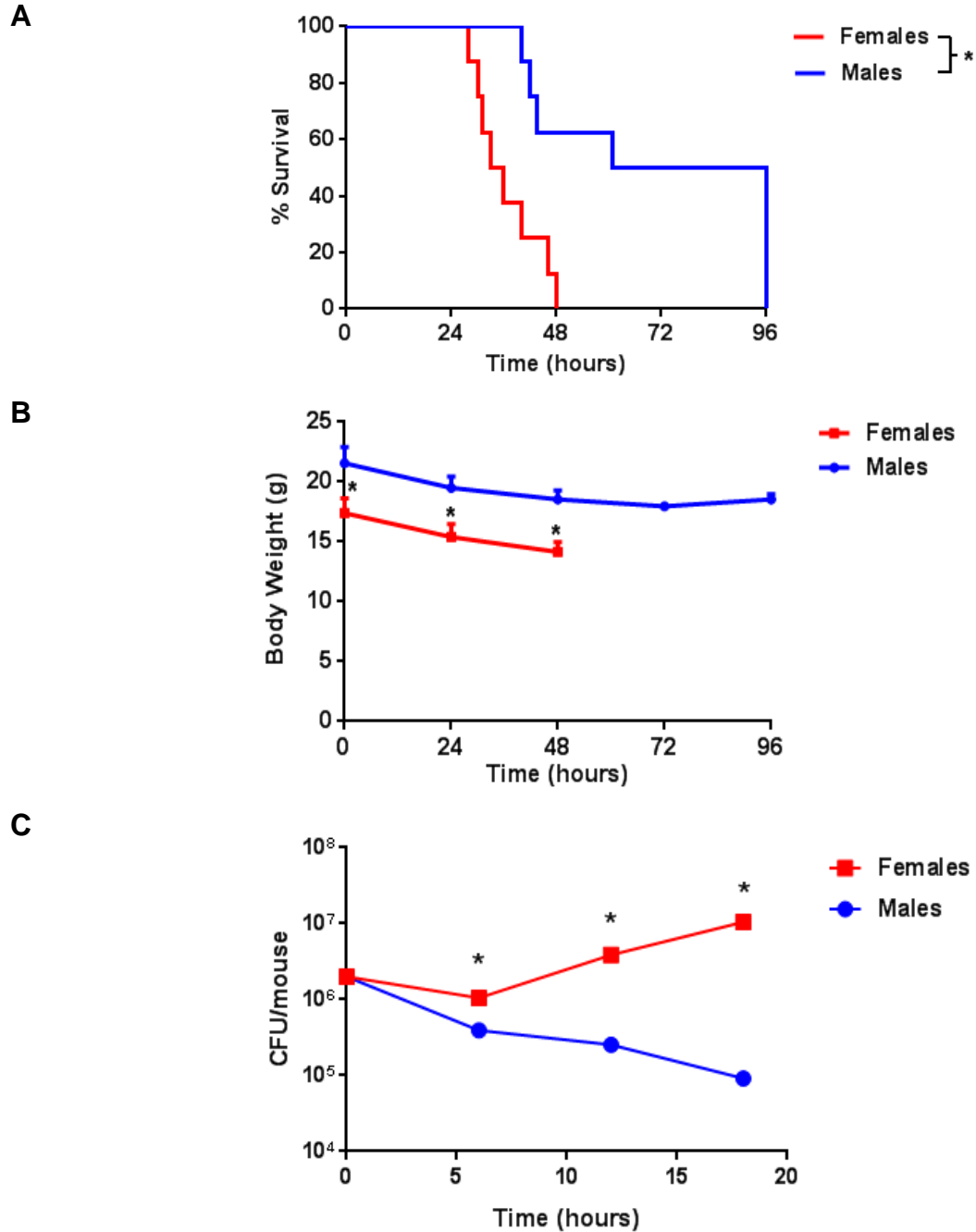
Subject Number	Age (yrs)	FEV1%	BMI (kg/m ²)	Genotype	Sputum bacteria
1	30	72	19.7	Neither	OSSA
2	33	117	22.6	delF508 homo	PA
3	22	51	17.4	Neither	AX
4	27	30	27.2	delF508 het	PA
5	30	42	19.8	delF508 homo	PA, OSSA, SM
6	32	53	22.7	Neither	OSSA, PA
7	22	62	18.2	delF508 het	PA, SM
8	27	69	24.2	delF508 homo	PA, MRSA
9	22	36	21.8	delF508 homo	PA, MRSA
10	22	35	17.1	delF508 het	PA
11	27	85	20.6	delF508 het	OSSA
12	30	54	20.8	delF508 het	PA, OSSA
13	42	118	23.3	Neither	MA
14	23	60	19.8	delF508 het	AX, MRSA
15	34	34	25.7	delF508 het	PA, MA, SM
16	19	101	23.7	Neither	OSSA
17	22	72	21.4	delF508 het	PA, SM, MAI
18	20	47	18.8	delF508 homo	PA
19	24	22	14.8	delF508 homo	PA, MRSA
20	21	67	19.2	delF508 het	PA
Mean ± SEM:	26.5 ± 0.3	61.4 ± 1.4	20.9 ± 3.1		

Definition of abbreviations:

Genotypes: delF508 homo – homozygous, delF508 het – heterozygous; neither – no delF508 mutations;

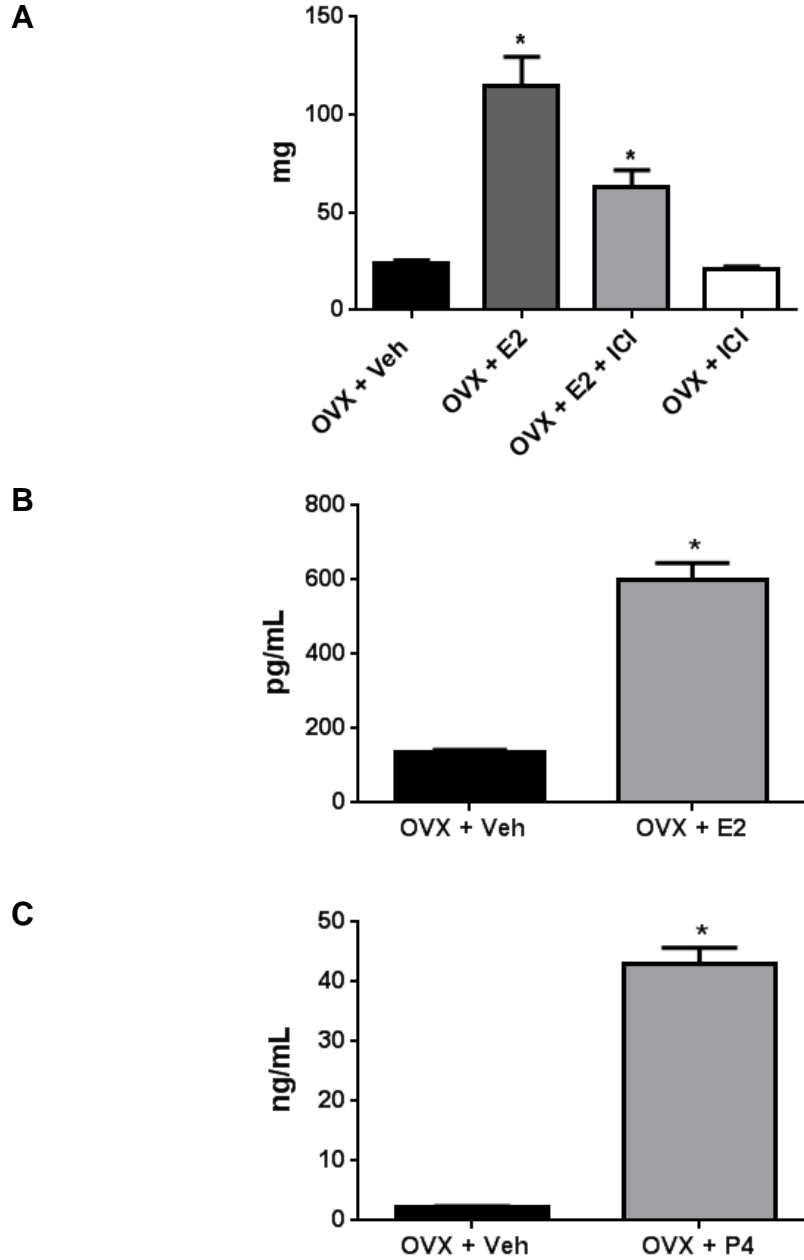
Sputum bacteria: OSSA - Oxacillin Sensitive *Staphylococcus aureus*; PA - *Pseudomonas aeruginosa*; AX - *Achromobacter xylosoxidans*; SM - *Stenotrophomonas maltophilia*; MRSA - Methicillin Resistant *Staphylococcus aureus*; MA - *Mycobacterium abscessus*; MAI - *Mycobacterium avium intracellulare*

Figure S1



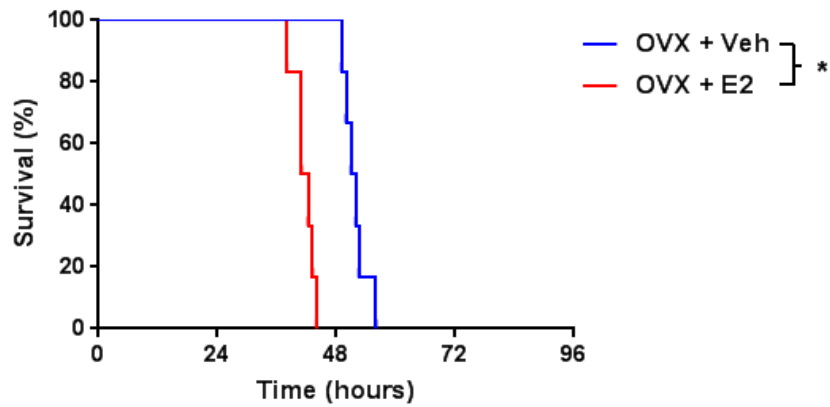
Supplemental Figure S1. Survival in male versus females when age matched. (A) Male and female C57BL/6 wild type mice were infected with *P. aeruginosa*, 8.5×10^5 CFU and followed for up to 96 hours (n = 8 per group). Displayed are Kaplan-Meier survival plots; *p < 0.05. (B) Body weights measured in experiment A. (C) Bacterial CFU remaining in the lungs of male versus female mice after infection with *P. aeruginosa* at 3×10^5 CFU, (n = 6 mice per time point per group), p < 0.05. Data in B and C are mean \pm SEM, *p < 0.05.

Figure S2



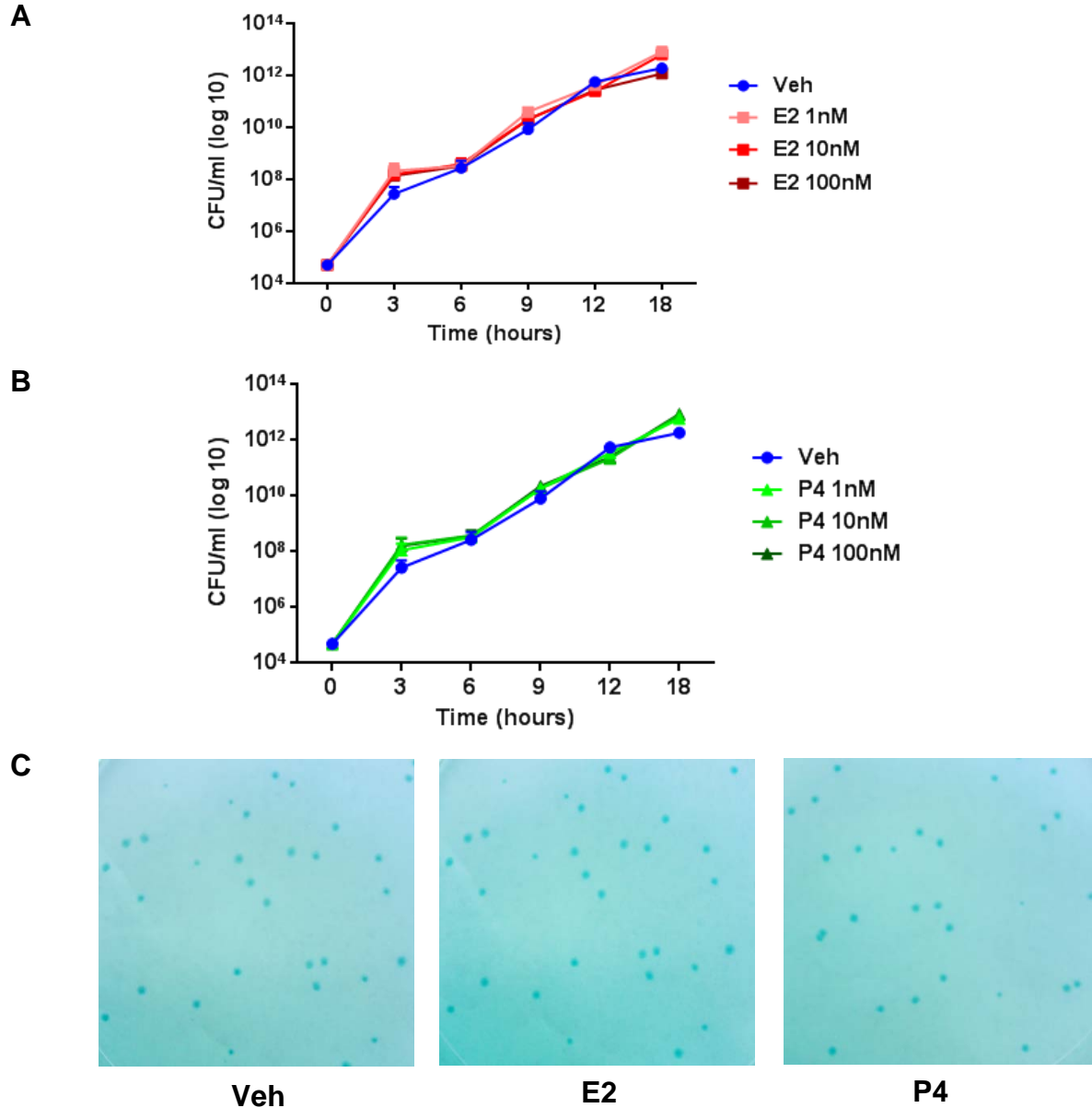
Supplemental Figure S2. Estrogen and progesterone treatments in mice are physiologic. (A) Uteri from C57BL/6 mice weighed 2 weeks after ovariectomy and hormone treatment (n = 8 per group). (B) 17β-estradiol levels measured from the blood of mice 2 weeks after ovariectomy and estrogen supplementation (n = 12 per group). (C) Progesterone levels measured from the blood of mice 2 weeks after ovariectomy and progesterone supplementation (n = 12 per group). Data in A, B, and C are displayed as mean ± SEM; *p < 0.05 relative to vehicle group.

Figure S3.



Supplemental Figure S3. Survival in ovariectomized female mice infected with mucoid *P. aeruginosa*, M57-15. Wild type C57BL/6 female mice were ovariectomized (OVX) and supplemented with physiologic doses of hormones: vehicle (Veh) or E2 (0.5ug/day) and infected with M57-15 at 1.2×10^6 CFU. Displayed are Kaplan-Meier survival plots; *p < 0.05.

Figure S4



Supplemental Figure S4. *P. aeruginosa* growth is insensitive to E2 or P4 stimulation.

(A) *P. aeruginosa* was grown *in vitro* and treated with vehicle or escalating doses of 17 β -estradiol (n = 3 per treatment group); p = 0.4687. (B) *P. aeruginosa* was grown *in vitro* and treated with vehicle or escalating doses of progesterone (n = 3 per treatment group); p = 0.448. (C) *P. aeruginosa* was grown for up to 72 hours with E2 (10 nM) or P4 (10 nM) on blue agar to evaluate the transformation from a non-mucoid to mucoid state. Shown are representative images at 72 hours (n = 3 treatment group).

Figure S5

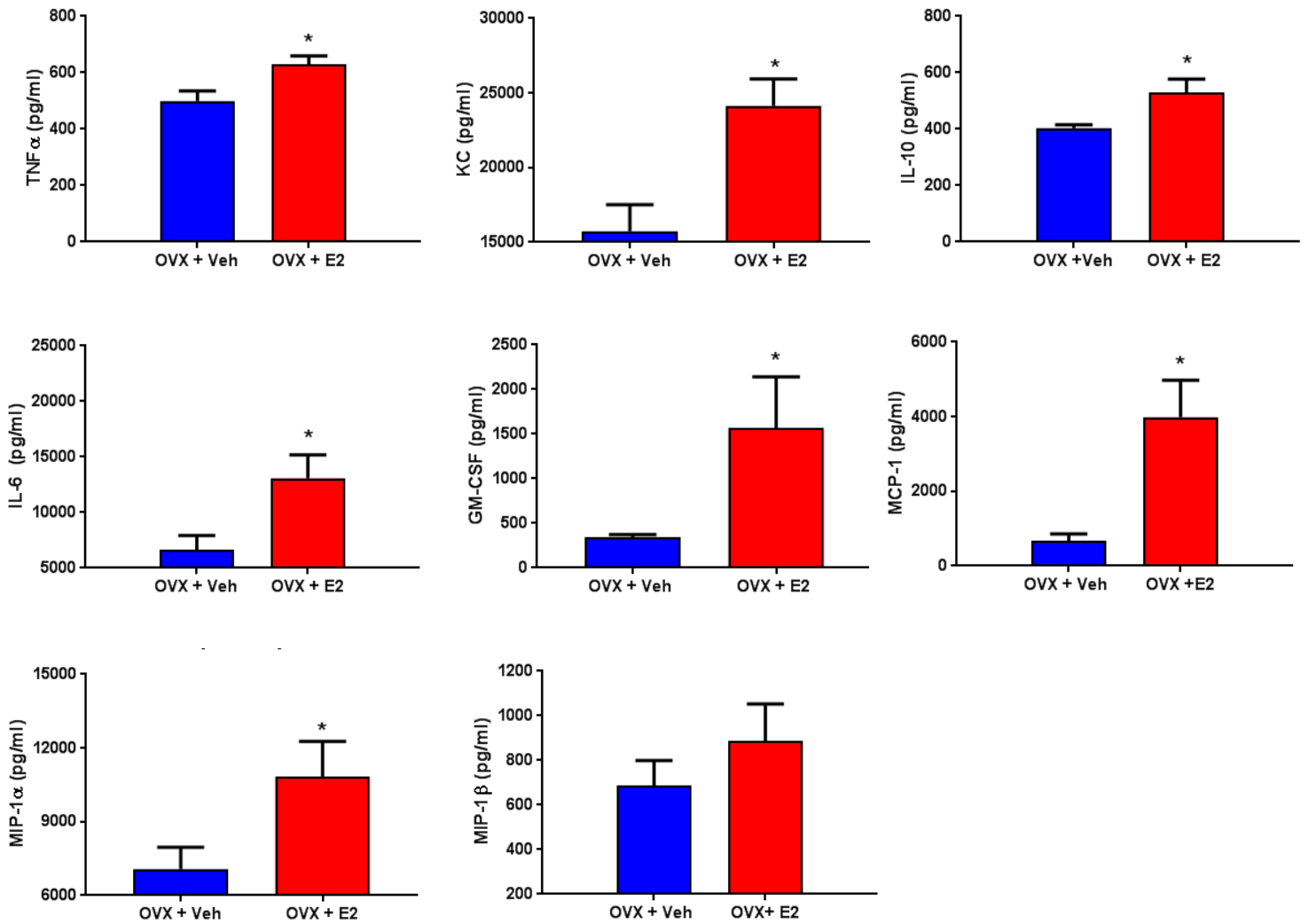
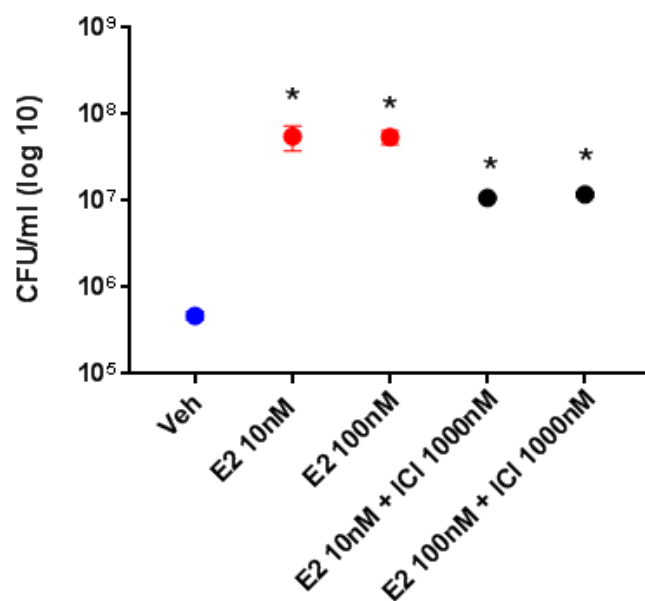


Figure S5. Estrogen induces inflammation in the setting of *P. aeruginosa* infection. Cytokine expression in whole lung samples of female mice ovariectomized and hormone treated as in Figure 1A taken 18 hours after infection (n = 7 mice per group). Data are expressed as mean \pm SEM, *p < 0.05.

Figure S6.



Supplemental Figure S6. 17β -estradiol impacts responses to *P. aeruginosa* infection of neutrophils from female subjects without CF. Neutrophils from female subjects without CF treated *ex vivo* with E2 and ICI 182,780 (ICI) at indicated doses in *P. aeruginosa* killing assays at an MOI of 1:4. CFU = Colony forming units of *P. aeruginosa* (n = 6). Shown are mean \pm SEM; *p < 0.05 relative to vehicle.

Supplementary References:

1. Chotirmall SH, Smith SG, Gunaratnam C, Cosgrove S, Dimitrov BD, O'Neill SJ, Harvey BJ, Greene CM, McElvaney NG. 2012. Effect of estrogen on pseudomonas mucoidy and exacerbations in cystic fibrosis. *N Engl J Med* 366:1978-1986.