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**Biological sex influences susceptibility to *Acinetobacter baumannii* pneumonia in mice**

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## **Abstract**

*Acinetobacter baumannii* is an extremely versatile multidrug resistant pathogen with a very high mortality rate therefore, it has become crucial to understand the host response during its infection. Given the importance of mice for modelling infection and their role in pre-clinical drug development equal emphasis should be placed on the utilization of both sexes. Through our studies using a murine model of acute pneumonia with *A. baumannii*, we observed that female mice were more susceptible to infection. Likewise, treatment of male mice with estradiol increased their susceptibility to infection. Analysis of the airway compartment revealed enhanced inflammation and reduced neutrophil and alveolar macrophage numbers compared to male mice. Depletion of either neutrophils or alveolar macrophages was important for bacterial clearance however, depletion of alveolar macrophages further exacerbated female susceptibility due to severe alterations in metabolic homeostasis. Our data highlights the importance of utilizing both sexes when assessing host immune pathways.

## INTRODUCTION

*Acinetobacter baumannii* is an emerging opportunistic and extremely versatile multidrug resistant pathogen that has become a serious global threat resulting in high mortality rates (1, 2). Its extreme genome plasticity and ability to quickly develop resistance to a plethora of antibiotics makes it an important pathogen and one of the reasons it has been designated as a serious threat and high priority target by the CDC (3-7). This has driven significant research in recent years to understand how the host senses and responds to *A. baumannii* infection (8, 9). However, given that the emergence of *A. baumannii* is relatively recent, there are still several aspects of the immune response that have not been fully investigated.

Differences in susceptibility between female and male have been observed in the context of vaccination, autoimmune and infectious diseases (10-13). However, sex-based differences and the host immune mechanisms behind this differential response have only recently begun to be dissected. Several studies have presented data showing differential immune response to infectious diseases according to gender (14-16). The innate immune response of females is typically stronger in the context of infection (12). Here we report that female mice have increased susceptibility to *A. baumannii* induced pneumonia and we demonstrate that this difference is due to differential innate immune responses. Alveolar macrophages were found to play a more significant role in clearance of *A. baumannii* from female mice compared to males. The exacerbation of this sex difference through alveolar macrophage depletion was found to be due to

severe mitochondrial alterations that resulted in a metabolic imbalance, leading to a defective response to the pathogen. Our data highlights the importance of utilizing both sexes when assessing host immune pathways to *A. baumannii* and points to a crucial metabolic role for alveolar macrophages in controlling infection in female mice.

## RESULTS

### **Female mice are more susceptible to *Acinetobacter baumannii* induced pneumonia**

While establishing a non-lethal acute pneumonia model with the *A. baumannii* highly virulent clinical isolate 5075 we noticed that female mice had increased susceptibility to infection (Figure S1). To confirm this observation, weight matched C57BL/6J female and male mice (Figure 1A) were intranasally infected with *A. baumannii* 5075 to account for potential differences in lung volume or upper respiratory tract dimensions. While 88% of male mice were able to survive only 12% of female mice survived ( $P < 0.001$ ) (Figure 1B). In a non-lethal model of infection female mice had bacterial counts 939-fold higher ( $P < 0.0001$ ) in bronchoalveolar lavage fluid (BALF), 2076-fold higher in lung tissue ( $P < 0.0001$ ) and over 40,000 times greater in the spleen ( $P < 0.0001$ ) compared to male mice 24 hours after infection (Figure 1C). Consistent with the increased bacterial numbers, female mice had decreased body temperatures (3.9°C decrease vs 0.7°C,

Figure 1D,  $P < 0.0001$ ) compared to male infected mice. Consistent with the poor prognosis, female mice had protein content levels 62% higher than male mice in their airways ( $P < 0.05$ ), as a sign of lung injury (Figure 1E). Pathological examination of infected lungs from both sexes further confirmed the exacerbated phenotype in females. Male infected lungs showed evidence of inflammatory infiltrates and largely interstitial inflammation (Figure 1F). Microabscesses composed of neutrophils were observed within the lung parenchyma (Figure S2A; arrow) while some hemorrhaging and edema was also observed. Lungs of female infected mice showed marked levels of pulmonary edema (Figure S2B), hemorrhaging and multifocal interstitial inflammation with regions of marked consolidation. Multiple lung lobes were involved. There was also evidence of fibrin within airways (Figure 1F; arrow) and neutrophils in airspaces (Figure S2C; arrow). The overwhelming increase in bacteria was also evident in female mice, with bacteria readily observed within regions of edema (Figure S2D; arrow).

Studies on the innate immune response to *A. baumannii* induced pneumonia can display conflicting results based on the virulence of the strain utilized (17, 18). We repeated our weight match studies (Figure S3) with a less virulent strain to assess if the sex difference was strain specific. Consistent with our previous observation, female mice were more susceptible to infection with *A. baumannii* strain ATCC 19606. Bacterial counts were 5-fold higher in BALF ( $P < 0.001$ ) and 19-fold higher in lung (Figure S3A&B,  $P < 0.0001$ ). Female mice infected with ATCC 19606 also had decreased body temperatures compared to male infected mice (Figure S1C). No differences in bacterial

counts was observed in the spleen between sexes (Figure S1B), presumably a result of less extrapulmonary dissemination given its decreased virulence.

Gut microbial differences and dysbiosis have been associated to several lung diseases, including bacterial infections (19-21). To assess if the gut microbiome was an underlying cause for the increased susceptibility, fecal samples from female and male mice were collected immediately before infection. Fecal microbiome analysis revealed similar beta diversity between the microbiome of female and male mice, leading to a similar clustering of bacterial strains between the sexes (Figure S4). This suggests that the gut microbiota is not a key contributing factor to the differences observed in response to *A. baumannii* lung infection between female and male.

### **Treatment with 17 $\beta$ -estradiol increases male mice susceptibility to *A. baumannii* lung infection**

To begin dissecting the sex differences, male mice were treated with 17 $\beta$ -estradiol, the major female sex hormone during adult life. Male mice treated with estradiol had bacterial counts that were 5.9-fold higher in BALF (Figure 1G; P=0.057), 2,800-fold higher in lung tissue (P<0.001; Figure 1G) and 317-fold higher in the spleen (P<0.001; Figure 1G) compared to vehicle controls. Concordant with the increases in bacteria, 17 $\beta$ -estradiol treated males had a significant decrease in body temperature compared to their control counterparts (3.3°C vs 1.2°C, Figure 1H). H&E staining of the lung revealed increased pathology in the infected males treated with estradiol when

compared to ones treated with the vehicle control (Figure S5). Lungs of male vehicle infected mice showed similar pathology to as described before with interstitial inflammation and inflammatory infiltrates across multiple lobes and microabscesses throughout. Males treated with estrogen prior to infection exhibited regions of significant consolidation and loss of alveolar architecture, dilated vessels and edema.

Sex steroid hormones have been described to directly impact bacterial growth and metabolism (22-24). To test if estradiol could be exerting a direct effect on *A. baumannii* growth, bacteria were grown in both LB and M9 minimal media supplemented with glucose, with and without estradiol. In the conditions tested, estradiol did not alter the growth of *A. baumannii* (Figure S6A&B). Given the importance now placed on utilizing both males and females in research, be it animal or human studies, we further characterized this phenotype by examining the immune responses of these mice.

### **Female mice display differential immune cell populations and cytokine production in response to respiratory *A. baumannii* infection**

It is now well established that sex hormones can directly act and exert an effect on immune cells (12, 25). To determine what may be influencing the enhanced bacterial burden in female over male mice, we analyzed the resident and recruited immune cells into the airway via flow cytometry. Both neutrophils and alveolar macrophages were significantly less in female infected mice. Neutrophils were reduced by 64% ( $P < 0.01$ ; Figure 2A) and alveolar macrophages were reduced by 68% ( $P < 0.01$ ; Figure 2A). This

is significant as both neutrophils (26) and alveolar macrophages (27) have been reported to play important roles in the clearance of *A. baumannii* from the airway. More subtle changes were observed with other cell populations (Figure S7).

The production of cytokines and chemokines by innate immune cells can also differ between the sexes in response to different stimuli including bacterial infections (12, 28). Analysis of the cytokine and chemokine levels in the airway showed that female mice had significantly increased cytokine production in response to *A. baumannii*, with the majority of the cytokines quantified being higher in female, compared to male *A. baumannii* infected mice (Figure 2B and Figure S8). Proinflammatory cytokines such as IL-6 (24-fold;  $P < 0.0001$ ), IL-1B (11.7-fold;  $P < 0.01$ ), IL-17 (24-fold;  $P < 0.001$ ) and TNF (2.9-fold;  $P < 0.0001$ ) were all significantly higher in female infected mice (Figure 2B). While neutrophil numbers were reduced in the airways of female mice, neutrophil chemokines such as MIP-2 (18-fold higher;  $P < 0.001$ ), KC (25.8-fold higher;  $P < 0.0001$ ) and MIP1A (3.3-fold higher,  $P < 0.01$ ) were all significantly increased (Figure 2B). These massive cytokine levels are a likely consequence of the higher bacterial burdens in the female mice, while the high bacterial burden is likely related to the reduced immune cell recruitment. In vitro killing assays revealed that male alveolar macrophages have increased capacity to control *A. baumannii* proliferation while female bone marrow isolated neutrophils showed a trend to increased proliferation, when compared to male mice following a 4 h and 1 h infection respectively (Figure 2C&D). This difference was more pronounced in alveolar macrophages (Figure 2C,  $P < 0.01$ ), serving as another indication of an important role of these immune cells in the sex-differential response to

*A. baumannii* infection. Assessment of immune cell function in vivo showed that neutrophils from infected female mice had a reduced capacity to produce reactive oxygen species (ROS) (36% reduction;  $P < 0.01$ ; Figure 2E) compared to males, indicative of maybe an inability to fight the infection. We also observed increased cell death (3-fold increase;  $P < 0.05$ ; Figure 2E) in neutrophils from female infected versus male infected mice. Lastly, we examined if exposure to estrogen had a direct impact on immune cell function in vitro. Treatment of both immortalized bone marrow derived macrophages (iBMM) and the RAW macrophage cell line with  $\beta$ -estradiol reduced their capacity to kill *A. baumannii* in an in vitro killing assay (Figure 2F&G).

### **Alveolar macrophage depletion exacerbates female susceptibility to *A. baumannii* induced pneumonia**

Evidence for a role for neutrophils and alveolar macrophages in response to *A. baumannii* has been observed (26, 27, 29, 30). The decreased recruitment of these cells observed in female mice (Figure 2A) is likely to contribute to the increased bacterial burden. We therefore tested the relative contribution of each population in male and female mice.

Neutrophils were depleted using a neutrophil specific Ly6G antibody, as opposed to previous studies that have used non-specific antibodies (Ly6C/G) towards neutrophils and on different strains of *A. baumannii*, where the virulence of an isolate can influence the outcome (9). Our data with neutrophil specific antibodies shows specific and

successful depletion (Figure 3A&B and Figure S9 for gating) that leads to increased bacterial burden irrespective of sex in the lung and spleen (Figure 3C and Figure S10). The magnitude of this increased bacterial burden compared to control mice was also equivalent between the sexes in the lung and spleen. Depletion of alveolar macrophages using clodronate loaded liposomes was also successful (Figure 3D&E and Figure S9) with no off-target cellular effects (Figure S11). In contrast to neutrophils, depletion of alveolar macrophages, although increasing bacterial burdens in both sexes, had a disparately higher effect in bacterial counts in the airways of female mice (Figure 3F), with 2500-fold and 400-fold higher bacterial counts in the BALF and lung respectively, compared to non-depleted controls, further highlighting the difference between sexes. Whereas male mice had 9, 8 and 4-fold changes in the same sample types. Previous reports have shown that ROS production by neutrophils is a key factor in killing *A. baumannii* (31). In alveolar macrophage depleted animals, males had significantly more ROS in the airway when compared to female mice (Figure 3G), which could contribute to their improved bacterial clearance compared to females. These results highlight an important role of alveolar macrophages in controlling *A. baumannii* pneumonia in female mice.

### **Female mice display metabolic changes in the absence of alveolar macrophages**

To understand the differential sex effect of alveolar macrophage depletion, RNA sequencing (RNA-seq) of whole lung homogenates of clodronate treated female and male mice following a 4 h infection was performed. We chose an early time point to

minimize transcriptional changes due to significant differences in bacterial burden between the sexes (Figure S12A). Lung tissue was used to examine the entire effect on the lung in the absence of alveolar macrophages. Differences in bacterial burden between sexes were already apparent in BALF at 4 h in clodronate treated mice (Figure S12A), which also correlated with a greater decrease in body temperature in female infected mice (Figure S12B). Alveolar macrophages were significantly depleted from mice irrespective of sex (Figure S12C&D). RNA-seq revealed significant changes between the sexes when alveolar macrophages were depleted (Figure 4A), with over 2,500 (fold change >1.5, P-value<0.05) transcript changes (Figure 4B). We observed significantly fewer gene changes in the absence of alveolar macrophage depletion at this time point (Figure S13). We observed an increase of 952 transcripts in female mice and 1,647 transcript decreases (Figure 4B), suggesting that alveolar macrophages in female mice contribute significantly to the transcriptional landscape.

Gene ontology analysis showed major reductions in the respiratory chain and ribosomal genes in female infected mice, indicating that alveolar macrophages from female mice contribute significantly to energetic requirements (Figure 4C). Alterations in mitochondrial complex function can propel to altered ROS generation. Total ROS levels were not altered at this time point (Figure S12F) however, transcription of several genes involved in the antioxidant response system such as *Sod1* and *Sod3* (data not shown), were upregulated in male mice, suggesting that male mice can have increase protection against ROS. LDH quantification revealed a trend to increased cytotoxicity and cell death in female mice, although this was not significant (Figure S12G,  $p=0.0976$ ). Given

that mitochondria are the primary energy producer; ATP levels were measured in BALF. Consistent with the transcriptional data, female mice displayed a significant decrease of 92% in total ATP levels when compared to male mice (Figure 4D,  $P < 0.05$ ). Lactate levels, which are a common readout for glycolysis (32), were increased by 42% in female mice (Figure 4E,  $P < 0.05$ ) 4 h following infection, indicating that due to reductions in mitochondrial oxidative phosphorylation (OXPHOS), female mice need to switch to a glycolytic state in order to generate ATP. We confirmed the importance of this observation by inhibiting cellular respiration in mice with rotenone, an inhibitor of mitochondrial electron transport. Treatment of male mice with this compound increased bacterial burdens by 16-fold ( $P < 0.05$ ; Figure 4F) in BALF and 5.3-fold in lung tissue ( $P < 0.01$ ; Figure 4F). These data suggest that in the absence of alveolar macrophages, female mice are unable to respond in a metabolically beneficial manner to *A. baumannii* infection.

## DISCUSSION

In this study we identified a sex-based innate immune response to *A. baumannii* respiratory tract infection. We showed that female mice are significantly more susceptible to infection, irrespective of *A. baumannii* virulence, which was also mimicked in male mice treated with  $17\beta$ -estradiol. We also demonstrated that the differential susceptibility to *A. baumannii* induced pneumonia between sexes is based on altered immune cell populations in the airway, specifically neutrophils, and depletion

of alveolar macrophages leads to a metabolic impairment of female mice following infection, also hindering their capacity to combat infection.

Previous work has pointed to an important role for alveolar macrophages in the early control of *A. baumannii* replication in the airway, possibly by contributing to the recruitment of neutrophils via production of inflammatory cytokines and chemokines (27). In this report we showed that their depletion in female mice resulted in a decrease of the eIF2 initiation machinery possibly resulting in decreased protein translation as well as severe alterations in mitochondria which lead to metabolic dysfunctions. A recent report raised the hypothesis of sex-dependent actin cytoskeleton regulation in alveolar macrophages (33), which has the potential to affect several processes such as phagocytosis. Here we reveal another layer on innate immune sex-based differences by demonstrating that alveolar macrophages in female mice infected with *A. baumannii* are important to maintain metabolic homeostasis and facilitate an effective response to infection.

The role of neutrophils in *A. baumannii* respiratory infection has up until now relied on non-specific antibodies (26, 30). In our model, neutrophil depletion eliminates the sex difference associated with higher bacterial burdens, suggesting that the decreased neutrophil numbers observed in the airway of the female mice is a crucial factor in mediating this phenotype. Moreover, clodronate treated mice female mice already had 35% less neutrophils in the airway than male mice 4 h after infection (Figure S12E,  $p=0.0952$ ), thus suggesting that alveolar macrophages can regulate neutrophil numbers

in the airway in response to *A. baumannii* infection. Neutrophil derived ROS has been described as a key factor in the killing of *A. baumannii* in respiratory tract infection (31). Despite conflicting results in the literature on the role of estradiol on neutrophils and ROS production, recent work showed that estradiol could lead to a reduction of superoxide anion production by neutrophils (34). We observed that ROS levels were increased in the airway of male mice 24 h after infection (Figure 3G) and their levels were higher in neutrophils from male mice (Figure 2G), thus contributing to the improved bacterial clearance. Also, while female mice had 64% less neutrophils than male mice they had a significant increase in neutrophil chemokines (Figure 2A&B) levels in the airway. When neutrophil transmigration to inflamed tissues is blocked IL-17 production is induced, which induces G-CSF production causing increased granulopoiesis and numbers of circulating neutrophils (35, 36). We observed a significant increase in the transcription of *Il17a* (2.5 fold) and *Csf3* (1.95 fold) in female mice (Figure S4H). All together our results suggest that neutrophil migration, ROS production and survival in the airway might be impaired in female mice.

The majority of studies do not report the use of both sexes of mice (37), including those understanding the innate immune response to *A. baumannii* pneumonia. Two previous reports made side observations of increased bacterial burden in female mice but the underlying causes were not pursued (38, 39). In one study, female mice infected similarly to our study displayed increased disease when compared to male mice and this was true for both C57BL/6 and BALB/c mice (38). C57BL/6 mice were more susceptible to *A. baumannii* than BALB/c mice, which has been attributed in other

infections to increased neutrophil influx and increased phagocytic capacity and oxidative burst of alveolar macrophages in BALB/c mice (40). Another open question and area for future investigation is how the female immune system directly impacts the immune response to *A. baumannii*. Studies in humans are limited. One study showed a significant predominance of infection with the male population however, this did not go into detail regarding severity of infection and patient outcome (41). Alternatively, this could be evidence of a fundamental difference that does not agree between mice and humans.

It has been seen in the human female population a bias exists towards several infectious diseases. These infections include *Legionella pneumophila*, HIV, malaria and influenza (12). In mice, greater mortality is observed in female mice for influenza infection, with estrogen leading to a higher inflammatory response (42), akin to what we observed in female mice infected with *A. baumannii*. In humans, evidence exists that females fair worse for several lung inflammatory conditions including cystic fibrosis, COPD, asthma and non-CF-related bronchiectasis (43, 44). Potential mechanisms behind these biases have included genetic predisposition, comorbidities and sex hormones. In female asthmatics for example, lung function is at its lowest during the luteal phase of the menstrual cycle when there is high estrogen and progesterone (45). Direct evidence as to how the hormones influence these conditions is not fully known. While our own respiratory studies with *Staphylococcus aureus*, *Klebsiella pneumoniae* or *Streptococcus pneumoniae* (46-49) have not indicated a significant susceptibility to any particular sex in mouse models, it has been shown that female mice

are more susceptible to respiratory *P. aeruginosa* infection (15). Consistent with our observations, female mice intranasally infected with *P. aeruginosa* have increased rates of mortality and levels of inflammatory cytokines, in addition to increased neutrophil necrosis (15). We observed that depletion of neutrophils led to increased bacterial burdens in our study irrespective of sex, so the observed increase in neutrophil necrosis in females both in our study and Abid *et al* (15) is a likely reason behind the sex bias. Why this is not observed in other bacterial respiratory models in mice could be due to differences in animal models and humans. Neutrophilic patients are at much greater risk of *S. aureus* pneumonia (50), yet depletion of neutrophils in mice does not change bacterial burden (51), unlike what we observed with *A. baumannii*.

Estrogen has also been shown to influence nutritional immunity. In the host iron homeostasis is controlled by the hepcidin-ferroportin axis, with hepcidin acting as the master regulator. It has been documented that there is an estrogen response element in the promoter region of hepcidin (52, 53), such that exogenous addition of estrogen leads to its repression (54). In mice lacking estrogen there is less hemoglobin and reduced iron levels in the serum. As iron is an essential nutrient this could be a mechanism behind sex bias in bacterial infections. It has also been observed that in male mice treated with estrogen, a significant reduction in lactoferrin was observed in the context of pulmonary *P. aeruginosa* infection (55). Lactoferrin is an important iron chelating antimicrobial compound as its absence increases the available free iron available to pathogens. Lactoferrin has also been shown to be able to inhibit *P. aeruginosa* biofilm formation (56), an important aspect of its pathogenic life cycle. While

we did not specifically test for lactoferrin, as with most organisms, iron is an important aspect of *A. baumannii* pathogenesis (57) and is a potential treatment strategy (58), and could also be a reason behind our observed sex phenotype.

Our findings reflect a fundamental difference in the innate immune response to *A. baumannii* infection in the airway between female and male mice. By gaining a better understanding of the host response we may identify elements of the immune system that could be targets for immunomodulatory therapy. Therefore, given these results, studies moving forward should address differences between the sexes. By identifying sex related immune differences, pre-clinical models can be designed appropriately to control for these difference and better develop therapeutics efficacious to both sexes, and has potential implications between sexes and males receiving hormone therapy in the general population.

## **METHODS**

### **Bacterial culture**

*Acinetobacter baumannii* strains 5075 and 19606 were grown in Luria-Bertani (LB) broth at 37°C to exponential phase (optical density of 1.0 at 600 nm). Growth curves were carried out in LB and M9 media with 2 mM MgSO<sub>4</sub>, 0.1 mM CaCl<sub>2</sub>, 0.1% casamino acids and 0.5% glucose with 20 µg/ml of β-estradiol or ethanol vehicle control.

### **Animal studies**

Six to eight week old female and male C57BL/6J WT mice were intranasally infected while under anesthesia with  $1 \times 10^7$  cfu of *A. baumannii* for 4 h or 24 h. For mortality studies C57BL/6 WT mice were intranasally infected with  $10^8$  cfu of *A. baumannii* and mortality assessed for 7 days. Weight matched mice were purchased from Jackson Laboratories. Male mice were treated with either  $17\beta$ -estradiol (10 $\mu$ g/mouse; Sigma) or vehicle control (sesame oil; Sigma) intraperitoneally, daily for 5 days before infection with *A. baumannii*. Respiration inhibition was conducted using 8 mg/kg of rotenone 4 h prior to infection (59). Lungs were fixed in 4% paraformaldehyde overnight to prepare paraffin blocks for hematoxylin and eosin (H&E)–stained slides. Images were taken using a Keyence BZ-X710 microscopy. Bronchoalveolar lavage fluid (BALF) was obtained from three successive washes with 1 ml of PBS. BALF was used to enumerate bacterial counts, quantify cytokine levels (mouse 32-plex; Eve Technologies), stain cells for flow cytometry and assess protein content using Bradford reagent. Lung and spleen tissue was homogenized in 400  $\mu$ L of PBS and used to enumerate bacterial counts. Temperature was quantified using an external infrared thermometer.

### **Flow cytometry**

Cells in BALF were stained with fluorescently labeled antibodies: CD45-AF700 (30-F11) CD11b-PE-Cy7 (M1/70), Ly6G-PerCP-Cy5.5 (1A8), CD11c-BV605 (N418), CD45-AF700 (30-F11), BV510-CD103 (2E7), Siglec-F-AF647 (E50-2440; BD Biosciences), MHCII-APC-Cy7 (M5/114.15.2), Ly6C-PE-Texas Red (AL-2; BD Biosciences) and DAPI to assess cell viability. Antibodies were from Biolegend unless otherwise stated. Cells in the airway were classified as followed: neutrophils-live CD45<sup>+</sup>/SiglecF<sup>-</sup>/MHCII<sup>-</sup>

/Ly6C<sup>+</sup>/CD11b<sup>+</sup>; alveolar macrophages-live CD45<sup>+</sup>/Ly6C<sup>-</sup>/CD11c<sup>+</sup>/SiglecF<sup>+</sup>; eosinophils-live CD45<sup>+</sup>/Ly6C<sup>-</sup>/CD11c<sup>-</sup>/SiglecF<sup>+</sup>/CD11b<sup>+</sup>; interstitial macrophages-live CD45<sup>+</sup>/Ly6C<sup>-</sup> SiglecF<sup>-</sup>CD11b<sup>+</sup>CD11c<sup>+</sup>MHCII<sup>+</sup>; Ly6C<sup>+</sup> monocytes-live CD45<sup>+</sup>/Ly6C<sup>+</sup>/CD11b<sup>+</sup>/MHCII<sup>-</sup>/Ly6G<sup>-</sup>; Ly6C<sup>-</sup> monocytes-live CD45<sup>+</sup>/Ly6C<sup>-</sup>/SiglecF<sup>-</sup>/CD11b<sup>+</sup>/CD11c<sup>+</sup>/MHCII<sup>-</sup>; plasmacytoid dendritic cells (DC)-live CD45<sup>+</sup>/Ly6C<sup>+</sup>/CD11b<sup>-</sup>/CD11c<sup>+</sup>/MHCII<sup>+</sup>; CD103 DC-live CD45<sup>+</sup>/Ly6C<sup>-</sup>/SiglecF<sup>-</sup>/CD11b<sup>-</sup>/MHCII<sup>+</sup>/CD103<sup>+</sup> and CD11b DC-live CD45<sup>+</sup>/Ly6C<sup>+</sup>/CD11b<sup>+</sup>/MHCII<sup>+</sup>/CD11c<sup>+</sup>. Reactive oxygen species production and cell death in cells was measured using CellRox (Life Technologies).

### **Cell depletion**

Alveolar macrophages were depleted by inoculating intranasally anesthetized mice with 50 µl of either liposome containing clodronate (5 mg/mL) or liposome only controls (Encapsula NanoSciences) 24 h before infection with *A. baumannii*. Efficiency of depletion was assessed by flow cytometry. To deplete neutrophils, mice were injected intraperitoneally either with 250 µg of α-Ly6G (clone 1A8; Bioxcell) or IgG2a, κ isotype control 24 h before infection. Efficiency of depletion was assessed by flow cytometry.

### **Cell culture**

Neutrophils were isolated from the bone marrow of the femurs and tibias of naïve female and male mice by density gradient centrifugation as previously described (48). Bone marrow derived neutrophils were used for experiments on the day of purification. To evaluate bacterial killing capacity, neutrophils were incubated with *A. baumannii* (MOI of 10) for 60 minutes with slow rotation at 37°C after which the reaction was

stopped at 4°C and cells were lysed with 1% saponin. Bacterial numbers were determined by serial dilutions. Alveolar macrophages were purified from the airway of naïve female and male mice by bronchoalveolar lavage (BAL). To evaluate bacterial killing capacity, alveolar macrophages were incubated with *A. baumannii* (MOI of 10) for 4 h, after which intracellular bacterial numbers were determined by serial dilutions. Immortalized bone marrow macrophages and RAW cells were cultured in RPMI 1640 media with penicillin, streptomycin and 10% heat inactivated fetal bovine serum. Cells were seed and treated with 100 nM of  $\beta$ -estradiol 2 h prior to incubation with *A. baumannii* (MOI 10) for 2 h at which point bacteria numbers were quantified.

### **ATP and Lactate assays**

ATP and lactate levels were measured in BALF samples from clodronate depleted female and male mice following a 4 h infection with *A. baumannii*. ATP levels were determined using the ATP colorimetric Assay Kit (BioVision, Inc.) following manufacturer's instructions. Lactate levels were determined using the fluorimetric EnzyFluo L-Lactate assay Kit (BioAssay Systems) following manufacturer's instructions. To avoid degradation of lactate by the presence of endogenous lactate dehydrogenase (LDH), samples were filtered through a 10Kd mw spin filter to remove all proteins.

### **LDH assay**

LDH was determined in BALF samples from clodronate depleted female and male mice following a 4 h infection with *A. baumannii* using the CytoSelect LDH Cytotoxicity assay kit (Cell Biolabs, Inc.) and following the manufacturer's instructions.

## **ROS measurement**

ROS levels were measured in BALF samples from clodronate depleted female and male mice following a 4 h and 24 h infection with *A. baumannii*. Carboxy-H<sub>2</sub>DCFDA (Molecular Probes) was added to 50 µL of BALF to a final concentration of 10 µM, and carboxydichlorofluorescein fluorescence was read at Ex495 nm/Em520 nm after 1 hour.

## **RNA-sequencing**

RNA was extracted from whole lungs using The Direct-zol™ RNA MiniPrep Kit (Zymo Research), following manufacturer's instructions. RNA-seq was performed by Novogene. mRNA was purified from 1 µg of total RNA using poly-T oligo-attached magnetic beads and then fragmented using fragmentation buffer. The first strand of cDNA was synthesized using a random hexamer and M-MuLV reverse transcriptase with the second strand of cDNA synthesized using DNA polymerase I and RNase H. cDNA was purified using AMPure XP beads (Beckman Coulter), converted to blunt ends, adenylated and New England Biolabs (NEB) adaptors ligated. Sequencing was performed on a Novoseq 6000 with 20M PE150 reads.

Sequencing data was analyzed using Partek. Reads were trimmed with Phred scores <30, 20 bp minimum read length and a maximum N of 10%. Adaptor sequences were removed. Trimmed reads were aligned using STAR and unaligned reads further aligned using TopHat 2. Combined alignments were quantified to the annotation model (Partek) and low read counts (N<10) were filtered. Filtered counts were normalized by adding 1

and using trimmed mean of M-values normalization (TMM). Differential analysis was conducted using ANOVA and Limma-voom. Genes with P-values < 0.05 and fold changes > 1.5 were used for gene set enrichment using Partek and Panther. Hierarchical clustering was based on the union of differentially expressed genes across all comparisons using an average linkage cluster metric and Euclidean point distance metric. RNA-seq data is deposited in GEO as GSE134805.

### **Microbiome analysis**

Mouse fecal pellet samples were thawed to room temperature from the  $-80^{\circ}\text{C}$  freezer and DNA extracted using the QIAamp PowerFecal DNA Kit according to the manufacturer's instructions. A column with no fecal pellet was included as a DNA free control. Extracted DNA was quantified using a Qubit 3.0 fluorometer and the Qubit dsDNA HS Assay kit (Thermo Fisher Scientific). Isolated mouse fecal pellet DNA samples were used to prepare libraries according to the Illumina protocol for 16S Metagenomics Sequencing Library Preparation (15044223 B). The V3 and V4 regions of the 16S rRNA were amplified using Illumina 16S amplicon primers (Forward Primer = 5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG and Reverse Primer = 5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATC C) using the 2X KAPA HiFi HotStart ReadyMix kit (Kapa Biosystems) and the 16S rRNA amplicons purified using AMPure XP beads (Beckman Coulter). Index PCR was performed using the Nextera™ DNA CD Indexes kit (Illumina) with 2X KAPA HiFi HotStart ReadyMix and indexed PCR products were purified as before using AMPure

beads. The indexed libraries were quantified using the Qubit 3.0 and Qubit dsDNA HS Assay kit and diluted in 10mM Tris-HCL to a final concentration of ~0.3ng/μl (1nM). A pool containing 5 μl of each library was generated and 5 μl of the pool was diluted in 95 μl of 10mM Tris-HCL to make a final pooled library concentration of 50 pM. Prior to sequencing on the iSeq, a 10% spike in of 50 pM PhiX was added to the pooled library. 20 μl of the pooled library with PhiX spike in was loaded onto the iSeq. Sequencing data was uploaded to the BaseSpace Sequence Hub and reads were demultiplexed using a FASTQ Generation script. The run yielded 2.05 Gbp with an average %Q30 of 91.8%. QIIME2 was used to assign taxonomic units to 16S rRNA sequences using DADA2 (Divisive Amplicon Denoising Algorithm) to filter and infer bacterial taxa to amplicons (60). Taxonomic units were assigned to DADA2 Feature IDs using the Silva taxonomy classifier (61). PCA analysis and extended bar plots representing the taxonomic diversity of samples was performed using statistical analysis of taxonomic and functional profiles (STAMP) (62). Processed sequence reads were submitted to SRA (Bioproject:PRJNA560086).

## **Statistics**

Animal data was assessed using a nonparametric Mann-Whitney test. In vitro experiments were assessed using an unpaired Student's 2 tail t-test. A P value less than 0.05 was considered significant. Statistics were performed with Prism software (GraphPad, La Jolla LA USA).

## **Study approval**

Animal work in this study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory animals of the National Institutes of Health, the Animal Welfare Act, and U.S. federal law. Protocols were approved by the Institutional Animal Care and Use Committee of Rutgers New Jersey Medical School of Newark New Jersey.

### **AUTHOR CONTRIBUTIONS**

S.P. and D.P. designed and conducted the experiments, analyzed the data and wrote the manuscript. A.P. conducted experiments. J.S. and D.S.S. extracted DNA from fecal matter and conducted the 16S rRNA analysis of the microbiome. D.P provided funding.

### **ACKNOWLEDGMENTS**

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## FIGURE LEGENDS

### **Figure 1. Female mice are more susceptible to pulmonary *A. baumannii* infection.**

A) Weights of mice before infection. B) Mice were intranasally infected with  $10^8$  cfu of *A. baumannii* 5075 and mortality assessed (n=26 female, n=8 male). C-E) Weight matched female and male C57BL/6J WT mice were intranasally infected with  $10^7$  cfu of *A. baumannii* 5075 for 24 h (n=12). B) Weight of mice before infection. C) BALF, lung and spleen homogenates were enumerated for bacterial counts 24 h after infection (n=9). D) Change in external temperature of female and male mice 24 h after intranasal infection. E) Protein content in BALF. n=4 and n=5 for uninfected female and male mice respectively. F) Representative hematoxylin and eosin-stained lung sections of mice at 24 h post infection (scale bar, 1 mm – left panel; 100  $\mu$ m – right panel). G&H) Male mice were treated with 17  $\beta$ -estradiol or vehicle control for five days prior to intranasal infection with  $10^7$  cfu of *A. baumannii* 5075 (n=11). G) BALF, lung and spleen homogenate were enumerated for bacterial counts 24 h after infection. H) Change in external temperature of mice after infection (n=12). Each point represents a mouse. Lines display medians. Data is from three independent experiments. \*\*\*\*P<0.0001, \*\*\*P<0.001, \*\*P<0.01 and \*P<0.05. Mortality data was assessed using a Fishers exact test. A nonparametric Mann-Whitney test was used to assess differences between groups.

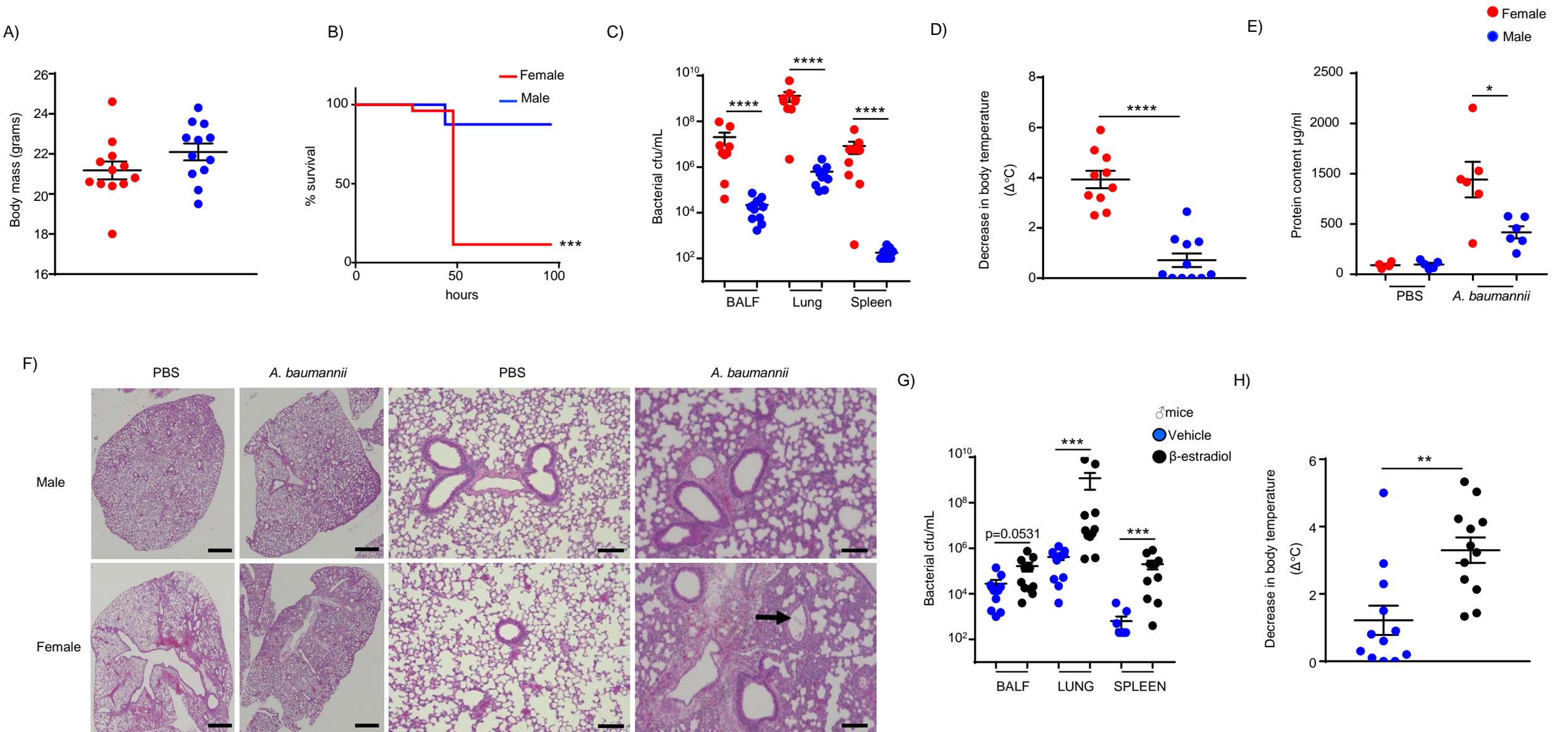
**Figure 2. Female mice have exacerbated inflammation and reduced immune cell recruitment to the airway in response to *A. baumannii*.** A) Flow cytometry analysis of neutrophils and alveolar macrophages present in the airway of female and male weight matched mice before and 24 h after intranasal infection with *A. baumannii*. Infected mice n= female-8; male-10; n=2 for uninfected. B) Cytokine levels in BALF (Infected mice n= female-8; male-10). Heat map is log<sub>10</sub> scale of cytokines in pg/ml, with female/male ratio shown and statistical significance. Select cytokines are shown individually. C) Bacterial numbers were assessed 4 h after infection of purified alveolar macrophages from both sexes of mice with *A. baumannii* (n=14). D) Bacterial numbers were assessed 1 h after infection of bone marrow derived neutrophils from female and male mice with *A. baumannii* (n=12). E) WT C57BL/6J mice were infected with 10<sup>7</sup> cfu of *A. baumannii* for 24 h and neutrophils were treated with CellRox reagent to assess ROS production and cell death. Male n=8 and female n=7. F) Immortalized bone marrow macrophages (iBMM) and G) RAW macrophages were incubated with β-estradiol or vehicle overnight before incubating with *A. baumannii* for 2 h before killing was assessed. iBMM, control n=4, β-estradiol n=6; RAW control n=6, β-estradiol n=5. Graphs show means with standard error. Data is from at least two independent experiments \*\*\*\*P<0.0001, \*\*\*P<0.001, \*\*P<0.01, \*P<0.05. A nonparametric Mann-Whitney test was used to assess differences between groups.

**Figure 3. Depletion of alveolar macrophages results in a significantly higher bacterial burden in female than male infected mice.** A) Representative flow cytometry contour plot showing neutrophils in BALF of mice that were treated with either

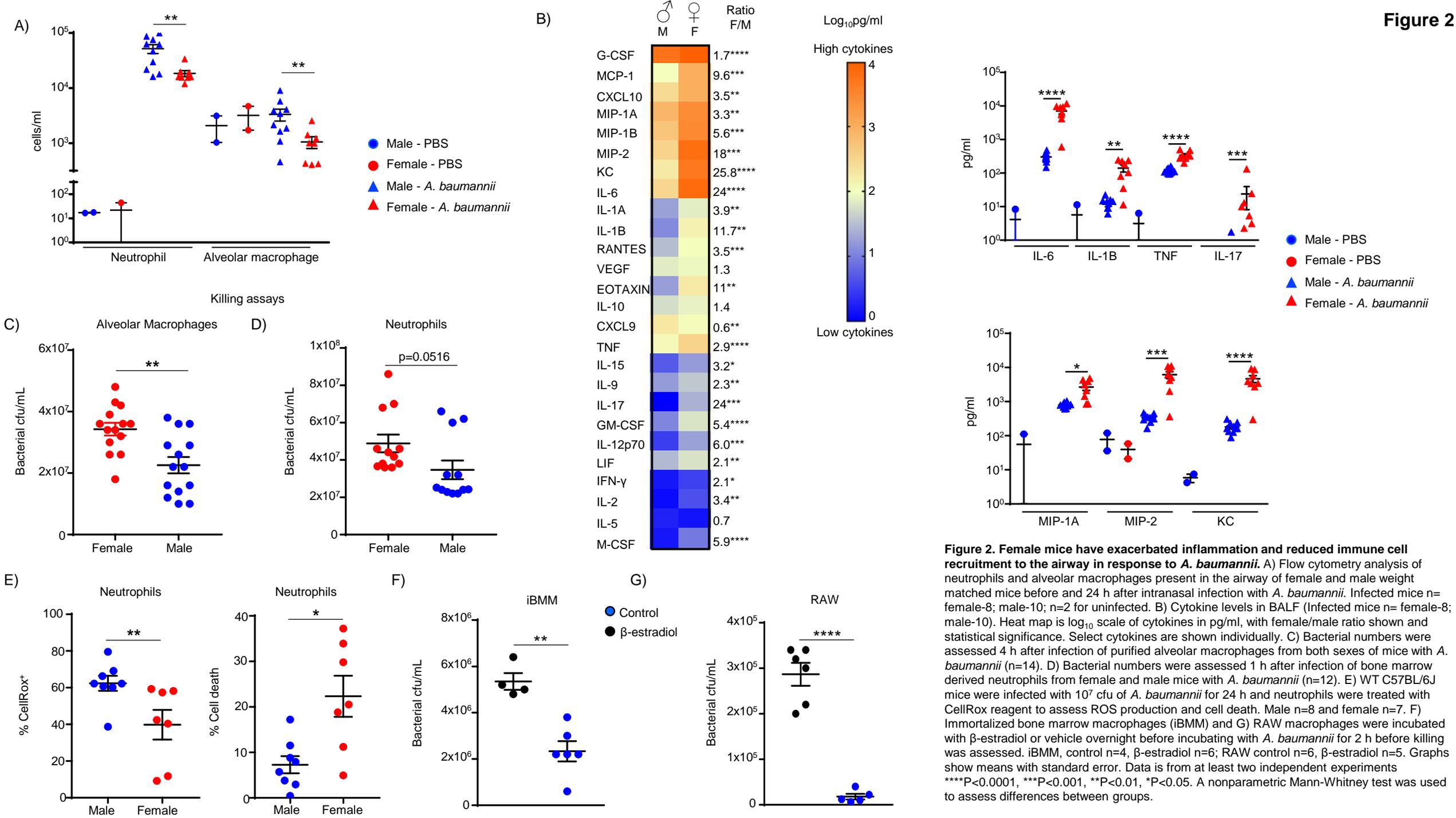
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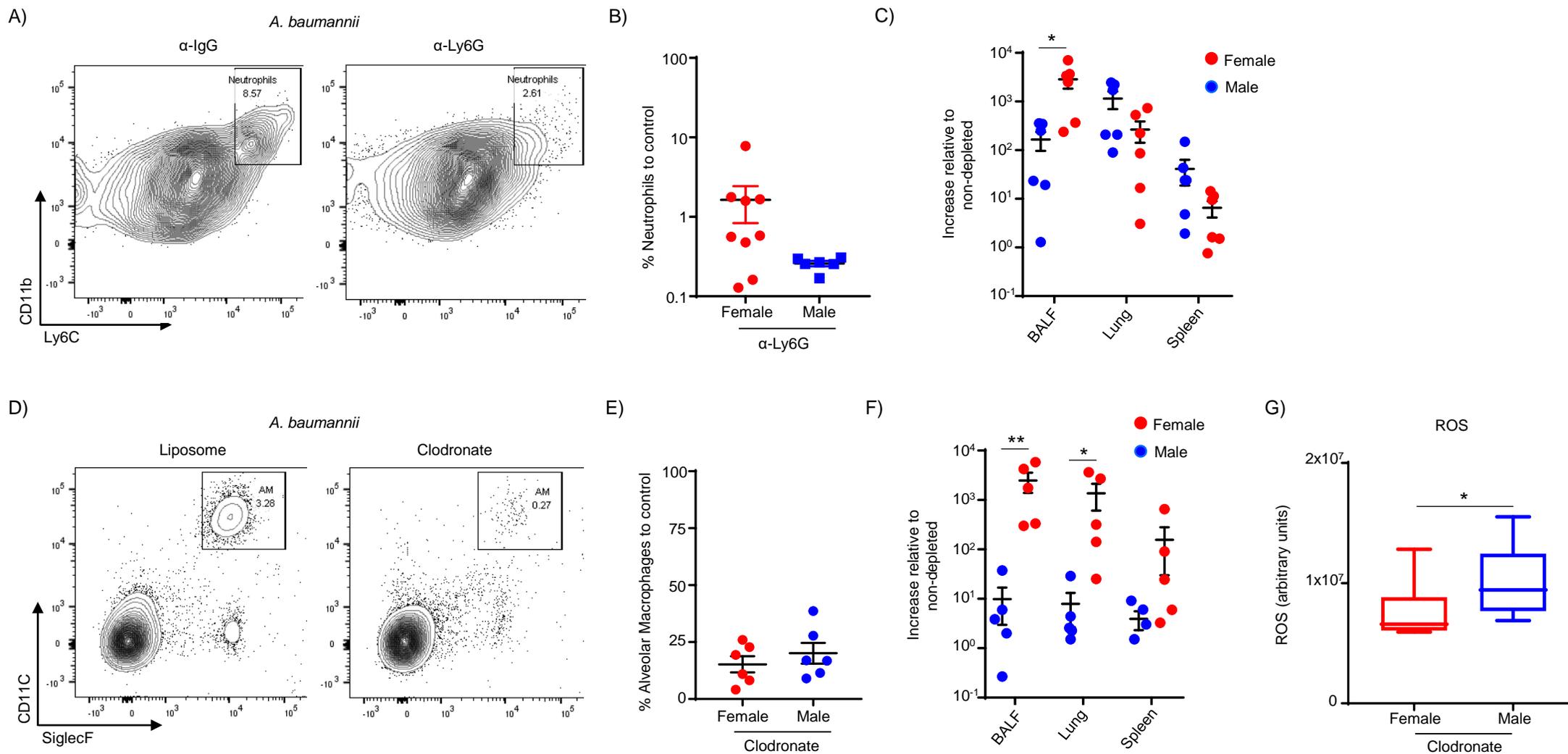
**Figure 4. Alveolar macrophage depletion results in mitochondrial defects and energetic imbalance in female infected mice.** A) Heat map of RNA-seq analysis of the comparison between female and male mice, depleted of alveolar macrophages, following a 4 h infection. Each column represents an individual mouse. B) Volcano plot of differentially expressed genes. C) Gene ontology analysis of differentially expressed

genes that are down regulated in female mice compared to males. D&E) ATP and lactate levels, respectively, in BALF following a 4 h infection in female and male depleted of alveolar macrophages. n=12. Box and whisker plots show mean with upper and lower quartiles, with whiskers at minimum and maximum values. F) Bacterial counts from BALF, lung and spleen of *A. baumannii* infected mice that had been treated with rotenone or vehicle control. Control n=8, rotenone n=11. Graphs show means with standard error. Data is from two independent experiments. \*\*\*\*P<0.0001, \*P<0.05. A nonparametric Mann-Whitney test was used to assess differences between groups.

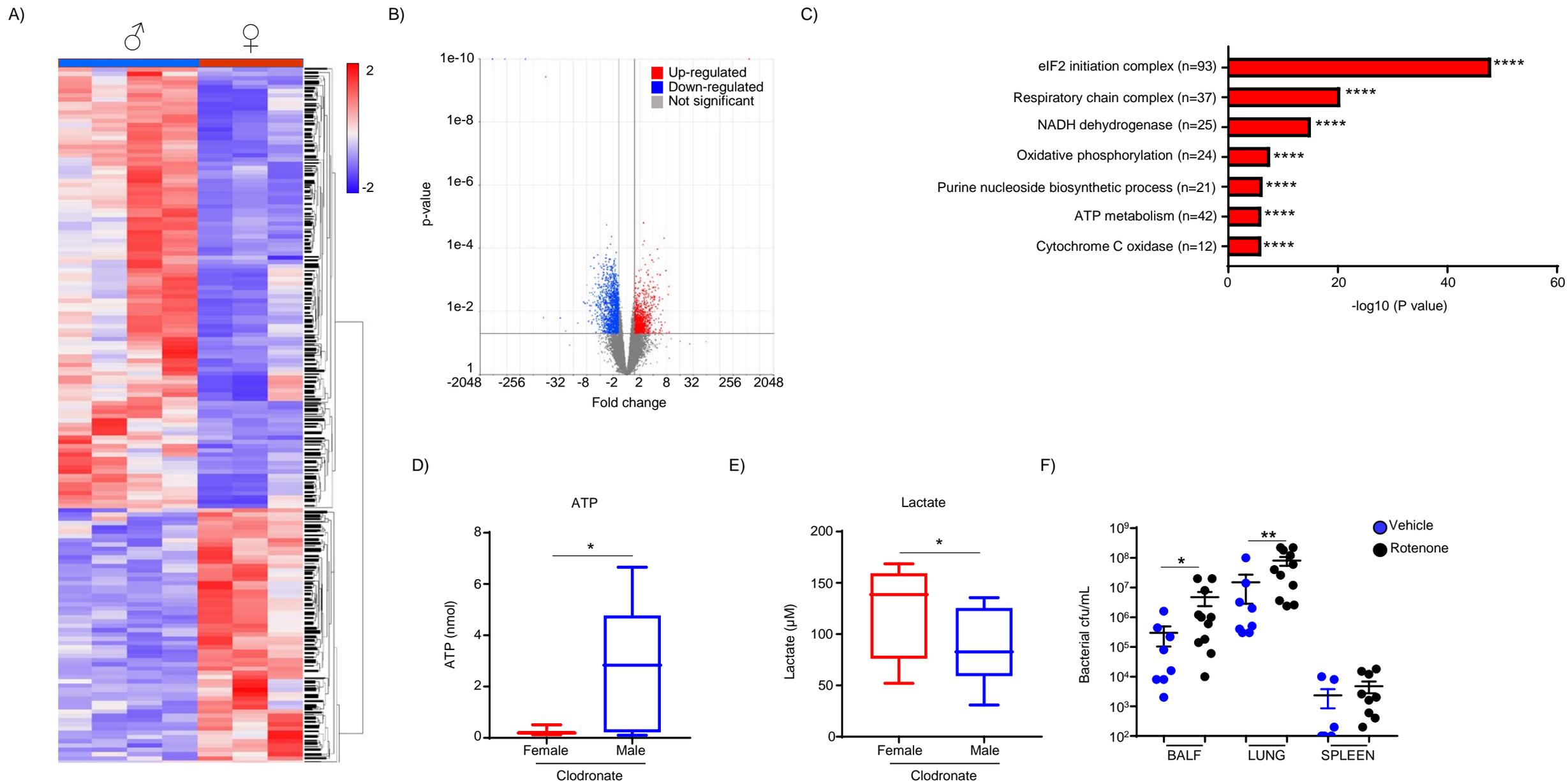
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**Figure 3**

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