Testosterone promotes Coxsackievirus B3 pathogenesis in an oral inoculation mouse model

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Running Title: CVB3 fecal shedding and pathogenesis is enhanced by testosterone

Abstract

Enteroviruses initiate infection in the gastrointestinal tract, and sex is often a biological variable that impacts infection. The role of sex hormones on enterovirus pathogenesis, however, is unclear. Previous data indicate that sex hormones can influence intestinal replication of Coxsackievirus B3 (CVB3), an enterovirus in the Picornavirus family. To determine if testosterone promotes CVB3 infection, male mice were castrated and provided placebo or testosterone-filled capsules. We found that testosterone-treated mice shed significantly more CVB3 in the feces and succumbed to CVB3-induced disease at a higher rate than castrated mice given a placebo. Treatment of male mice with an androgen receptor antagonist, flutamide, protected male mice from CVB3-induced lethality, further confirming the role of testosterone in viral pathogenesis. We also observed higher viral loads in peripheral tissues of testosterone-treated mice and an increase in the cytokine and chemokine response. Finally, we found that testosterone treatment in female mice increased fecal CVB3 shedding but had no impact on viral lethality. Overall, these data indicate that testosterone and androgen receptor signaling can promote CVB3 replication in the intestine and enhance CVB3 lethality in a sex-dependent manner.

47 **Importance**

48 Biological sex plays a significant role in the outcome of various infections and diseases. The
49 impact of sex hormones on intestinal replication and dissemination of Coxsackievirus B3
50 remains limited. Using an oral inoculation model, we found that testosterone enhances CVB3
51 shedding and lethality in male mice. Further, testosterone can promote CVB3 shedding in female
52 mice. This work highlights the role of testosterone in intestinal replication of CVB3 and suggests
53 that sex hormones can impact the replication of enteric viruses.

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Introduction

Sex plays a significant role in human disease. Males are often more susceptible to pathogens, while females are more predisposed to autoimmune diseases (1-4). This disparity is linked to the immune system as sex hormones can significantly affect immune responses. Androgens, such as testosterone, can suppress immunity, delaying the elimination of pathogens (5-9). On the other hand, estrogens can enhance both the cell-mediated and humoral immune response, and there is evidence supporting estrogen's influence on disease severity in cardiovascular diseases and traumatic brain injuries (10-16). However, the mechanism and consequences of sex hormones on infectious diseases remain limited.

Coxsackievirus is a non-enveloped RNA virus in the *Picornaviridae* family and implicated in several human diseases, including viral myocarditis, HFMD, and meningitis (17-19). Infants, children, and immunocompromised individuals are the most susceptible to these diseases, and Coxsackievirus is associated with an 11% fatality rate in neonates (20-22). Coxsackievirus B3 (CVB3) is one of the leading causes of viral myocarditis. There is a strong sex bias in viral myocarditis as it affects more males than females, with a mortality rate of 2:1 in individuals infected under the age of 40 (23, 24). Animal models of CVB3 have provided further evidence of this sex bias. Our laboratory, and others, have shown that sex hormones contribute to CVB3 pathogenesis. Castration of male mice before infection reduces CVB3-induced myocarditis and mortality (25, 26). In CVB3-infected mice, dysregulation of the immune response is hypothesized to contribute to mortality. Testosterone and estradiol influence the $CD4^+$ T cell response to CVB3 to either promote or protect against viral-induced myocarditis (27-29). Castration of male mice also significantly reduces anti-inflammatory M2 macrophages resulting

in more severe myocarditis following CVB3 infection, suggesting other immune cells contribute to myocarditis (30). Finally, genes on the Y chromosome also likely contribute to CVB3-induced disease, indicating that viral myocarditis is multifactorial in males (31, 32). Therefore, many questions remain as to the mechanism of the sex bias in Coxsackievirus infections.

While animal models have provided vital information on CVB3 pathogenesis, many of these models mimic systemic infection through intraperitoneal injections of the virus. However, Coxsackievirus is a member of a group of viruses transmitted through the fecal-oral route, termed enteroviruses. Very few studies have shown successful oral inoculation of mice with Coxsackievirus (33-37). Recently we established an oral inoculation model to examine CVB3 replication in the intestine. Using this model, we also observed a sex bias in infection. We found that male mice support robust intestinal replication and succumb to CVB3 induced disease. Female mice, however, are mainly resistant to CVB3 with limited viral replication in the intestine (38). Gonadectomy before CVB3 infection impacted fecal shedding in mice and protected male mice from CVB3-induced lethality, suggesting that sex hormones contribute to viral pathogenesis. Here, we show that testosterone enhances CVB3 shedding in male and female mice. Testosterone also enhanced lethality in male mice, but not in female mice. Overall, these data highlight the importance of examining sex bias in CVB3 induced disease.

Results

Testosterone and androgen receptor signaling enhances CVB3 lethality in male mice following oral inoculation. We have previously established an oral inoculation model for CVB3 using C57BL/6 *Ifnar*$^{-/-}$ (deficient for that interferon α/β receptor) mice (38). We found that

116 castration of male *Ifnar*$^{-/-}$ mice prior to oral infection with CVB3 protected against CVB3-
117 induced lethality and significantly reduced CVB3 fecal shedding. To determine if testosterone,
118 specifically, could impact CVB3 pathogenesis, we surgically castrated male *Ifnar*$^{-/-}$ mice to
119 deplete endogenous testosterone. As a control, we also performed mock castrations on male
120 mice. One week after surgery, we implanted mice with placebo or exogenous testosterone
121 capsules. To assess hormone replacement, we determined serum concentrations of testosterone
122 by ELISA. Castrated mice that received the placebo capsule had a significantly lower serum
123 testosterone concentration than mock-castrated and castrated male mice that received
124 testosterone (Fig. 1A). Further, mock castrated and castrated mice receiving exogenous
125 testosterone had similar amounts of serum testosterone, confirming successful hormone
126 treatment. One week following hormone treatment, male mice were orally inoculated with $5x10^7$
127 PFU CVB3 and monitored for survival for 14 days post-inoculation (dpi). We found that, like
128 our previous study (38), testosterone-depleted mice (castrated + placebo) were protected from
129 CVB3-induced lethality (Fig 1B). Further, mock castrated mice and testosterone-treated
130 (castrated + testosterone) mice were significantly more likely to die from CVB3 inoculation than
131 testosterone-depleted mice. These data indicate that testosterone contributes to CVB3-induced
132 lethality in male mice.
133
134 To confirm the effects of testosterone on male *Ifnar*$^{-/-}$ mice, we hypothesized that treatment with
135 an androgen receptor antagonist, flutamide, would also confer similar protection against CVB3
136 infection. To test this hypothesis, we administered flutamide (25µg/kg) by intraperitoneal
137 injections to male *Ifnar*$^{-/-}$ mice prior to oral inoculation with CVB3 and monitored mice for
138 survival for 14 dpi. We found that mice treated with flutamide were protected entirely from

CVB3-induced lethality compared to mice provided vehicle control injections (Fig. 1C). Taken together, these data indicate that testosterone and androgen receptor signaling in orally CVB3 inoculated male *Ifnar*$^{-/-}$ mice is a significant contributor to viral-induced lethality.

Testosterone promotes CVB3 fecal shedding and viral dissemination to peripheral tissues. CVB3 is spread through the fecal-oral route; therefore, we next examined CVB3 fecal shedding, indicative of viral replication in the intestine (38). Following oral inoculation, we collected feces at 1, 2, and 3 dpi and quantified fecal CVB3 using a plaque assay on HeLa cells. We found that at 2 and 3 dpi, testosterone-depleted mice shed significantly less CVB3 in the feces than mock-castrated male mice (Fig. 2A and 2B). Further, castrated mice receiving exogenous testosterone shed similar levels of CVB3 as compared to mock-castrated mice and significantly more CVB3 than testosterone-depleted mice. Since we observed higher fecal shedding in mice with testosterone, we hypothesized that these mice with testosterone would also have higher viral titers in the peripheral tissues following oral inoculation. To examine this hypothesis, we harvested the heart, liver, kidney, spleen, and pancreas at three dpi and quantified CVB3 tissue titers by a plaque assay. Similar to fecal shedding, we observed higher tissue CVB3 titers for mock castrated and testosterone-treated mice than testosterone-depleted mice (Fig. 2C). Taken together, these data suggest that testosterone enhances fecal shedding and promotes viral dissemination of CVB3 to peripheral tissues following replication in the intestine.

The inflammatory response to CVB3 is enhanced in mice with testosterone. The immune response plays a pivotal role in tissue damage following CVB3 infection (27, 39-43). Previously, we observed a systemic immune response to CVB3 in male mice as measured by serum cytokine

and chemokine levels (38). To test whether testosterone altered the magnitude of the cytokine and chemokine response, we measured the serum concentrations of 32 cytokine and chemokines at 3 dpi. In mock castrated mice, we saw an overall increase in the serum concentrations of pro-inflammatory cytokines and chemokines IL-6, IFN-γ, TNF-α, IP-10, MCP-1, and RANTES compared to uninfected males (Fig 3). The only cytokines and chemokines significantly altered by testosterone were KC and G-CSF, which were found in greater serum concentrations in mock castrated and testosterone-treated mice than uninfected mice. In contrast, testosterone-depleted mice had a decrease in serum cytokine and chemokine concentration levels consistent with lower levels of viral replication in the peripheral tissues. These data indicate that male *Ifnar*$^{-/-}$ mice with testosterone elicit a more robust immune response than testosterone-depleted mice following oral inoculation of CVB3.

CVB3 does not induce liver damage or cause pancreatitis in *Ifnar*$^{-/-}$ mice following oral inoculation. We found that CVB3 tissue titers were the highest in the liver and pancreas following CVB3 inoculation; therefore, we hypothesized that testosterone could enhance liver necrosis and/or pancreatitis in *Ifnar*$^{-/-}$ mice. First, to test this hypothesis, we examined alanine aminotransferase (ALT) and lipase levels in the serum by ELISA at 3 dpi to determine liver and pancreas damage, respectively. We observed no significant increase in ALT levels or lipase levels in the serum of mock or castrated male mice following oral inoculation (Fig. 4A and 4B). Next, we examined histological sections of the liver and the pancreas from male mice sacrificed at 3 dpi. Similar to ELISA results, we observed no evidence of hepatic necrosis or inflammation in the liver (Fig. 4C). Further, we did not observe a loss of acinar cells, beta cells, or

inflammation in the pancreas (Fig. 4D). These data indicate that CVB3-Nancy does not induce liver damage or pancreatitis after oral inoculation of *Ifnar*$^{-/-}$ mice.

Testosterone does not enhance CVB3-induced lethality in female mice but does enhance fecal shedding and viral dissemination. Previously we found that orally CVB3 inoculated female *Ifnar*$^{-/-}$ mice were protected entirely from CVB3-induced lethality and had limited viral fecal shedding (38). Since exogenous testosterone treatment restored fecal shedding and lethality in male mice, we hypothesized that testosterone might enhance pathogenesis in female mice. To examine this hypothesis, we provided exogenous testosterone or placebo capsules to female mice before oral inoculation with CVB3. Like our castration studies, female mice with testosterone had significantly higher serum testosterone concentrations than placebo controls (Fig. 5A). One week after hormone treatment, female mice were orally inoculated with $5x10^7$ PFUs of CVB3 and monitored for survival. In contrast to males, both testosterone- and placebo-treated infected females were protected from CVB3-induced lethality (Fig. 5B). Next, to examine fecal CVB3 shedding, feces were collected from infected mice at 1, 2, and 3 dpi, processed, and quantified by a standard plaque assay. We observed significantly more CVB3 in the feces at 3 dpi in female mice that received testosterone than female mice that received placebo capsules (Fig. 5C). Finally, we examined CVB3 titers in peripheral tissues at 3 dpi to test if testosterone enhanced viral dissemination. We found a significant increase in the viral loads of the heart, kidney, and spleen in testosterone-treated female mice (Fig. 5D). The viral load in the liver was higher in testosterone-treated mice but did not reach statistical significance. In contrast to the other organs, the viral load in the pancreas was similar between placebo- and testosterone-treated female mice. Overall, these data indicate that testosterone can enhance fecal CVB3 shedding and viral

207 dissemination to the heart, kidney, and spleen; however, testosterone does not contribute to
208 CVB3-induced lethality in female mice.
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210 **Discussion**
211 Sexual dimorphism is commonly observed in various infectious, autoimmune, and
212 cardiovascular diseases. Males are often more susceptible to diseases caused by bacteria, viruses,
213 fungi, or parasites. Females, however, are more predisposed to autoimmune diseases like
214 systemic lupus erythematosus, multiple sclerosis, and rheumatoid arthritis. Additionally, clinical
215 and epidemiological studies have highlighted the disparity in cardiovascular diseases with an
216 increased mortality rate in men. CVB3 also displays a sex bias in human infections where males
217 are twice as likely to have severe sequelae. Previously, we observed a sex bias in CVB3 orally
218 inoculated male and female *Ifnar*$^{-/-}$ mice, consistent with human infections (38). Here we show
219 that testosterone, the primary sex hormone in males, impacts CVB3 pathogenesis following oral
220 infection.
221
222 Our data indicate that testosterone enhances intestinal replication of CVB3. We observed that
223 testosterone could promote fecal shedding of CVB3 in both male and female *Ifnar*$^{-/-}$ mice (Figs.
224 2A and 5C). To our knowledge, this is the first time that testosterone has been demonstrated to
225 influence the intestinal replication of an enteric virus. These data differ from previous studies
226 using systemic models of CVB3 infection. In this model, castration of wild-type, immune-
227 competent male mice did not impact viral replication in the heart (30). This difference may be
228 due to a sex bias in organ-specific replication of CVB3 and dependent on the type I IFN
229 response. In agreement, we have previously shown that while CVB3 replicates better in the

intestine of wild-type male mice, CVB3 replicated equally in the organs of wild-type male and female mice following systemic infection. Further, the loss of type 1 IFN also facilitates sex-dependent replication in the liver and spleen (38). Overall, these data suggest that organ-specific androgen signaling may play a significant role in CVB3 replication.

Unfortunately, the mechanism for the sex hormone modulation of intestinal replication is unclear. Previous data indicate that sex hormones, including testosterone, can enhance CVB3 attachment to cardiomyocytes (26); therefore, testosterone may alter the expression of viral receptors on intestinal cells. Additionally, testosterone can interact with immune cells and suppress inflammation (30, 44-47). It is possible that testosterone dampens the innate immune response in the intestine, promoting initial viral replication in the intestine. Our data indicate that the loss of testosterone broadly reduces the systemic cytokine and chemokine response to CVB3 (Fig. 3). However, it is unclear if this reduction is due to loss of testosterone or limited CVB3 replication and dissemination. Whether testosterone influences CVB3 binding to intestinal epithelial cells or alters the intestinal immune response during initial viral replication warrants further study.

Recent data also show that intestinal bacteria can influence the intestinal replication of enteric viruses, including CVB3 (48-50). We have recently demonstrated that specific intestinal bacteria in male mice play a role in promoting CVB3 infectivity and viral stability (51). While the mechanism is unclear for CVB3, a similar enterovirus, poliovirus, has been found to use bacteria to enhance attachment to the poliovirus receptor (49, 52). Interestingly, sex-specific differences in intestinal bacteria can account for the sex bias in mouse models of type-1 diabetes (53, 54).

Since bacteria can also metabolize hormones that alter bacterial growth (55), it is intriguing to speculate that sex hormones may change the overall intestinal ecosystem to directly or indirectly impact CVB3 replication.

With a systemic infection model of CVB3, previous studies have found that testosterone enhances lethality in male mice (25), consistent with our data. Castrating male mice, or by using flutamide, an androgen receptor antagonist, we were also able to rescue mice from CVB3-induced lethality (Fig. 1B and 1C). Interestingly, testosterone increased CVB3 shedding and viral dissemination in female mice in our model, but testosterone did not enhance lethality (Fig. 5). In agreement with our data, systemic models of CVB3 in infection have shown that while testosterone treatment promoted cardiac inflammation in female mice following CVB3 infection, testosterone did not increase mortality in female mice (25, 27). These data suggest that other sex-dependent factors may play a role in clearing the viral infection in female mice. Previous studies have shown that the Y chromosome contributes to CVB3 pathogenesis (31, 32); therefore, genes on the sex chromosomes may represent one of these critical factors. Future studies are required to identify Y-specific genes and other potential correlates of immune protection that may promote or limit CVB3 pathogenesis in mice.

The cause of death in our oral inoculation model remains unknown. CVB3 is the primary cause of viral myocarditis. However, in C57BL/6 mice, CVB3 also causes hepatic damage and pancreatitis (56-59). Since we observed high viral loads in the liver and pancreas of testosterone-treated animals (Fig 2C), we hypothesized that the male mice died of hepatitis or pancreatitis. However, contrary to our hypothesis, we did not observe inflammation or tissue damage in the

liver or pancreas following infection (Fig 4C and 4D). We confirmed these histology data by serum ALT and Lipase concentrations at 3 dpi (Fig 4A and 4B). The cause of death is still under investigation; however, enteric viruses, including poliovirus and Coxsackievirus, can cross the blood-brain barrier, infect the central nervous system, and cause viral meningitis and encephalitis (60, 61). Further studies are required to determine viral loads in the central nervous system and resolve the determinants of lethality in the oral inoculation model.

In conclusion, we found that testosterone promotes intestinal CVB3 replication in orally inoculated male and female *Ifnar*$^{-/-}$ mice. Further, testosterone enhances viral-induced lethality in a sex-dependent manner. The exact mechanism of how testosterone aggravates disease is unclear, but future studies will be necessary to determine how this sex hormone promotes intestinal CVB3 infection. Overall, these data reinforce the importance of sex as a biological variable in enteric viral infections.

Materials and Methods

Cells and virus

HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum and 1% penicillin-streptomycin at 37˚C with 5% CO_2. The CVB3-Nancy infectious clone was obtained from Marco Vignuzzi (Pasteur Institute, Paris, France) and propagated in HeLa cells as described previously (38). CVB3 was quantified by a standard plaque assay using HeLa cells.

Mouse experiments

All animals were handled according to the Guide for the Care of Laboratory Animals of the National Institutes of Health. All mouse studies were performed at Indiana University School of Medicine using protocols approved by the local Institutional Animal Care and Use Committee in a manner designated to minimalize pain, and any animals that exhibited severe disease were euthanized immediately by CO_2 inhalation. C57BL/6 $PVR^{+/+}$ $Ifnar^{-/-}$ mice were obtained from S. Koike (Tokyo, Japan) (62). One week post hormone treatment, mice were orally infected with 5x10^7 PFU of CVB3 IC Nancy. All adult experimental mice were 10-15 weeks old at the time of infection. Feces from infected mice were collected 1, 2, and 3 days post-infection, processed as previously described, and the fecal virus was quantified by a standard plaque assay (38).

Castration and hormone manipulation

Eight-week to ten-week-old male mice were put under anesthesia, and their testes were surgically removed or mock castrated as a surgical control. Testosterone implants were

constructed using silastic tubing (inner diameter-.078", outer diameter-.125"; Dow Chemical Company). After placing 7.5mm of Crystalline Testosterone (Sigma Aldrich) in the tubing, the ends of the tubing were sealed with 2.5 mm of medical adhesive (732 Multi-Purpose Sealant, Dowsil). After the medical adhesive dried, the implants were incubated at 37°C overnight in sterile phosphate-buffered saline. To ensure osmoregulation, implants that were found floating were discarded. Due to the light sensitivity of sex steroid hormones, the testosterone implants were concealed from light. One-week post castration, castrated mice were administered either testosterone or placebo capsules subcutaneously under the right shoulder. Mock-castrated mice were given placebo capsules. Testosterone levels of mouse serum were quantified using a rat/mouse testosterone ELISA following the manufacturer's instructions (MP Biomedical). Flutamide (TCI America, Portland, OR) was dissolved in 100% DMSO and diluted in double-distilled water. As a vehicle control, 100% DMSO was also diluted in double-distilled water before administration. Male mice were given 25µg/kg of flutamide or vehicle control by intraperitoneal injection for three consecutive days before oral inoculation with CVB3-Nancy.

Tissue collection and histological analysis

The heart, liver, spleen, kidneys, and pancreas were aseptically collected 3 days post-infection and homogenized in phosphate-buffered saline using 0.9-2.0 mm stainless steel beads in a Bullet Blender (Next Advance). Cellular debris was removed by centrifugation at 12,000xg for 10 min at 4°C, supernatants were collected, and CVB3 was quantified by plaque assay on HeLa cells. Additionally, a portion of the aseptically removed organs were cut, washed in 1XPBS, put in histology cassettes, and fixed overnight using 10% Neutral buffered formalin (NBF). The

samples were transferred to 70% Ethanol. The tissues were then paraffin-embedded, sectioned, and stained with hematoxylin and eosin (H&E) by the Indiana University Histology Core.

Serum collection and analysis

Blood was collected from the inferior vena cava from infected or uninfected male and female mice 3 days post-infection and incubated at room temperature for 30 mins to initiate coagulation. Samples were centrifuged at 2000 rpm for 15 mins, and separated serum was collected and stored at -20 C for downstream analysis. Alanine transaminase (EN0207Mu-1, CusaBio Technologies) and Pancreatic lipase (E91453Mu-1, Cusabio Technologies) were measured by ELISA. Serum cytokine levels were measured by the Indiana University Multiplex Analysis Core using a Millipore Milliplex MAP Mouse Cytokine/Chemokine Magnetic Kit (Millipore Sigma, Burlington, MA).

Statistical Analysis

Comparisons between control and study groups were analyzed using either a Mann-Whitney U test or a one-way analysis of variance (ANOVA). A Log-rank test was used for survival curve analysis. Error bars in the figures represent the standard errors of the means. A p-value <0.05 was considered significant. All analyses were performed using GraphPad Prism 9 (GraphPad Software, La Jolla, CA).

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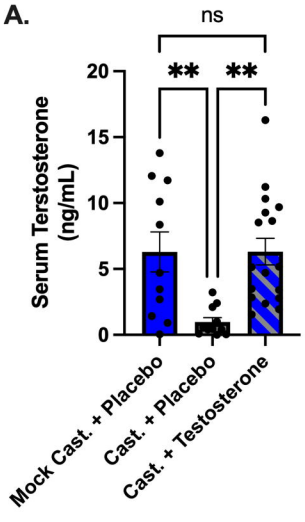
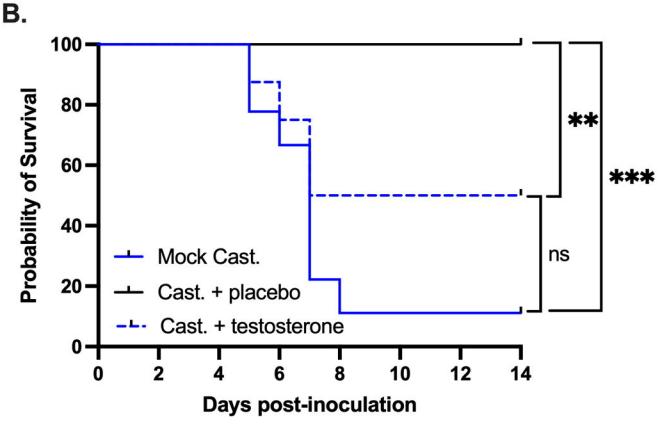
562 **Figure Legends**

563 **Figure 1.** CVB3-induced lethality is enhanced by testosterone. (A) Serum testosterone
564 concentrations in mock or castrated male C57BL/6 *Ifnar*$^{-/-}$ provided either placebo or
565 testosterone capsules. **p<0.01, one-way ANOVA. (B) Survival of mock castrated + placebo,
566 castrated + placebo, and castrated + testosterone mice after oral inoculation with 5x10^7 PFU of
567 CVB3-Nancy. n=8-9 mice per group. **p<0.01, ***p<0.001, Log-rank test. (C) Survival of male
568 *Ifnar*$^{-/-}$ mice treated with flutamide or vehicle control after oral inoculation with 5x10^7 PFU of
569 CVB3-Nancy. n=8-9 mice per group. *p<0.05 Log-rank test.
570
571 **Figure 2.** CVB3 fecal shedding and tissue titers following oral inoculation. Mice were orally
572 inoculated with 5x10^7 PFU of CVB3-Nancy. (A) CVB3-Nancy fecal titers in mock castrated,
573 castrated + placebo, and castrated + mice. (B) CVB3-Nancy fecal titers in mock castrated,
574 castrated + placebo, and castrated + mice at days 2 and 3 post-inoculation. *p<0.05, **p<0.01,
575 Kruskal-Wallis test. (C) CVB3-Nancy tissue titers. Mice were euthanized at 3 dpi, and tissues
576 were collected in mock castrated (blue), castrated + placebo (gray), and castrated + testosterone
577 (blue and gray) *Ifnar*$^{-/-}$ mice.
578
579 **Figure 3.** The inflammatory response to CVB3 is enhanced in mice with testosterone. Serum
580 cytokine quantification in uninfected, mock castrated, castrated + placebo, and castrated +
581 testosterone *Ifnar*$^{-/-}$ mice. Infected mice were orally inoculated with 5x10^7 PFUs of CVB3-
582 Nancy, and serum was collected 3 dpi.
583

584 **Figure 4.** Oral inoculation of *Ifnar*$^{-/-}$ mice with CVB3-Nancy does not induce liver damage or
585 cause pancreatitis. Serum levels of (A) ALT and (B) pancreatic lipase were quantified by ELISA
586 at 3 dpi. One-way ANOVA. H&E stained (C) liver and (D) pancreas collected at 3 dpi following
587 oral inoculation of mock castrated, castrated + placebo, and castrated + testosterone male *Ifnar*$^{-/-}$
588 mice. Histology is representative of two experiments and at least 4 mice per group.
589
590 **Figure 5.** Testosterone promotes CVB3 shedding and dissemination in female *Ifnar*$^{-/-}$ mice, but
591 not lethality. (A) Serum testosterone concentrations in female C57BL/6 *Ifnar*$^{-/-}$ provided either
592 placebo or testosterone capsules. (B) Survival of female C57BL/6 *Ifnar*$^{-/-}$ provided either placebo
593 or testosterone capsules after oral inoculation with $5x10^7$ PFU of CVB3-Nancy. (C) CVB3-
594 Nancy fecal titers in female C57BL/6 *Ifnar*$^{-/-}$ provided either placebo or testosterone. *$p<0.05$,
595 Mann-Whitney test. (D) CVB3-Nancy tissue titers in female C57BL/6 *Ifnar*$^{-/-}$ provided either
596 placebo or testosterone. *$p<0.05$, **$p<0.01$, Mann-Whitney test.
597
598

Figure 1


A.
ns
**
**
Serum Terstosterone (ng/mL)
20
15
10
5
0
Mock Cast. + Placebo
Cast. + Placebo
Cast. + Testosterone


B.
Probability of Survival
100
80
60
40
20
0
0
2
4
6
8
10
12
14
Days post-inoculation
Mock Cast.
Cast. + placebo
Cast. + testosterone
**
***
ns

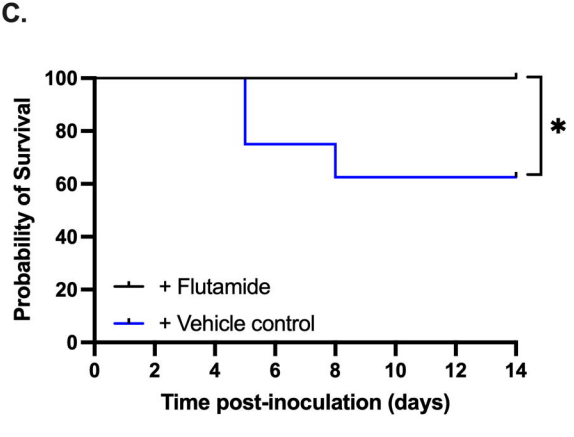

C.
Probability of Survival
100
80
60
40
20
0
0
2
4
6
8
10
12
14
Time post-inoculation (days)
+ Flutamide
+ Vehicle control
*

Figure 2

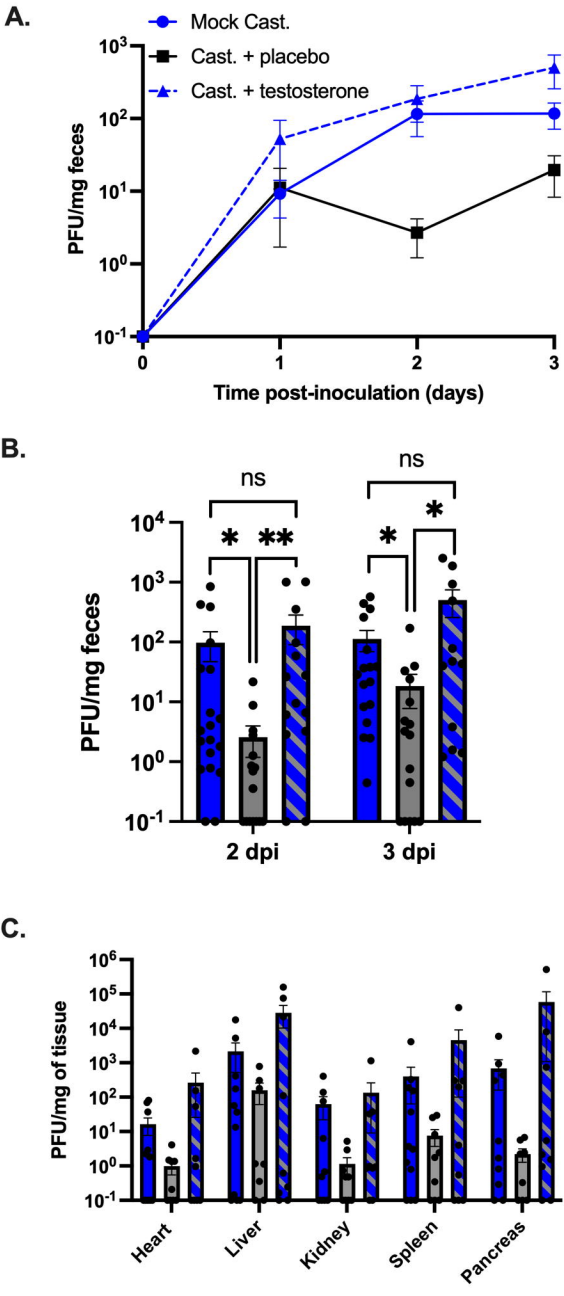

A.
Mock Cast.
Cast. + placebo
Cast. + testosterone
PFU/mg feces
Time post-inoculation (days)
B.
ns
ns
*
**
*
*
PFU/mg feces
2 dpi
3 dpi
C.
PFU/mg of tissue
Heart
Liver
Kidney
Spleen
Pancreas

Figure 3

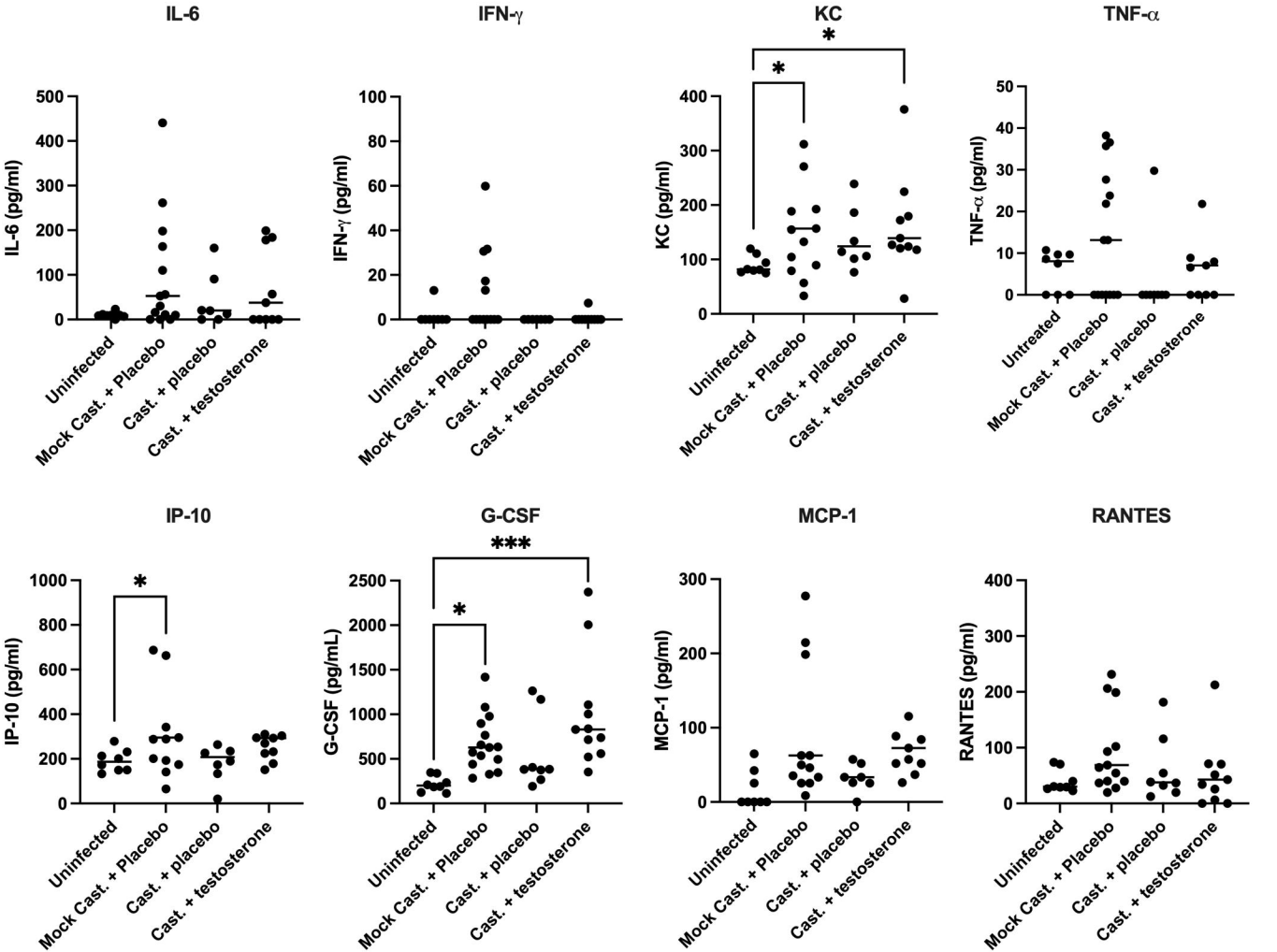
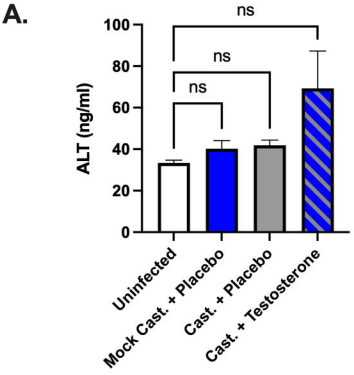
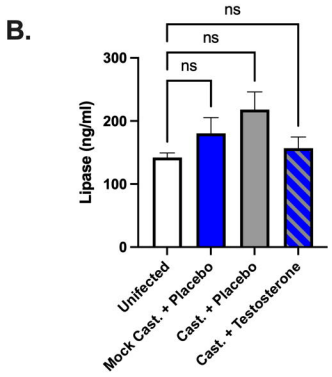
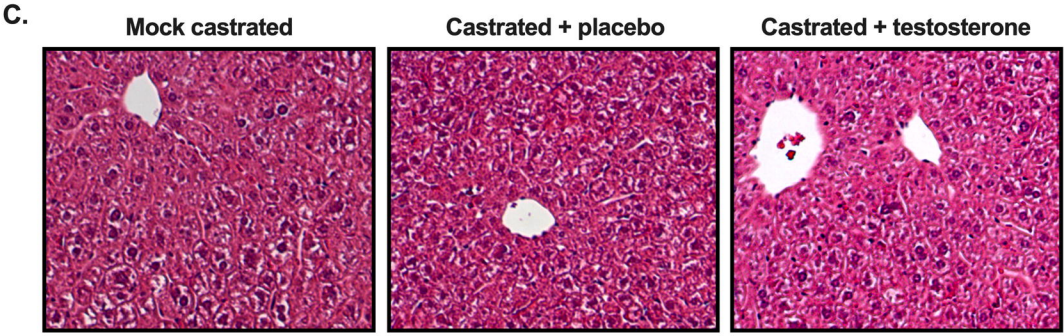

IL-6
IL-6 (pg/ml)
500
400
300
200
100
0
Uninfected
Mock Cast. + Placebo
Cast. + placebo
Cast. + testosterone
IFN-γ
IFN-γ (pg/ml)
100
80
60
40
20
0
KC
KC (pg/ml)
400
300
200
100
0
*
*
TNF-α
TNF-α (pg/ml)
50
40
30
20
10
0
Untreated
IP-10
IP-10 (pg/ml)
1000
800
600
400
200
0
G-CSF
G-CSF (pg/mL)
2500
2000
1500
1000
500
0
***
MCP-1
MCP-1 (pg/ml)
300
200
100
0
RANTES
RANTES (pg/ml)
400
300
200
100
0

Figure 4


A.
ALT (ng/ml)
100
80
60
40
20
0
ns
ns
ns
Uninfected
Mock Cast. + Placebo
Cast. + Placebo
Cast. + Testosterone


B.
Lipase (ng/ml)
300
200
100
0
ns
ns
ns
Unifected
Mock Cast. + Placebo
Cast. + Placebo
Cast. + Testosterone


C.
Mock castrated
Castrated + placebo
Castrated + testosterone

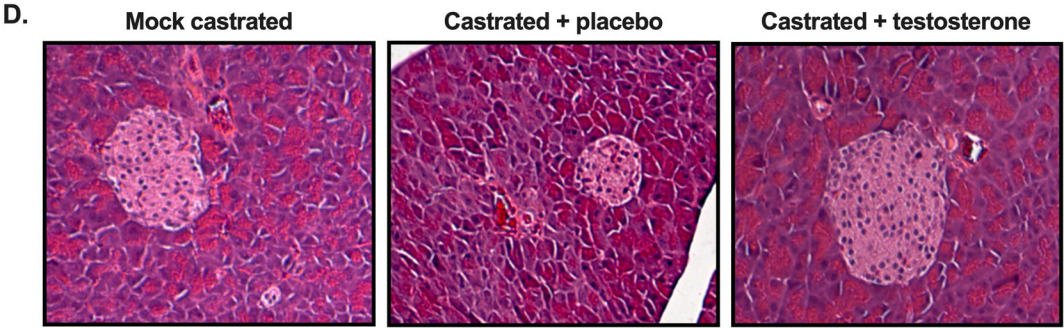
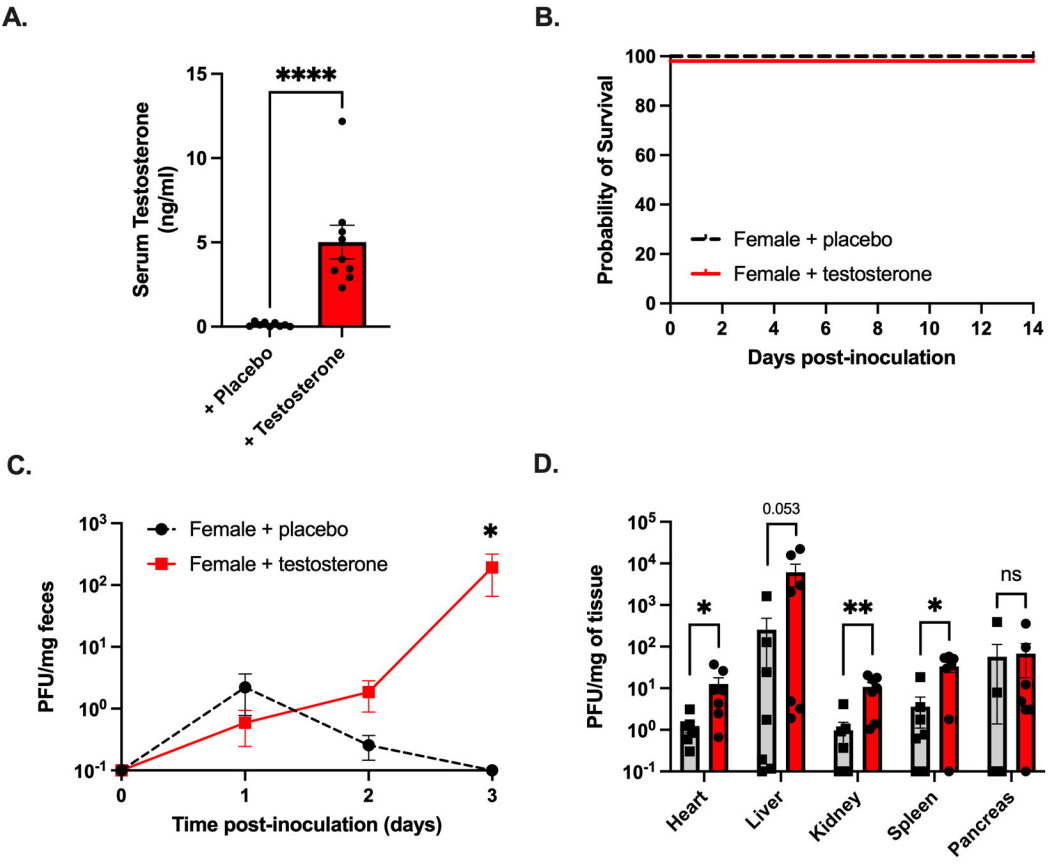

D.
Mock castrated
Castrated + placebo
Castrated + testosterone

Figure 5


A.
Serum Testosterone (ng/ml)
15
10
5
0
****
+ Placebo
+ Testosterone
B.
Probability of Survival
100
80
60
40
20
0
Female + placebo
Female + testosterone
0
2
4
6
8
10
12
14
Days post-inoculation
C.
PFU/mg feces
10^3
10^2
10^1
10^0
10^{-1}
Female + placebo
Female + testosterone
*
0
1
2
3
Time post-inoculation (days)
D.
PFU/mg of tissue
10^5
10^4
10^3
10^2
10^1
10^0
10^{-1}
0.053
*
**
*
ns
Heart
Liver
Kidney
Spleen
Pancreas