Testosterone promotes intestinal replication and dissemination of Coxsackievirus B3 in an oral inoculation mouse model

Adeeba H. Dhalech[#], Caleb M. Corn[#], Vrushali Mangale, Fahim Syed, Stephanie A. Condotta, Martin J. Richer, and Christopher M. Robinson*

Department of Microbiology and Immunology, Indiana University School of Medicine, Indianapolis, IN, 46202, USA.

[#]These authors contributed equally to this work.

*To whom correspondence should be addressed.

Mailing address: 635 Barnhill Drive, Indianapolis, IN, 46202

Phone: 317-274-7822

Fax: 317-274-3331

Running Title: Testosterone enhances CVB3 pathogenesis

Abstract

Enteroviruses initiate infection in the gastrointestinal tract, and sex is often a biological variable that impacts pathogenesis. Previous data suggest that sex hormones can influence intestinal replication of Coxsackievirus B3 (CVB3), an enterovirus in the *Picornaviridae* family. However, the specific sex hormone(s) that regulate intestinal CVB3 replication is poorly understood. To determine if testosterone promotes intestinal CVB3 replication, we orally inoculated male and female *Ifnar*$^{-/-}$ mice that were treated with either placebo or testosterone-filled capsules. Following oral inoculation, we found that testosterone-treated male and female mice shed significantly more CVB3 in the feces than placebo-treated mice indicating that testosterone enhances intestinal replication. Similarly, testosterone enhanced viral dissemination in both sexes as we observed higher viral loads in peripheral tissues following infection. Further, male mice treated with testosterone also had a higher mortality rate than testosterone-depleted male mice. Finally, we observed that testosterone significantly affected the immune response to CVB3. We found that testosterone broadly increased pro-inflammatory cytokines and chemokines while decreasing the number of splenic B cells and dendritic cells following CVB3 infection. Moreover, while testosterone did not affect the early CD4 T cell response to CVB3, testosterone reduced the activation of CD8 T cells. These data indicate that testosterone can promote intestinal CVB3 replication and dissemination while impacting the subsequent viral immune response.

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 poorly understood. Using an oral inoculation model, we found that testosterone
51 enhances CVB3 shedding and dissemination in male and female mice. Further, testosterone can
52 alter the immune response to CVB3. This work highlights the role of testosterone in CVB3
53 pathogenesis and suggests that sex hormones can impact the replication and dissemination of
54 enteric viruses.

55

56

57

58

59

60

61

62

63

64

65

66

67

68

69

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 and delay 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, 11). 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 a member of a group of viruses transmitted through the fecal-oral route termed enteroviruses. Enteroviruses are some of the most common viruses infecting humans worldwide, with an estimated 10-15 million infections per year in the United States (12). Coxsackievirus is often the most isolated among enteroviruses and causes viral myocarditis, hand, foot, and mouth disease, and meningitis (13-15). Infants, children, and immunocompromised individuals are the most susceptible to these diseases, and Coxsackievirus is associated with an 11% fatality rate in neonates (16-18). To date, there are no vaccines or treatments available for Coxsackievirus infections.

Among Coxsackievirus serotypes, Coxsackievirus B3 (CVB3) is one of the leading causes of viral myocarditis, with nearly 40,000 symptomatic cases reported in the United States each year (19). 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 (20, 21). Animal models of

93 CVB3 have provided further evidence of this sex bias. Our laboratory, and others, have shown
94 that sex hormones contribute to CVB3 pathogenesis. Castration of male mice before infection
95 reduces CVB3-induced myocarditis and mortality (22, 23). Further, these hormones contribute to
96 dysregulation of the immune response, which is hypothesized to contribute to mortality.
97 Testosterone and estradiol influence the $CD4^+$ T cell response to promote or protect against
98 CVB3-induced myocarditis (24-26). Castration of male mice significantly reduces anti-
99 inflammatory M2 macrophages in the heart, suggesting a possible role in CVB3-induced
100 myocarditis (27). Along with T cells and macrophages, mast cells have also been implicated in
101 heart disease, suggesting that multiple immune cells contribute to myocarditis (28). Finally,
102 genes on the Y chromosome also likely contribute to CVB3-induced disease, indicating that viral
103 myocarditis in males is multifactorial (29, 30). Therefore, many questions remain as to the
104 mechanism of the sex bias in Coxsackievirus infections.
105
106 While animal models have provided vital information on CVB3 pathogenesis, many of these
107 models mimic systemic infection through intraperitoneal injections of the virus. However, this is
108 not representative of the natural route of infection as Coxsackievirus is transmitted through the
109 fecal-oral route. Few studies have shown successful oral inoculation of mice with
110 Coxsackievirus (31-35). Recently we established an oral inoculation model to examine CVB3
111 replication in the intestine using C57BL/6 *Ifnar*$^{-/-}$ (deficient for interferon α/β receptor) mice
112 (36). Using this model, we observed a sex bias in infection where male mice support robust
113 intestinal replication and succumb to CVB3-induced disease. Female mice, however, are mainly
114 resistant to CVB3 with limited viral replication in the intestine (36). Gonadectomy before CVB3
115 infection impacted fecal shedding in male and female mice and protected male mice from CVB3-

116 induced lethality. These data suggest that sex hormones contribute to viral pathogenesis in our
117 model; however, the role of specific hormones is unclear. Here, we show that testosterone
118 enhances CVB3 shedding and dissemination to peripheral tissues in male and female mice.
119 Testosterone also enhanced lethality in male mice but not in female mice. Finally, we found that
120 testosterone impacts the immune response, which may limit CVB3 pathogenesis. Overall, these
121 data highlight the importance of examining sex bias in CVB3-induced disease.
122

123 **Results**

124 **Testosterone enhances CVB3 lethality, fecal shedding, and dissemination in male *Ifnar*$^{-/-}$**
125 **mice following oral inoculation.** Previously, we found that castration of male *Ifnar*$^{-/-}$ mice prior
126 to oral infection with CVB3 protected against CVB3-induced lethality and significantly reduced
127 CVB3 fecal shedding. These data implicated a role for testosterone, the primary sex hormone in
128 males. To determine if testosterone could impact CVB3 replication and lethality, we surgically
129 castrated male *Ifnar*$^{-/-}$ mice to deplete endogenous testosterone. We also performed mock
130 castrations on male mice as a control to confirm testosterone treatments. One week after surgery,
131 we implanted mice with placebo or exogenous testosterone capsules. To assess hormone
132 replacement, we determined serum concentrations of testosterone by ELISA. Castrated mice that
133 received the placebo capsule had a significantly lower serum testosterone concentration than
134 mock-castrated and castrated male mice that received testosterone (Fig. 1A). Further, mock-
135 castrated and castrated mice receiving exogenous testosterone had similar amounts of serum
136 testosterone, confirming successful hormone treatment. One week following hormone treatment,
137 male mice were orally inoculated with $5x10^7$ PFU CVB3 and monitored for survival for 14 days
138 post-inoculation (dpi). We found that, like our previous study (36), testosterone-depleted mice

139 (castrated + placebo) were protected from CVB3-induced lethality (Fig 1B). However,
140 testosterone-treated (castrated + testosterone) mice were significantly more likely to die from
141 CVB3 inoculation than testosterone-depleted mice, with 50% of mice succumbing to disease.
142 The survival rate and kinetics were similar to oral inoculation of gonad-intact C57BL/6 *Ifnar*$^{-/-}$
143 and mock-castrated mice, as we previously demonstrated (36). Thus, these data indicate that
144 testosterone contributes to CVB3-induced lethality in male mice.
145
146 CVB3 is spread through the fecal-oral route; therefore, we next examined CVB3 fecal shedding,
147 indicative of viral replication in the intestine (36). Following oral inoculation, we collected feces
148 at 1, 2, and 3 dpi and quantified fecal CVB3 using a standard plaque assay. We found that at 2
149 and 3 dpi, testosterone-depleted mice shed significantly less CVB3 in the feces than testosterone-
150 treated male mice (Fig. 1C). Since fecal shedding in testosterone-treated mice was higher, we
151 hypothesized testosterone would also increase viral loads in the peripheral tissues following oral
152 inoculation. To test this hypothesis, we harvested the heart, liver, kidney, spleen, and pancreas at
153 3 dpi and quantified CVB3 tissue titers by a plaque assay. We observed significantly higher
154 tissue CVB3 titers for testosterone-treated mice compared to testosterone-depleted mice in the
155 spleen and pancreas (Fig. 1D). Further, we found higher titers in the heart and kidney in
156 testosterone-treated mice that reached near significance ($p=0.0684$ and 0.0586, respectively).
157 Taken together, these data suggest that testosterone promotes intestinal CVB3 replication and
158 viral dissemination in male mice following oral inoculation.
159
160 **Testosterone promotes pro-inflammatory cytokine and chemokine expression to CVB3.** The
161 immune response plays a pivotal role in tissue damage following CVB3 infection (24, 37-41).

162 Previously, we observed an increase in serum cytokine and chemokine concentrations in CVB3-
163 infected male but not female mice (36). To test whether testosterone altered the magnitude of the
164 cytokine and chemokine response, we measured the serum concentrations of a broad panel of 25
165 cytokines and chemokines at 3 dpi. In mice with testosterone, we saw a general increase in the
166 serum concentrations of pro-inflammatory cytokines and chemokines IL-6, IFN-γ, IL-15, IP-10,
167 MIP-1β, G-CSF, KC, MCP-1, RANTES, and TNF-α compared to uninfected males (Fig. 2A).
168 Further, there were significantly higher serum concentrations of IP-10, C-CSF, MCP-1, and
169 TNF-α in testosterone-treated mice than in testosterone-depleted mice (Fig. 2B – 2E). Finally,
170 we analyzed cytokine and chemokine response by uniform manifold approximation and
171 projected (UMAP). This dimension reduction approach has been used to analyze complex data
172 sets and visualize relationships between experimental groups (42, 43). We found that UMAP
173 could separate the cytokine and chemokine profile into three clusters that correlated with CVB3
174 infection and the presence of testosterone (Fig. 2F). These data suggest that testosterone alters
175 the cytokine and chemokine profile in CVB3-infected males. Overall, these data indicate that
176 testosterone in CVB3 infected male *Ifnar*$^{-/-}$ mice promotes a pro-inflammatory response
177 characterized by an induction of select cytokines and chemokines.
178
179 **Testosterone decreases the frequency of B cells and the number of dendritic cells in the**
180 **spleen.** Since immune cells have been shown to greatly alter CVB3 pathogenesis, we next
181 assessed the impact of testosterone on immune cells from the spleen. We harvested the spleen
182 from infected testosterone-treated and testosterone-depleted male *Ifnar*$^{-/-}$ mice at 5 dpi and
183 performed flow cytometry on splenocytes. We chose to investigate the immune cell response at 5
184 dpi since this represents the time when males begin to succumb to disease in our model (Fig.

185 1B). We found that testosterone decreased the total number of splenocytes in both uninfected and
186 infected male mice (Table 1); however, this had no impact on the frequency and numbers of
187 splenic macrophages, monocytes, and neutrophils between infected testosterone-treated and
188 testosterone-depleted mice (Table 1). Contrary to macrophages, monocytes, and neutrophils,
189 testosterone significantly decreased the frequency and number of $CD19^+$ B cells in the spleen
190 following CVB3 infection (Table 1). However, the decrease in splenic B cells is testosterone-
191 dependent, as a similar reduction in B cells was observed in uninfected mice. These data indicate
192 that testosterone reduces the number of B cells in the spleen; however, these differences are not
193 due to CVB3 infection.
194
195 Next, we assessed the impact of testosterone on $CD11c^+$, MHC II^+ dendritic cells. In contrast, to
196 B cells, no significant difference in the frequency and numbers of dendritic cells were observed
197 between uninfected mice regardless of testosterone treatment (Fig. 3A and 3B). Following CVB3
198 infection, we observed a significant increase in the frequency of splenic dendritic cells in both
199 testosterone-depleted and testosterone-treated mice compared to uninfected mice. However, we
200 only observed a significant increase in the numbers of splenic dendritic cells in testosterone-
201 depleted mice infected with CVB3 (Fig. 3C). Further, following infection, we found that
202 testosterone-depleted mice had significantly higher numbers of dendritic cells in the spleen
203 compared to testosterone-treated mice. Overall, these data indicate that while CVB3 infection
204 increases the frequency of splenic dendritic cells, in the absence of testosterone, the number of
205 dendritic cells in the spleen is significantly reduced following oral inoculation.
206

207 **Testosterone limits the activation of CD8$^+$ T cells following oral CVB3 infection.** CD4$^+$ and
208 CD8$^+$ T cells have been implicated in limiting CVB3 replication; however, they may also
209 contribute to disease in myocarditis models (39). Since we observed a significant difference in
210 the numbers of splenic dendritic cells between testosterone-treatment groups, we hypothesized
211 that testosterone might alter the T cell response to CVB3 in our model. Following CVB3
212 inoculation, we harvested the spleen at 5 dpi and performed flow cytometry on splenocytes to
213 examine our hypothesis. We found a significant decrease in the proportion of splenic CD4$^+$ and
214 CD8$^+$ T cells in infected mice compared to uninfected mice from both testosterone-depleted and
215 testosterone-treated groups (Fig. 4A – 4D). Testosterone did not appear to impact the difference
216 in the CD4$^+$ T cell response, as no significant difference in the frequency between testosterone
217 groups was observed in uninfected and infected animals (Fig. 4C). In contrast, we found that
218 testosterone-depleted mice had a significant reduction in the frequency of CD8$^+$ T cells in both
219 uninfected and infected male mice compared to testosterone-treated mice (Fig. 4D). These data
220 indicate that oral inoculation with CVB3 leads to a contraction in splenic CD4$^+$ and CD8$^+$ T
221 cells, and testosterone increases the frequency of CD8$^+$, but not CD4$^+$, T cells in the spleen.
222
223 CVB3 infection and testosterone impacted the frequency of splenic T cells; however, these
224 results do not distinguish between naïve and activated T cells. Unfortunately, few CVB3-specific
225 CD4$^+$ and CD8$^+$ T cell epitopes have been identified, which limits the ability to track viral-
226 specific, activated T cells. However, previous studies have established that activated T cells can
227 be followed regardless of their specificity using surrogate markers (44-49). To investigate the
228 impact of testosterone on activated T cells, we next examined the expression of CD11a and
229 CD49d on CD4$^+$ T cells as a measure of activated, antigen-experienced CD4$^+$ T cells (Fig. 5A).

230 Similar to the frequency of total $CD4^+$ T cells, we found no significant difference in
231 $CD11a^{hi}CD49d^{hi}$ $CD4^+$ T cells between testosterone-treated and testosterone-depleted mice (Fig.
232 5B). Further, in contrast to total $CD4^+$ T cells, CVB3 infection did not alter the frequency of
233 splenic $CD11a^{hi}CD49d^{hi}$ $CD4^+$ T cells compared to uninfected mice. Taken together, these data
234 indicate that testosterone does not impact the $CD4^+$ T cell response at 5 dpi in male mice.
235
236 Next, we assessed the impact of testosterone on activated $CD8^+$ T cells. Naïve $CD8^+$ T cells are
237 $CD62L^{hi}$, and activated CD8+ T cells differentiate into effector subtypes during acute infections.
238 During this effector phase, CD62L is downregulated (45, 50, 51). Therefore, we next assessed if
239 testosterone affects the activation of $CD8^+$ T cells following CVB3 infection by expression of
240 CD62L. We found that CVB3-infected mice had a significantly higher frequency of $CD62L^{lo}$
241 $CD8^+$ T cells in the spleen than uninfected mice in both testosterone-treated and testosterone-
242 depleted groups (Fig. 6A and 6B). Intriguingly, in infected mice, we observed a trending
243 reduction in the frequency of splenic $CD62L^{lo}$ $CD8^+$ T cells in testosterone-treated mice
244 compared to testosterone-depleted mice; however, this did not reach statistical significance
245 ($p=0.0754$, unpaired t-test) (Fig. 6B). Since testosterone nearly decreased the frequency of
246 $CD62L^{lo}$ $CD8^+$ T cells in infected mice, we next examined if $CD62L^{lo}$ $CD8^+$ T cells from
247 infected mice were undergoing blast transformation. Within a few hours of antigen activation,
248 $CD8^+$ T cells can undergo blast transformation, which results in larger lymphocytes as they
249 develop into mature effector cells. Since changes in the light scatter can reveal blast
250 transformation and reflect a measure of T cell activation (52), we assessed the mean fluorescence
251 intensity (gMFI) for the forward (FSC) and side scatter area (SSC) of the $CD62L^{lo}$ $CD8^+$ T cell
252 population. We found that $CD62L^{lo}$ $CD8^+$ T cells from testosterone-depleted mice had a

253 significant increase in size and granularity, as measured by FSC and SSC, compared to CD62L^{lo}
254 CD8^{+} T cells from testosterone-treated mice (Fig. 6C and 6D). These data suggest that
255 testosterone reduces early blast transformation in CD8^{+} T cells in response to CVB3.
256
257 To confirm our results, we next examined the expression of integrin molecule CD11a on CD8^{+} T
258 cells. Following CD8^{+} T cell activation, increased expression of CD11a can distinguish naïve
259 CD8^{+} T cells from antigen-experienced effector and memory CD8^{+} T cells (45). We observed no
260 difference in the proportion of splenic CD11a^{hi}CD62L^{lo} CD8^{+} T cells between infected and
261 uninfected male mice treated with testosterone (Fig. 6E and 6F). In contrast, we found a
262 significant increase in the frequency of splenic CD11a^{hi}CD62L^{lo} CD8^{+} T cells between infected
263 and uninfected male mice that were testosterone-depleted. Overall, these data suggest that
264 testosterone limits the frequency of splenic CD11a^{hi}CD62L^{lo} CD8^{+} T cells following CVB3
265 infection, indicative of dampening early activation of CD8^{+} T cells.
266
267 **Testosterone does not enhance CVB3-induced lethality in female mice but does enhance**
268 **fecal shedding and viral dissemination.** Previously we found that orally CVB3 inoculated
269 female *Ifnar*$^{-/-}$ mice were protected entirely from CVB3-induced lethality and had limited viral
270 fecal shedding (36). Since exogenous testosterone treatment restored fecal shedding and lethality
271 in male mice, we hypothesized that testosterone might enhance pathogenesis in female mice. To
272 examine this hypothesis, we provided exogenous testosterone or placebo capsules to female mice
273 before oral inoculation with CVB3. Like our castration studies, female mice with testosterone
274 had significantly higher serum testosterone concentrations than placebo controls (Fig. 7A). One
275 week after hormone treatment, female mice were orally inoculated with 5x10^{7} PFUs of CVB3

276 and monitored for survival. In contrast to males, both testosterone- and placebo-treated infected
277 females were protected from CVB3-induced lethality (Fig. 7B). Next, to examine fecal CVB3
278 shedding, feces were collected from infected mice at 1, 2, and 3 dpi, processed, and quantified by
279 a standard plaque assay. We observed significantly more CVB3 in the feces at 3 dpi in female
280 mice that received testosterone than in female mice that received placebo capsules (Fig. 7C).
281 Finally, we examined CVB3 titers in peripheral tissues at 3 dpi to test if testosterone enhanced
282 viral dissemination. We found a significant increase in the viral loads of the heart, kidney, and
283 spleen in testosterone-treated female mice (Fig. 7D). The viral load in the liver was higher in
284 testosterone-treated mice but did not reach statistical significance. In contrast to the other organs,
285 the viral load in the pancreas was similar between placebo- and testosterone-treated female mice.
286 Overall, these data indicate that testosterone can enhance fecal CVB3 shedding and viral
287 dissemination to the heart, kidney, and spleen; however, testosterone is not sufficient to increase
288 CVB3-induced lethality in female mice.
289
290 **Discussion**
291 Sexual dimorphism is commonly observed in various infectious, autoimmune, and
292 cardiovascular diseases. Males are often more susceptible to diseases caused by bacteria, viruses,
293 fungi, or parasites. Females, however, are more predisposed to autoimmune diseases like
294 systemic lupus erythematosus, multiple sclerosis, and rheumatoid arthritis. Additionally, clinical
295 and epidemiological studies have highlighted the disparity in cardiovascular diseases with an
296 increased mortality rate in men. CVB3 also displays a sex bias in human infections where males
297 are twice as likely to have severe sequelae. Previously, we established an oral inoculation model
298 for CVB3 using male and female *Ifnar*$^{-/-}$ mice. *Ifnar*$^{-/-}$ mice were chosen for this model based on

299 previous work showing that lack of a type I IFN response enhances the susceptibility to human
300 enteric viruses by the oral route (53-55). Using this model, we observed a sex bias in CVB3
301 orally inoculated male and female *Ifnar*$^{-/-}$ mice, consistent with human infections (36). Here we
302 show that testosterone, the primary sex hormone in males, impacts CVB3 pathogenesis following
303 oral infection.
304
305 Our data indicate that testosterone enhances intestinal replication and dissemination of CVB3.
306 We observed that testosterone could promote fecal shedding and higher viral loads of CVB3 in
307 tissues of both male and female *Ifnar*$^{-/-}$ mice following infection (Figs. 1C, 1D, 7C, and 7D). To
308 our knowledge, this is the first time that testosterone has been demonstrated to influence the
309 intestinal replication and dissemination of an enteric virus. These data differ from previous
310 studies examining the impact of sex hormones on CVB3 and other viruses, such as influenza. In
311 these studies, testosterone modulates the immune response rather than directly impacting viral
312 replication to influence disease (56-58). Similarly, in CVB3 models, castration of wild-type,
313 immune-competent male mice did not directly affect viral replication in the heart (27). Further,
314 testosterone treatment in males only led to a modest increase in the CVB3 load in the heart (24).
315 The discrepancy between these data and ours is likely due to the different inoculation routes.
316 However, in our oral inoculation model, our data suggest that sex differences in intestinal CVB3
317 replication and viral dissemination are likely a significant contributor to the sex bias in mortality.
318
319 The mechanism for the sex hormone modulation of intestinal replication and dissemination is
320 currently unclear. Previous data indicate that sex hormones, including testosterone, can enhance
321 CVB3 attachment to cardiomyocytes (23); therefore, testosterone may alter the expression of

322 viral receptors on intestinal cells. It is also intriguing to speculate that sex hormones may change
323 the overall intestinal ecosystem to directly or indirectly impact CVB3 replication. Recent data
324 show intestinal bacteria can influence the intestinal replication of enteric viruses, including
325 CVB3 (59-61). Our lab recently demonstrated that specific intestinal bacteria in male mice
326 promote CVB3 infectivity and viral stability (62). While the mechanism is unclear for CVB3, a
327 similar enterovirus, poliovirus, has been found to use bacteria to enhance attachment to the
328 poliovirus receptor (63). Interestingly, sex-specific differences in intestinal bacteria can account
329 for the sex bias in mouse models of type 1 diabetes (64, 65). Since bacteria can also metabolize
330 hormones that alter bacterial growth, future studies are required to examine a link between
331 bacteria and sex hormones as enhancers of CVB3 attachment and replication in the intestine.
332
333 With a systemic infection model of CVB3, previous studies have found that testosterone
334 enhances lethality in male mice (22), consistent with our data. Further, systemic models of
335 CVB3 infection have shown that while testosterone treatment promoted cardiac inflammation in
336 female mice following CVB3 infection, testosterone did not increase mortality (22, 24). In
337 agreement, we also found that testosterone was insufficient to enhance lethality in female mice
338 following oral inoculation. However, we found that testosterone enhances fecal CVB3 shedding
339 and CVB3 dissemination, suggesting that other unknown immune correlates of protection may
340 help limit mortality in females. Further, it is possible that predominant female hormones,
341 estrogen, and progesterone, also offer protection for females in our model. This current study
342 focused on gonad-intact females; therefore, a limitation in our current study is that we cannot
343 rule out the potential protective effects of estrogen and progesterone. Experiments examining the

344 role of these hormones are currently underway in our laboratory and may reveal additional sex-
345 dependent factors that protect female mice from CVB3-induced mortality.
346
347 In contrast to directly enhancing viral replication, testosterone may indirectly enhance viral
348 replication and pathogenesis by modulating the immune response following infection. Previous
349 studies have shown that testosterone acts as an anti-inflammatory hormone (66); however, our
350 data indicate that testosterone broadly increases the pro-inflammatory cytokine and chemokine
351 response to CVB3 (Fig. 2A – 2C). In testosterone-treated mice, we saw an overall increase in the
352 serum concentrations for various cytokines and chemokines, including IL-6, Il-15, IP-10, MIP-
353 1β, G-CSF, KC, MCP-1, RANTES, and TNF-α. Interestingly, we only found that IP-10, G-CSF,
354 MCP-1, and TNF-α were significantly upregulated in the presence of testosterone. Using UMAP
355 analysis to recognize significant trends in the cytokine/chemokine data, we found that mice
356 clustered into three distinct groups. From our data, we discovered that testosterone-treated mice
357 responded differently than testosterone-depleted mice and uninfected mice. Interestingly, the
358 testosterone-depleted mice grouped closest to uninfected mice (Fig. 2F). However, whether this
359 reduction is due to loss of testosterone or limited CVB3 replication and dissemination in
360 testosterone-depleted mice is unclear. These data, intriguingly, mirror our previous findings
361 where serum concentrations of pro-inflammatory cytokines and chemokines between infected
362 and uninfected female mice were not significantly different (36). Taken together, these data
363 suggest that the improved outcome of CVB3 pathogenesis may be associated with limited
364 cytokine and chemokine expression. However, we recognize the limitations of our data as the
365 time at which we analyzed the inflammatory response may not be representative of all the
366 cytokines and chemokines investigated. Further, our model's variability in viral dissemination

367 may account for cytokine and chemokine differences in mice that fell outside the groups tested in
368 our UMAP analysis. Additionally, the lack of a type I IFN response in certain cell types may
369 impair the ability to produce specific pro-inflammatory cytokines. The kinetics of the cytokine
370 and chemokine response and their induction in orally inoculated wild-type C57BL/6 mice
371 warrants further studies.
372
373 Along with the cytokine and chemokine response, testosterone can also influence the function
374 and phenotype of many immune cells (67). We observed a significant increase in total
375 splenocytes in testosterone-depleted male mice in uninfected and infected mice (Table 1). We
376 found that B cells likely accounted for this increase in overall splenic cells (Table 1). These data
377 confirm previous reports that show that testosterone dampens the number of B cells in the spleen
378 (68). However, it is unclear if the difference in B cell dynamics impacts antibody production and
379 maturation since we observed no significant difference between infected and uninfected mice
380 regardless of testosterone treatment. We previously demonstrated that surviving male mice
381 mount a neutralizing antibody response to CVB3 (36); therefore, it is possible that testosterone
382 could limit neutralizing antibodies that provide protection. Future work will be required to
383 examine testosterone's effect on B cell function and antibody production during infection.
384
385 Previous studies have shown that other immune cells, including T cells, can contribute to CVB3
386 clearance but can also contribute to CVB3-induced disease (39). In the current study, we
387 observed that CVB3 infection decreased CD4+ and CD8+ T cell frequency in the spleen (Fig. 4C
388 – 4D). However, testosterone only affected the frequency of $CD8^+$ T cells. Interestingly, when
389 we further investigated the T cell response, we found no difference in the frequency and number

390 of antigen-experienced CD49d^{hi}CD11a^{hi} CD4^{+} T cells (Figure 5). This contrasts with previous
391 studies in systemic models of CVB3 infection. In those models, testosterone can affect the CD4
392 T helper (Th) cell phenotype to promote myocarditis (24, 26). Differences between our findings
393 and others may be due to the limited time point selected for our study, and CD4^{+} T cells may be
394 activated later during infection. However, given that male mice succumb to disease starting at
395 5dpi, even if CD4^{+} T cells are induced later during infection, our data indicates that CD4^{+} T cells
396 do not contribute to the mortality associated with our model.
397
398 In contrast to CD4^{+} T cells, we observed that testosterone might dampen the activation of CD8^{+}
399 T cells following oral inoculation. CD62L is a cell-adhesion molecule that is downregulated
400 during the activation of CD8^{+} T cells. Interestingly, we observed a decrease in the frequency of
401 CD62L^{lo} CD8^{+}s in the testosterone-treated mice, which trended towards statistical significance.
402 To further investigate potential CD8^{+} T cell activation, we measured the size of CD62L^{lo} CD8^{+} T
403 cells from both testosterone-depleted and testosterone-treated mice. As measured by FSC and
404 SSC, we found that CD62L^{lo} CD8^{+} T cells from testosterone-depleted mice differed in shape and
405 were larger than CD62L^{lo} CD8^{+} T cells in testosterone-treated mice. These data suggest that
406 CD62L^{lo} CD8s from testosterone-depleted mice are activated and in the early stages of blast
407 transformation (Fig 6C – 6D). In support of this finding, we found a significant increase in the
408 frequency of CD11a^{hi}CD62L^{lo} CD8s in testosterone-depleted mice (Fig. 6F) . Since the
409 expression of CD11a is upregulated in activated CD8^{+} T cells, these data indicate that
410 testosterone may dampen the CD8^{+} T cell response to CVB3. These data are surprising
411 considering that previous studies have shown a limited *in vivo* CD8^{+} T cell response to CVB3
412 (69-71). Our data confirm that CVB3 fails to elicit a robust CD8^{+} T cell response in male mice

413 with testosterone; however, our findings suggest that in the absence of testosterone, male mice
414 may mount a CD8 T cell response to CVB3 that could be protective. The size and breadth of this
415 response in testosterone-depleted mice require further studies. Additionally, identifying CVB3-
416 specific epitopes is necessary to determine if the $CD8^+$ T cell response is viral-specific or due to
417 bystander activation.
418
419 In conclusion, we found that testosterone promotes intestinal CVB3 replication and viral
420 dissemination in orally inoculated male and female *Ifnar*$^{-/-}$ mice. Further, testosterone enhances
421 viral-induced lethality in a sex-dependent manner. The exact mechanism of how testosterone
422 aggravates disease is currently unclear, but our data indicates that alterations to the host immune
423 response may play an important role. Future studies will be necessary to determine how
424 testosterone directly or indirectly promotes intestinal CVB3 infection. Overall, these data
425 reinforce the importance of sex as a biological variable in enteric viral infections.
426
427
428
429
430
431
432
433
434
435

436 **Materials and Methods**

437

438 ***Cells and virus***

439 HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with
440 10% calf serum and 1% penicillin-streptomycin at 37˚C with 5% CO_2. The CVB3-Nancy
441 infectious clone was obtained from Marco Vignuzzi (Pasteur Institute, Paris, France) and
442 propagated in HeLa cells as described previously (36). CVB3 was quantified by a standard
443 plaque assay using HeLa cells.

444

445 ***Mouse experiments***

446 All animals were handled according to the Guide for the Care of Laboratory Animals of the
447 National Institutes of Health. All mouse studies were performed at Indiana University School of
448 Medicine using protocols approved by the local Institutional Animal Care and Use Committee in
449 a manner designated to minimalize pain, and any animals that exhibited severe disease were
450 euthanized immediately by CO_2 inhalation. C57BL/6 *PVR*$^{+/+}$ *Ifnar*$^{-/-}$ mice were obtained from S.
451 Koike (Tokyo, Japan) (54). One week post hormone treatment, mice were orally infected with
452 $5x10^7$ PFU of CVB3 IC Nancy. All adult experimental mice were 10-15 weeks old at the time of
453 infection. Feces from infected mice were collected 1, 2, and 3 days post-infection, processed as
454 previously described, and the fecal virus was quantified by a standard plaque assay (36).

455

456 ***Castration and hormone manipulation***

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

459 constructed using silastic tubing (inner diameter-.078", outer diameter-.125"; Dow Chemical
460 Company). After placing 7.5mm of Crystalline Testosterone (Sigma Aldrich catalog #T1500) in
461 the tubing, the ends of the tubing were sealed with 2.5 mm of medical adhesive (732 Multi-
462 Purpose Sealant, Dowsil). After the medical adhesive dried, the implants were incubated at 37°C
463 overnight in sterile phosphate-buffered saline. To ensure osmoregulation, implants that were
464 found floating were discarded. Due to the light sensitivity of sex steroid hormones, the
465 testosterone implants were concealed from light. One-week post castration, castrated mice were
466 administered either testosterone or placebo capsules subcutaneously under the right shoulder.
467 Mock-castrated mice were given placebo capsules. Testosterone levels of mouse serum were
468 quantified using a rat/mouse testosterone ELISA following the manufacturer's instructions (MP
469 Biomedical).
470
471 ***Tissue collection and processing***
472 The heart, liver, spleen, kidneys, and pancreas were aseptically collected 3 dpi and homogenized
473 in phosphate-buffered saline using 0.9-2.0 mm stainless steel beads in a Bullet Blender (Next
474 Advance). Cellular debris was removed by centrifugation at 12,000xg for 10 min at 4°C,
475 supernatants were collected, and CVB3 was quantified by plaque assay on HeLa cells.
476
477 ***Flow cytometry analysis***
478 The spleen was collected at 5 dpi from testosterone-depleted (placebo) or testosterone-treated
479 mice. The spleen was mechanically disrupted to generate single-cell suspension, and
480 erythrocytes were lysed using RBC lysis buffer as previously described (49). Cells were washed
481 and stained with antibodies for indicated immune cells, then fixed using IC fixation buffer

482 (eBioscience). Samples were analyzed on a BD LSRFortessa flow cytometer and FlowJo
483 software (BD Biosciences). The following mouse antibodies were used in an appropriate
484 combination of fluorochromes: CD4 (clone GK1.5, BioLegend, catalog #100412), CD8α (clone
485 53-6.7, BioLegend, catalog #100725), MHC II (clone M5/114.15.2, BioLegend, catalog
486 #107619), CD11a (clone N418, BioLegend, catalog #117327) , CD19 (clone 6D5, BioLegend,
487 catalog #115507), CD62L (clone MEL-14, BioLegend, catalog # 104438), and CD49d (clone
488 R1-2, BioLegend, catalog # 103607).
489
490 ***Serum collection and analysis***
491 Blood was collected from the inferior vena cava of infected and uninfected male and female
492 mice 3 dpi and incubated at room temperature for 30 mins to initiate coagulation. Samples were
493 centrifuged at 2000 rpm for 15 mins, and separated serum was collected and stored at -20 C for
494 downstream analysis. Alanine transaminase (EN0207Mu-1, CusaBio Technologies) and
495 Pancreatic lipase (E91453Mu-1, Cusabio Technologies) were measured by ELISA. Serum
496 cytokine levels were measured by the Indiana University Multiplex Analysis Core using a
497 Millipore Milliplex MAP Mouse Cytokine/Chemokine Magnetic Kit (Millipore Sigma,
498 Burlington, MA). Undiluted serum was provided to the Indiana University Multiplex Analysis
499 Core and ran according to the manufacturer's protocol. The heatmap was generated using the
500 log2-fold change of average serum concentration of each cytokine and chemokine from the
501 infected groups compared to uninfected control mice. UMAP analysis on serum cytokine and
502 chemokine levels across the three groups was carried out using the R UMAP 0.2.8.0 package.
503 UMAP is a method to reduce high-dimensional data to recognize significant trends in similarity.
504 This technique assumes all the data points are connected and that these data points approximate

505 the total data points. The data set was Z-score normalized prior to UMAP analysis. UMAP
506 default parameters, as well as a minimum Euclidean distance of 0.1, were used.
507
508 ***Statistical Analysis***
509 Comparisons between control and study groups were analyzed using either an unpaired t-test,
510 Mann-Whitney U test, or a one-way analysis of variance (ANOVA). A Log-rank test was used
511 for survival curve analysis. Error bars in the figures represent the standard errors of the means. A
512 p-value <0.05 was considered significant. All analyses were performed using GraphPad Prism 9
513 (GraphPad Software, La Jolla, CA).
514
515 **Acknowledgments**
516 We thank Samantha Scholz and Keely Szilagyi for their help and input with the mouse castration
517 and testosterone experiments. We would also like to thank the members of the Indiana
518 University Melvin and Bren Simon Cancer Center Flow Cytometry Resource Facility for their
519 outstanding technical support. This work was funded by a K01 DK110216, R03 DK124749, a
520 Showalter Trust Award, and a Biomedical Research Grant from the Indiana Clinical and
521 Translational Sciences Institute to CMR. The Indiana University Melvin and Bren Simon
522 Comprehensive Cancer Center Flow Cytometry Resource Facility (FCRF) is funded in part by
523 NIH, National Cancer Institute (NCI) grant P30 CA082709 and National Institute of Diabetes
524 and Digestive and Kidney Diseases (NIDDK) grant U54 DK106846. The FCRF is supported in
525 part by NIH instrumentation grant 1S10D012270.
526
527

528 **References**

529 1. Fuller AC, Kang B, Kang HK, Yahikozowa H, Dal Canto MC, Kim BS. 2005. Gender bias in
530 Theiler's virus-induced demyelinating disease correlates with the level of antiviral
531 immune responses. J Immunol 175:3955-63.
532 2. Shah VA, Chong CY, Chan KP, Ng W, Ling AE. 2003. Clinical characteristics of an outbreak
533 of hand, foot and mouth disease in Singapore. Ann Acad Med Singapore 32:381-7.
534 3. Aminu M, Ahmad AA, Umoh JU, de Beer MC, Esona MD, Steele AD. 2007. Adenovirus
535 infection in children with diarrhea disease in Northwestern Nigeria. Ann Afr Med 6:168-
536 73.
537 4. Curiel RE, Miller MH, Ishikawa R, Thomas DC, Bigley NJ. 1993. Does the gender
538 difference in interferon production seen in picornavirus-infected spleen cell cultures
539 from ICR Swiss mice have any in vivo significance? J Interferon Res 13:387-95.
540 5. Hou J, Zheng WF. 1988. Effect of sex hormones on NK and ADCC activity of mice. Int J
541 Immunopharmacol 10:15-22.
542 6. Rettew JA, Huet-Hudson YM, Marriott I. 2008. Testosterone reduces macrophage
543 expression in the mouse of toll-like receptor 4, a trigger for inflammation and innate
544 immunity. Biol Reprod 78:432-7.
545 7. McKay LI, Cidlowski JA. 1999. Molecular control of immune/inflammatory responses:
546 interactions between nuclear factor-kappa B and steroid receptor-signaling pathways.
547 Endocr Rev 20:435-59.
548 8. Furman D, Hejblum BP, Simon N, Jojic V, Dekker CL, Thiebaut R, Tibshirani RJ, Davis MM.
549 2014. Systems analysis of sex differences reveals an immunosuppressive role for
550 testosterone in the response to influenza vaccination. Proc Natl Acad Sci U S A 111:869-
551 74.
552 9. Trigunaite A, Dimo J, Jorgensen TN. 2015. Suppressive effects of androgens on the
553 immune system. Cell Immunol 294:87-94.
554 10. Brotfain E, Gruenbaum SE, Boyko M, Kutz R, Zlotnik A, Klein M. 2016. Neuroprotection
555 by Estrogen and Progesterone in Traumatic Brain Injury and Spinal Cord Injury. Curr
556 Neuropharmacol 14:641-53.
557 11. Murphy E. 2011. Estrogen signaling and cardiovascular disease. Circ Res 109:687-96.
558 12. Strikas RA, Anderson LJ, Parker RA. 1986. Temporal and geographic patterns of isolates
559 of nonpolio enterovirus in the United States, 1970-1983. J Infect Dis 153:346-51.
560 13. Kosrirukvongs P, Kanyok R, Sitritantikorn S, Wasi C. 1996. Acute hemorrhagic
561 conjunctivitis outbreak in Thailand, 1992. Southeast Asian J Trop Med Public Health
562 27:244-9.
563 14. He SJ, Han JF, Ding XX, Wang YD, Qin CF. 2013. Characterization of enterovirus 71 and
564 coxsackievirus A16 isolated in hand, foot, and mouth disease patients in Guangdong,
565 2010. Int J Infect Dis 17:e1025-30.
566 15. Andreoletti L, Leveque N, Boulagnon C, Brasselet C, Fornes P. 2009. Viral causes of
567 human myocarditis. Arch Cardiovasc Dis 102:559-68.
568 16. Khetsuriani N, Lamonte A, Oberste MS, Pallansch M. 2006. Neonatal enterovirus
569 infections reported to the national enterovirus surveillance system in the United States,
570 1983-2003. Pediatr Infect Dis J 25:889-93.

571 17. Khetsuriani N, Lamonte-Fowlkes A, Oberst S, Pallansch MA, Centers for Disease C,
572 Prevention. 2006. Enterovirus surveillance--United States, 1970-2005. MMWR Surveill
573 Summ 55:1-20.
574 18. Abedi GR, Watson JT, Nix WA, Oberste MS, Gerber SI. 2018. Enterovirus and
575 Parechovirus Surveillance - United States, 2014-2016. MMWR Morb Mortal Wkly Rep
576 67:515-518.
577 19. Gebhard JR, Perry CM, Harkins S, Lane T, Mena I, Asensio VC, Campbell IL, Whitton JL.
578 1998. Coxsackievirus B3-induced myocarditis: perforin exacerbates disease, but plays no
579 detectable role in virus clearance. Am J Pathol 153:417-28.
580 20. Fairweather D, Cooper LT, Jr., Blauwet LA. 2013. Sex and gender differences in
581 myocarditis and dilated cardiomyopathy. Curr Probl Cardiol 38:7-46.
582 21. Woodruff JF. 1980. Viral myocarditis. A review. Am J Pathol 101:425-84.
583 22. Huber SA, Job LP, Auld KR. 1982. Influence of sex hormones on Coxsackie B-3 virus
584 infection in Balb/c mice. Cell Immunol 67:173-9.
585 23. Lyden DC, Olszewski J, Feran M, Job LP, Huber SA. 1987. Coxsackievirus B-3-induced
586 myocarditis. Effect of sex steroids on viremia and infectivity of cardiocytes. Am J Pathol
587 126:432-8.
588 24. Huber SA, Kupperman J, Newell MK. 1999. Hormonal regulation of CD4(+) T-cell
589 responses in coxsackievirus B3-induced myocarditis in mice. J Virol 73:4689-95.
590 25. Huber SA. 2008. Coxsackievirus B3-induced myocarditis: infection of females during the
591 estrus phase of the ovarian cycle leads to activation of T regulatory cells. Virology
592 378:292-8.
593 26. Huber SA, Pfaeffle B. 1994. Differential Th1 and Th2 cell responses in male and female
594 BALB/c mice infected with coxsackievirus group B type 3. J Virol 68:5126-32.
595 27. Frisancho-Kiss S, Coronado MJ, Frisancho JA, Lau VM, Rose NR, Klein SL, Fairweather D.
596 2009. Gonadectomy of male BALB/c mice increases Tim-3(+) alternatively activated M2
597 macrophages, Tim-3(+) T cells, Th2 cells and Treg in the heart during acute
598 coxsackievirus-induced myocarditis. Brain Behav Immun 23:649-57.
599 28. Fairweather D, Frisancho-Kiss S, Gatewood S, Njoku D, Steele R, Barrett M, Rose NR.
600 2004. Mast cells and innate cytokines are associated with susceptibility to autoimmune
601 heart disease following coxsackievirus B3 infection. Autoimmunity 37:131-45.
602 29. Robinson DP, Huber SA, Moussawi M, Roberts B, Teuscher C, Watkins R, Arnold AP, Klein
603 SL. 2011. Sex chromosome complement contributes to sex differences in coxsackievirus
604 B3 but not influenza A virus pathogenesis. Biol Sex Differ 2:8.
605 30. Case LK, Toussaint L, Moussawi M, Roberts B, Saligrama N, Brossay L, Huber SA,
606 Teuscher C. 2012. Chromosome y regulates survival following murine coxsackievirus b3
607 infection. G3 (Bethesda) 2:115-21.
608 31. Bopegamage S, Kovacova J, Vargova A, Motusova J, Petrovicova A, Benkovicova M,
609 Gomolcak P, Bakkers J, van Kuppeveld F, Melchers WJ, Galama JM. 2005. Coxsackie B
610 virus infection of mice: inoculation by the oral route protects the pancreas from
611 damage, but not from infection. J Gen Virol 86:3271-80.
612 32. Bopegamage S, Borsanyiova M, Vargova A, Petrovicova A, Benkovicova M, Gomolcak P.
613 2003. Coxsackievirus infection of mice. I. Viral kinetics and histopathological changes in

614 mice experimentally infected with coxsackieviruses B3 and B4 by oral route. Acta Virol
615 47:245-51.
616 33. Harrath R, Bourlet T, Delezay O, Douche-Aourik F, Omar S, Aouni M, Pozzetto B. 2004.
617 Coxsackievirus B3 replication and persistence in intestinal cells from mice infected orally
618 and in the human CaCo-2 cell line. J Med Virol 74:283-90.
619 34. Loria RM, Kibrick S, Broitman SA. 1974. Peroral infection with group B coxsackievirus in
620 the adult mouse: protective functions of the gut. J Infect Dis 130:539-43.
621 35. Loria RM, Kibrick S, Broitman SA. 1974. Peroral infection with group B coxsackievirus in
622 the newborn mouse: a model for human infection. J Infect Dis 130:225-230.
623 36. Robinson CM, Wang Y, Pfeiffer JK. 2017. Sex-Dependent Intestinal Replication of an
624 Enteric Virus. J Virol 91.
625 37. Huber SA, Job LP, Woodruff JF. 1981. Sex-related differences in the pattern of
626 coxsackievirus B-3-induced immune spleen cell cytotoxicity against virus-infected
627 myofibers. Infect Immun 32:68-73.
628 38. Estrin M, Huber SA. 1987. Coxsackievirus B3-induced myocarditis. Autoimmunity is
629 L3T4+ T helper cell and IL-2 independent in BALB/c mice. Am J Pathol 127:335-41.
630 39. Henke A, Huber S, Stelzner A, Whitton JL. 1995. The role of CD8+ T lymphocytes in
631 coxsackievirus B3-induced myocarditis. J Virol 69:6720-8.
632 40. Frisancho-Kiss S, Davis SE, Nyland JF, Frisancho JA, Cihakova D, Barrett MA, Rose NR,
633 Fairweather D. 2007. Cutting edge: cross-regulation by TLR4 and T cell Ig mucin-3
634 determines sex differences in inflammatory heart disease. J Immunol 178:6710-4.
635 41. Fairweather D, Rose NR. 2007. Coxsackievirus-induced myocarditis in mice: a model of
636 autoimmune disease for studying immunotoxicity. Methods 41:118-22.
637 42. Becht E, McInnes L, Healy J, Dutertre CA, Kwok IWH, Ng LG, Ginhoux F, Newell EW.
638 2018. Dimensionality reduction for visualizing single-cell data using UMAP. Nat
639 Biotechnol doi:10.1038/nbt.4314.
640 43. Herr C, Mang S, Mozafari B, Guenther K, Speer T, Seibert M, Srikakulam SK, Beisswenger
641 C, Ritzmann F, Keller A, Mueller R, Smola S, Eisinger D, Zemlin M, Danziger G, Volk T,
642 Hoersch S, Krawczyk M, Lammert F, Adams T, Wagenpfeil G, Kindermann M, Marcu C,
643 Ataya ZWD, Mittag M, Schwarzkopf K, Custodis F, Grandt D, Schaefer H, Eltges K, Lepper
644 PM, Bals R, Group CS. 2021. Distinct Patterns of Blood Cytokines Beyond a Cytokine
645 Storm Predict Mortality in COVID-19. J Inflamm Res 14:4651-4667.
646 44. McDermott DS, Varga SM. 2011. Quantifying antigen-specific CD4 T cells during a viral
647 infection: CD4 T cell responses are larger than we think. J Immunol 187:5568-76.
648 45. Rai D, Pham NL, Harty JT, Badovinac VP. 2009. Tracking the total CD8 T cell response to
649 infection reveals substantial discordance in magnitude and kinetics between inbred and
650 outbred hosts. J Immunol 183:7672-81.
651 46. Butler NS, Schmidt NW, Vaughan AM, Aly AS, Kappe SH, Harty JT. 2011. Superior
652 antimalarial immunity after vaccination with late liver stage-arresting genetically
653 attenuated parasites. Cell Host Microbe 9:451-62.
654 47. Butler NS, Moebius J, Pewe LL, Traore B, Doumbo OK, Tygrett LT, Waldschmidt TJ,
655 Crompton PD, Harty JT. 2011. Therapeutic blockade of PD-L1 and LAG-3 rapidly clears
656 established blood-stage Plasmodium infection. Nat Immunol 13:188-95.

657 48. Kimura D, Miyakoda M, Kimura K, Honma K, Hara H, Yoshida H, Yui K. 2016. Interleukin-
658 27-Producing CD4(+) T Cells Regulate Protective Immunity during Malaria Parasite
659 Infection. Immunity 44:672-682.
660 49. Pardy RD, Rajah MM, Condotta SA, Taylor NG, Sagan SM, Richer MJ. 2017. Analysis of
661 the T Cell Response to Zika Virus and Identification of a Novel CD8+ T Cell Epitope in
662 Immunocompetent Mice. PLoS Pathog 13:e1006184.
663 50. Schlub TE, Badovinac VP, Sabel JT, Harty JT, Davenport MP. 2010. Predicting CD62L
664 expression during the CD8+ T-cell response in vivo. Immunol Cell Biol 88:157-64.
665 51. Nolz JC, Starbeck-Miller GR, Harty JT. 2011. Naive, effector and memory CD8 T-cell
666 trafficking: parallels and distinctions. Immunotherapy 3:1223-33.
667 52. Bohmer RM, Bandala-Sanchez E, Harrison LC. 2011. Forward light scatter is a simple
668 measure of T-cell activation and proliferation but is not universally suited for doublet
669 discrimination. Cytometry A 79:646-52.
670 53. Ohka S, Igarashi H, Nagata N, Sakai M, Koike S, Nochi T, Kiyono H, Nomoto A. 2007.
671 Establishment of a poliovirus oral infection system in human poliovirus receptor-
672 expressing transgenic mice that are deficient in alpha/beta interferon receptor. J Virol
673 81:7902-12.
674 54. Ida-Hosonuma M, Iwasaki T, Yoshikawa T, Nagata N, Sato Y, Sata T, Yoneyama M, Fujita
675 T, Taya C, Yonekawa H, Koike S. 2005. The alpha/beta interferon response controls
676 tissue tropism and pathogenicity of poliovirus. J Virol 79:4460-9.
677 55. Wells AI, Grimes KA, Coyne CB. 2022. Enterovirus Replication and Dissemination Are
678 Differentially Controlled by Type I and III Interferons in the Gastrointestinal Tract. mBio
679 doi:10.1128/mbio.00443-22:e0044322.
680 56. vom Steeg LG, Klein SL. 2016. SeXX Matters in Infectious Disease Pathogenesis. PLoS
681 Pathog 12:e1005374.
682 57. Klein SL, Flanagan KL. 2016. Sex differences in immune responses. Nat Rev Immunol
683 doi:10.1038/nri.2016.90.
684 58. Fairweather D, Stafford KA, Sung YK. 2012. Update on coxsackievirus B3 myocarditis.
685 Curr Opin Rheumatol 24:401-7.
686 59. Robinson CM, Woods Acevedo MA, McCune BT, Pfeiffer JK. 2019. Related Enteric
687 Viruses Have Different Requirements for Host Microbiota in Mice. J Virol 93.
688 60. Kuss SK, Best GT, Etheredge CA, Pruijssers AJ, Frierson JM, Hooper LV, Dermody TS,
689 Pfeiffer JK. 2011. Intestinal microbiota promote enteric virus replication and systemic
690 pathogenesis. Science 334:249-52.
691 61. Baldridge MT, Nice TJ, McCune BT, Yokoyama CC, Kambal A, Wheadon M, Diamond MS,
692 Ivanova Y, Artyomov M, Virgin HW. 2015. Commensal microbes and interferon-lambda
693 determine persistence of enteric murine norovirus infection. Science 347:266-9.
694 62. Dhalech AH, Fuller TD, Robinson CM. 2021. Specific Bacterial Cell Wall Components
695 Influence the Stability of Coxsackievirus B3. J Virol 95:e0142421.
696 63. Robinson CM, Jesudhasan PR, Pfeiffer JK. 2014. Bacterial lipopolysaccharide binding
697 enhances virion stability and promotes environmental fitness of an enteric virus. Cell
698 Host Microbe 15:36-46.
699 64. Markle JG, Frank DN, Mortin-Toth S, Robertson CE, Feazel LM, Rolle-Kampczyk U, von
700 Bergen M, McCoy KD, Macpherson AJ, Danska JS. 2013. Sex differences in the gut

701 microbiome drive hormone-dependent regulation of autoimmunity. Science 339:1084-
702 8.
703 65. Yurkovetskiy L, Burrows M, Khan AA, Graham L, Volchkov P, Becker L, Antonopoulos D,
704 Umesaki Y, Chervonsky AV. 2013. Gender bias in autoimmunity is influenced by
705 microbiota. Immunity 39:400-12.
706 66. Maggio M, Blackford A, Taub D, Carducci M, Ble A, Metter EJ, Braga-Basaria M, Dobs A,
707 Basaria S. 2006. Circulating inflammatory cytokine expression in men with prostate
708 cancer undergoing androgen deprivation therapy. J Androl 27:725-8.
709 67. Trigunaite A, Dimo J, Jørgensen TN. 2015. Suppressive effects of androgens on the
710 immune system. Cellular Immunology 294:87-94.
711 68. Wilhelmson AS, Lantero Rodriguez M, Stubelius A, Fogelstrand P, Johansson I, Buechler
712 MB, Lianoglou S, Kapoor VN, Johansson ME, Fagman JB, Duhlin A, Tripathi P,
713 Camponeschi A, Porse BT, Rolink AG, Nissbrandt H, Turley SJ, Carlsten H, Martensson IL,
714 Karlsson MCI, Tivesten A. 2018. Testosterone is an endogenous regulator of BAFF and
715 splenic B cell number. Nat Commun 9:2067.
716 69. Slifka MK, Pagarigan R, Mena I, Feuer R, Whitton JL. 2001. Using recombinant
717 coxsackievirus B3 to evaluate the induction and protective efficacy of CD8+ T cells
718 during picornavirus infection. J Virol 75:2377-87.
719 70. Cornell CT, Kiosses WB, Harkins S, Whitton JL. 2007. Coxsackievirus B3 proteins
720 directionally complement each other to downregulate surface major histocompatibility
721 complex class I. J Virol 81:6785-97.
722 71. Kemball CC, Harkins S, Whitmire JK, Flynn CT, Feuer R, Whitton JL. 2009. Coxsackievirus
723 B3 inhibits antigen presentation in vivo, exerting a profound and selective effect on the
724 MHC class I pathway. PLoS Pathog 5:e1000618.
725
726
727
728
729
730
731
732
733
734
735

736 **Figure Legends**

737

738 **Figure 1.** CVB3-induced lethality, fecal shedding, and dissemination are enhanced by
739 testosterone. (A) Serum testosterone concentrations in mock or castrated male C57BL/6 *Ifnar*$^{-/-}$
740 provided either placebo or testosterone capsules. **p<0.01, one-way ANOVA. (B) Survival of
741 castrated + placebo (testosterone-depleted) and castrated + testosterone (testosterone-treated)
742 mice after oral inoculation with $5x10^7$ PFU of CVB3-Nancy. n=8-9 mice per group. **p<0.01,
743 ***p<0.001, Log-rank test. (C) CVB3-Nancy fecal titers in testosterone-depleted (gray) and
744 testosterone-treated (blue and gray) mice at days 2 and 3 post-inoculation. n=15-16 mice per
745 group. **p<0.01, ***p<0.001, Mann-Whitney test. (D) CVB3-Nancy tissue titers. Mice were
746 euthanized at 3 dpi, and tissues were collected in testosterone-depleted (gray) and testosterone-
747 treated (blue with diagonal lines) *Ifnar*$^{-/-}$ mice. n=8-9 mice per group. *p<0.05, Mann-Whitney
748 test. Data are shown as mean ± SEM. LOD = limit of detection.

749

750 **Figure 2.** Testosterone promotes pro-inflammatory cytokines and chemokines in response to
751 CVB3. Infected mice were orally inoculated with $5x10^7$ PFUs of CVB3-Nancy, and serum was
752 collected at 3 dpi. (A) Heatmap of the serum levels of the indicated cytokine or chemokines at 3
753 dpi between CVB3-infected testosterone-depleted and testosterone-treated male *Ifnar*$^{-/-}$ mice.
754 The log_2-fold change was normalized to uninfected mice. (B-E) The serum concentration of
755 indicated cytokine and chemokines from infected mice. The serum concentration for each
756 cytokine is represented by a box and whisker plot. The box represents the 25th and 75th
757 percentiles of the data and the center line in the box represents the median value. The black
758 whiskers mark the 5th and 95th percentiles of the data. *p<0.05, **p<0.01, ***p<0.001, Kruskal-

759 Wallis test. (F) UMAP analysis of chemokine and cytokine serum concentrations in uninfected
760 (black circles), testosterone-depleted (gray circles), and testosterone-treated (blue circles) male
761 *Ifnar*$^{-/-}$ mice. Data are representative of two independent experiments with n=7-11 mice per
762 group.
763
764 **Figure 3.** The effect of testosterone on the frequency and number of dendritic cells following
765 oral CVB3 inoculation. (A) Representative flow cytometry plots of CD11c^{+}, MHC II splenic
766 dendritic cells at 5dpi in uninfected and CVB3-infected mice that are testosterone-treated or
767 testosterone-depleted (placebo). The frequency (B) and number (C) of splenic dendritic cells in
768 testosterone-treated (blue with diagonal lines) and testosterone-depleted (gray) mice following
769 infection. *$p<0.05$, **$p<0.01$, ****$p<0.0001$, two-way ANOVA. All data are from two
770 independent experiments with n = 4-6 mice per group and are shown as mean ± SEM.
771
772 **Figure 4**. CVB3 and testosterone impact the proportion of splenic CD4^{+} and CD8^{+} T cells
773 following oral inoculation. (A) Representative gating strategies to identify CD4^{+} and CD8^{+} T
774 cells. (B) Representative flow cytometry plots of CD4^{+} and CD8^{+} T cells in uninfected and
775 infected mice that are testosterone-treated or testosterone-depleted (placebo). (C) The frequency
776 of CD4^{+} T cells in the spleen in testosterone-treated (blue with diagonal lines) and testosterone-
777 depleted (gray) mice. (D) The frequency of CD8^{+} T cells in the spleen in testosterone-treated
778 (blue with diagonal lines) and testosterone-depleted (gray) mice. *$p<0.05$, **$p<0.01$,
779 ****$p<0.0001$, two-way ANOVA. All data are from two independent experiments with n = 4-6
780 mice per group and are shown as mean ± SEM.
781

782 **Figure 5.** Testosterone does not affect the early $CD4^+$ T cell response to CVB3. (A)
783 Representative gating strategy for $CD11a^{hi}CD49d^{hi}$ $CD4^+$ T cells. (B) The frequency of
784 $CD49d^{hi}CD11a^{hi}$ $CD4^+$ T cells in the spleen at 5 dpi. All data are from two independent
785 experiments, n=5-6 mice per group. Two-way ANOVA.
786
787 **Figure 6.** Testosterone dampens the activation of $CD8^+$ T cells following oral CVB3 infection.
788 (A) Representative flow cytometry plot of the gating strategy to identify $CD62L^{lo}$ $CD8^+$ T cells.
789 (B) The frequency of $CD62L^{lo}$ $CD8^+$ T cells in the spleen of testosterone-treated (blue with
790 diagonal lines) and testosterone-depleted (gray) male mice. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ns
791 = not significant, two-way ANOVA. (C) The geometric mean fluorescence intensity (gMFI) FSC
792 of $CD62L^{lo}$ $CD8^+$ T cells **$p<0.01$, unpaired t-test. (D) Representative of the gMFI SSC of
793 $CD62L^{lo}$ $CD8^+$ T cells from two independent experiments. *$p<0.05$, unpaired t-test. (E)
794 Representative flow cytometry plots of the expression of CD11a and CD62L $CD8^+$ T cells in the
795 spleen of testosterone-treated (blue with diagonal lines) and testosterone-depleted (gray) male
796 mice. (F) The frequency of $CD11a^{hi}CD62L^{lo}$ $CD8^+$ T cells in the spleen following CVB3
797 infection *$p<0.05$, two-way ANOVA. All data are from two independent experiments with n =
798 5-6 mice per group and are shown as mean ± SEM.
799
800 **Figure 7.** Testosterone promotes CVB3 shedding and dissemination in female *Ifnar*$^{-/-}$ mice, but
801 not lethality. (A) Serum testosterone concentrations in female C57BL/6 *Ifnar*$^{-/-}$ provided either
802 placebo or testosterone capsules. (B) Survival of female C57BL/6 *Ifnar*$^{-/-}$ provided either placebo
803 or testosterone capsules after oral inoculation with $5x10^7$ PFU of CVB3-Nancy. (C) CVB3-
804 Nancy fecal titers in female C57BL/6 *Ifnar*$^{-/-}$ provided either placebo or testosterone. *$p<0.05$,

805 Mann-Whitney test. (D) CVB3-Nancy tissue titers in female C57BL/6 *Ifnar*$^{-/-}$ provided either

806 placebo or testosterone. All data are from two-three independent experiments, n=5-8 mice per

807 group. *p<0.05, **p<0.01, Mann-Whitney test. LOD = limit of detection.

808

809

810 **Table 1.**

	Frequency of cells (%) per spleen		Total number of cells (x10^3) per spleen		Frequency of cells (%) per spleen		Total number of cells (x10^3) per spleen	
Infection	**Uninfectd male mice**				**CVB3-infected male mice**			
Cell type	**Placebo**	**Testosterone**	**Placebo**	**Testosterone**	**Placebo**	**Testosterone**	**Placebo**	**Testosterone**
Total Splenocytes	100	100	**51375±5134.57***	**29250±2721.57***	100	100	**33166.667±2216.10***	**21200±2527.85***
Macrophages	1.62±0.10	2.55±0.35	840.0±114.8	653.8±51.15	1.55±0.15	2.08±0.32	451.9±90.6	521.8±79.4
Monocytes	0.7538±0.13	0.7111±0.06	402.763±102.02	197.672±25.17	0.783±0.08	1.07±0.17	258.3±27.4	218.1±29.3
Neutrophils	0.435±0.09	1.25±0.36	205.025±41.35	306.4±74.07	0.2±0.06	0.374±0.08	66.4±20.6	84.1±21.5
B cells	**58.86±1.76***	**45.72±3.95***	**30553.875±3730.96***	**12776.25±1380.84***	**58.18±2.05***	**41.3±2.73***	**19592±1434.7***	**8928.4±1329.4***

Data are presented as mean +/- SEM from two independnent experiments (n=5-6 per group).

811 Significant differences between groups are bolded and denoted by asterisks (*p<0.05).

Figure 1

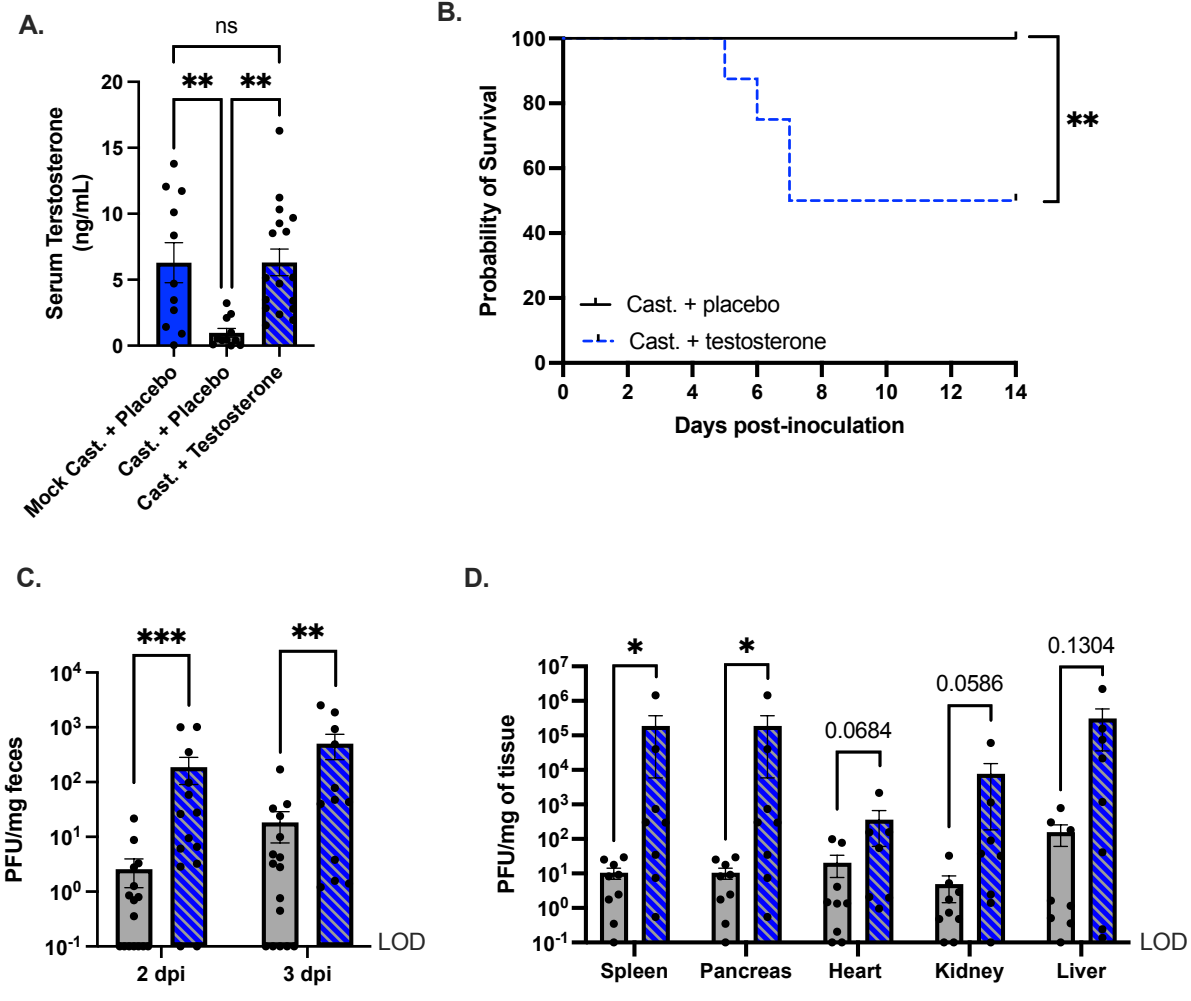

A.
ns
**
**
Serum Terstosterone (ng/mL)
20
15
10
5
0
Mock Cast. + Placebo
Cast. + Placebo
Cast. + Testosterone
B.
Probability of Survival
100
80
60
40
20
0
**
Cast. + placebo
Cast. + testosterone
0
2
4
6
8
10
12
14
Days post-inoculation
C.
***
**
PFU/mg feces
10^4
10^3
10^2
10^1
10^0
10^{-1}
LOD
2 dpi
3 dpi
D.
*
*
0.0684
0.0586
0.1304
PFU/mg of tissue
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
10^{-1}
LOD
Spleen
Pancreas
Heart
Kidney
Liver

Figure 2

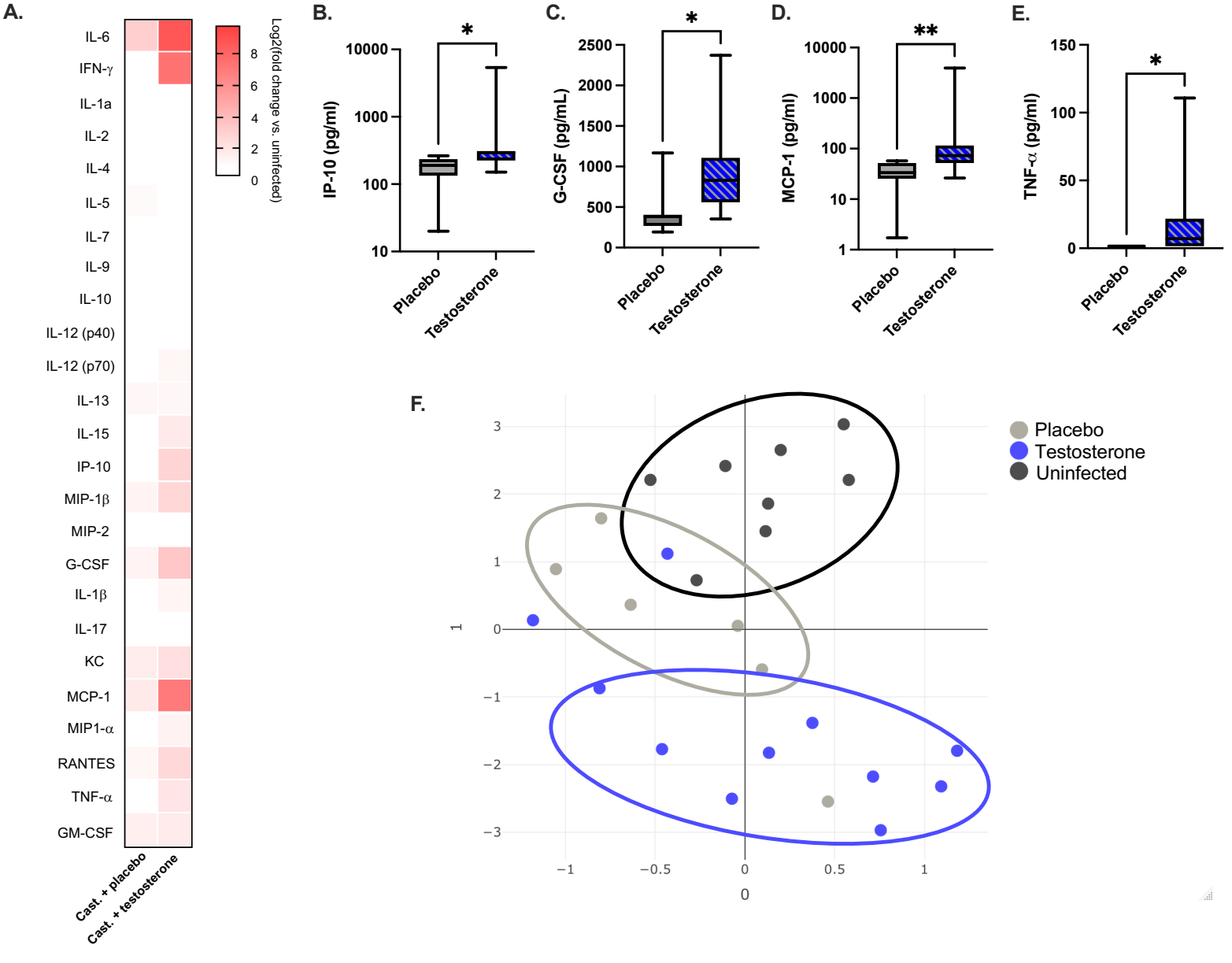

A.
IL-6
IFN-γ
IL-1a
IL-2
IL-4
IL-5
IL-7
IL-9
IL-10
IL-12 (p40)
IL-12 (p70)
IL-13
IL-15
IP-10
MIP-1β
MIP-2
G-CSF
IL-1β
IL-17
KC
MCP-1
MIP1-α
RANTES
TNF-α
GM-CSF
Cast. + placebo
Cast. + testosterone
Log2(fold change vs. uninfected)
8
6
4
2
0
B.
IP-10 (pg/ml)
10000
1000
100
10
*
Placebo
Testosterone
C.
G-CSF (pg/mL)
2500
2000
1500
1000
500
0
*
Placebo
Testosterone
D.
MCP-1 (pg/ml)
10000
1000
100
10
1
**
Placebo
Testosterone
E.
TNF-α (pg/ml)
150
100
50
0
*
Placebo
Testosterone
F.
Placebo
Testosterone
Uninfected
1
3
2
1
0
−1
−2
−3
−1
−0.5
0
0.5
1
0

Figure 3

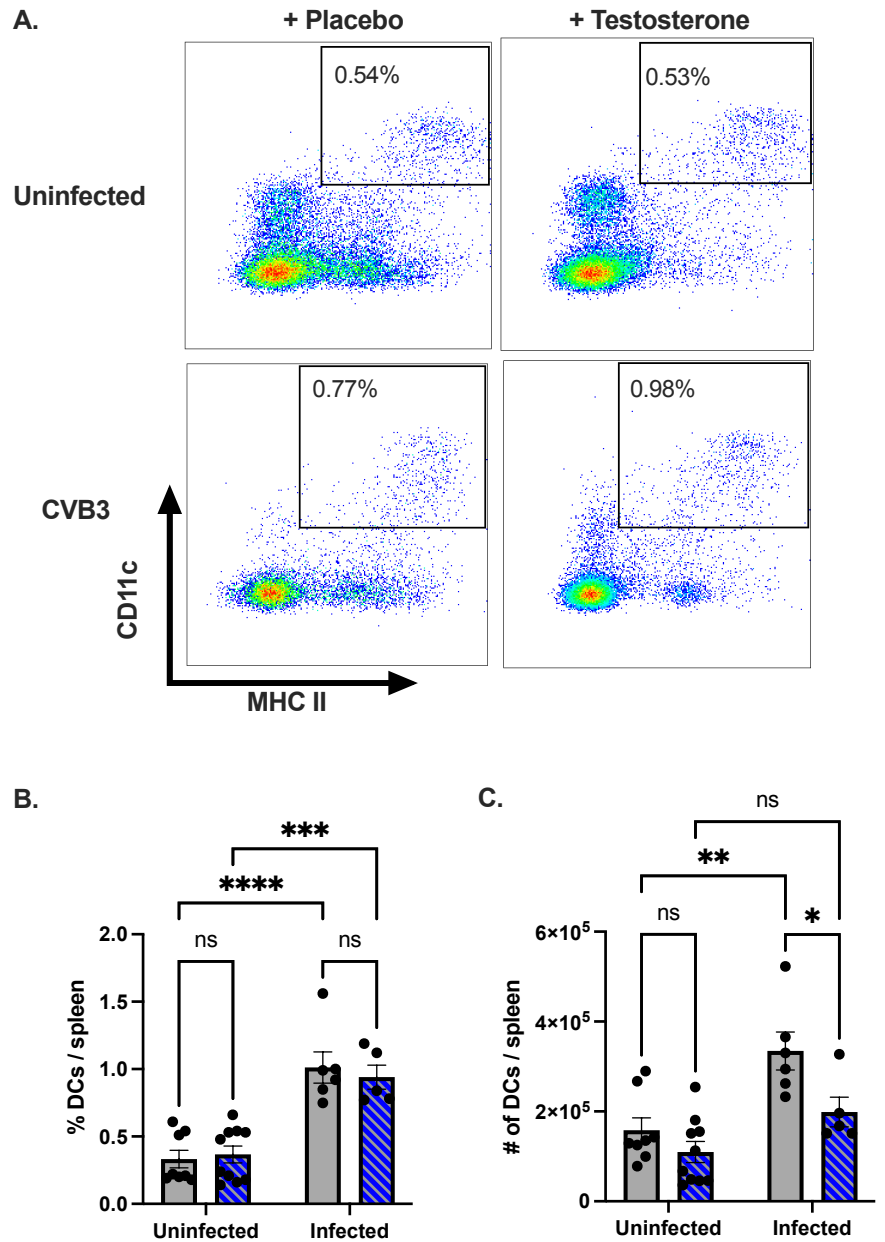

A.
+ Placebo
+ Testosterone
Uninfected
CVB3
0.54%
0.53%
0.77%
0.98%
CD11c
MHC II
B.
% DCs / spleen
2.0
1.5
1.0
0.5
0.0
ns
ns
****
***
Uninfected
Infected
C.
# of DCs / spleen
6×10^5
4×10^5
2×10^5
0
ns
ns
**
*
Uninfected
Infected

Figure 4

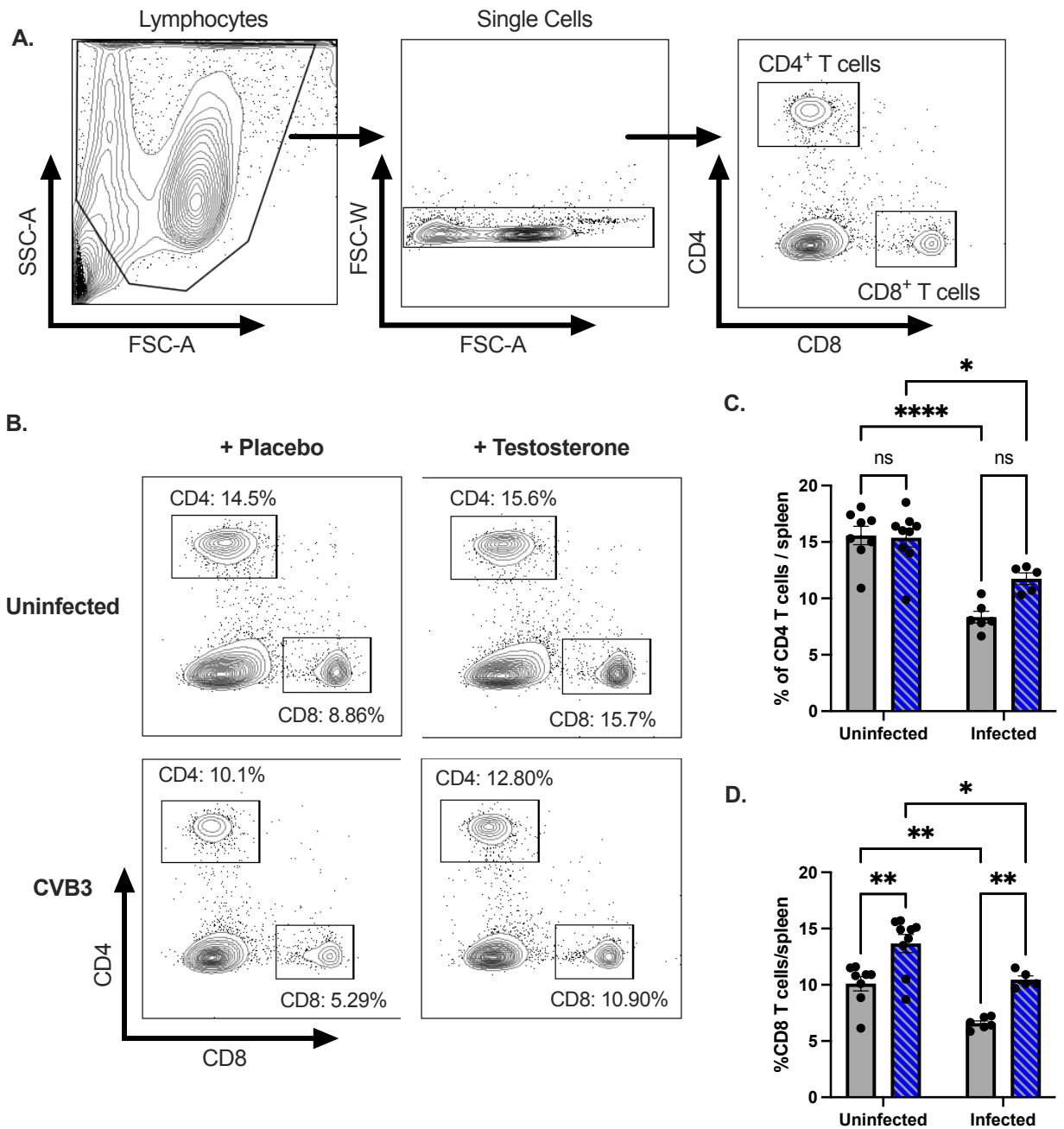
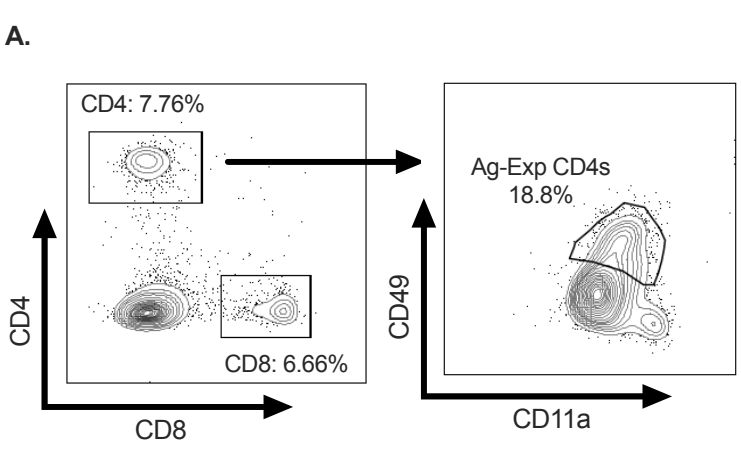

A.
Lymphocytes
SSC-A
FSC-A
Single Cells
FSC-W
FSC-A
CD4+ T cells
CD8+ T cells
CD4
CD8
B.
+ Placebo
+ Testosterone
Uninfected
CVB3
CD4: 14.5%
CD8: 8.86%
CD4: 15.6%
CD8: 15.7%
CD4: 10.1%
CD8: 5.29%
CD4: 12.80%
CD8: 10.90%
CD4
CD8
C.
% of CD4 T cells / spleen
ns
ns
****
*
Uninfected
Infected
D.
%CD8 T cells/spleen
**
**
**
*
Uninfected
Infected

Figure 5


A.
CD4: 7.76%
CD8: 6.66%
CD4
CD8
Ag-Exp CD4s
18.8%
CD49
CD11a

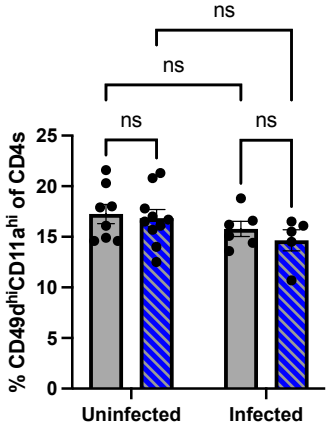

B.
ns
ns
ns
ns
% CD49d^hiCD11a^hi of CD4s
25
20
15
10
5
0
Uninfected
Infected

Figure 6

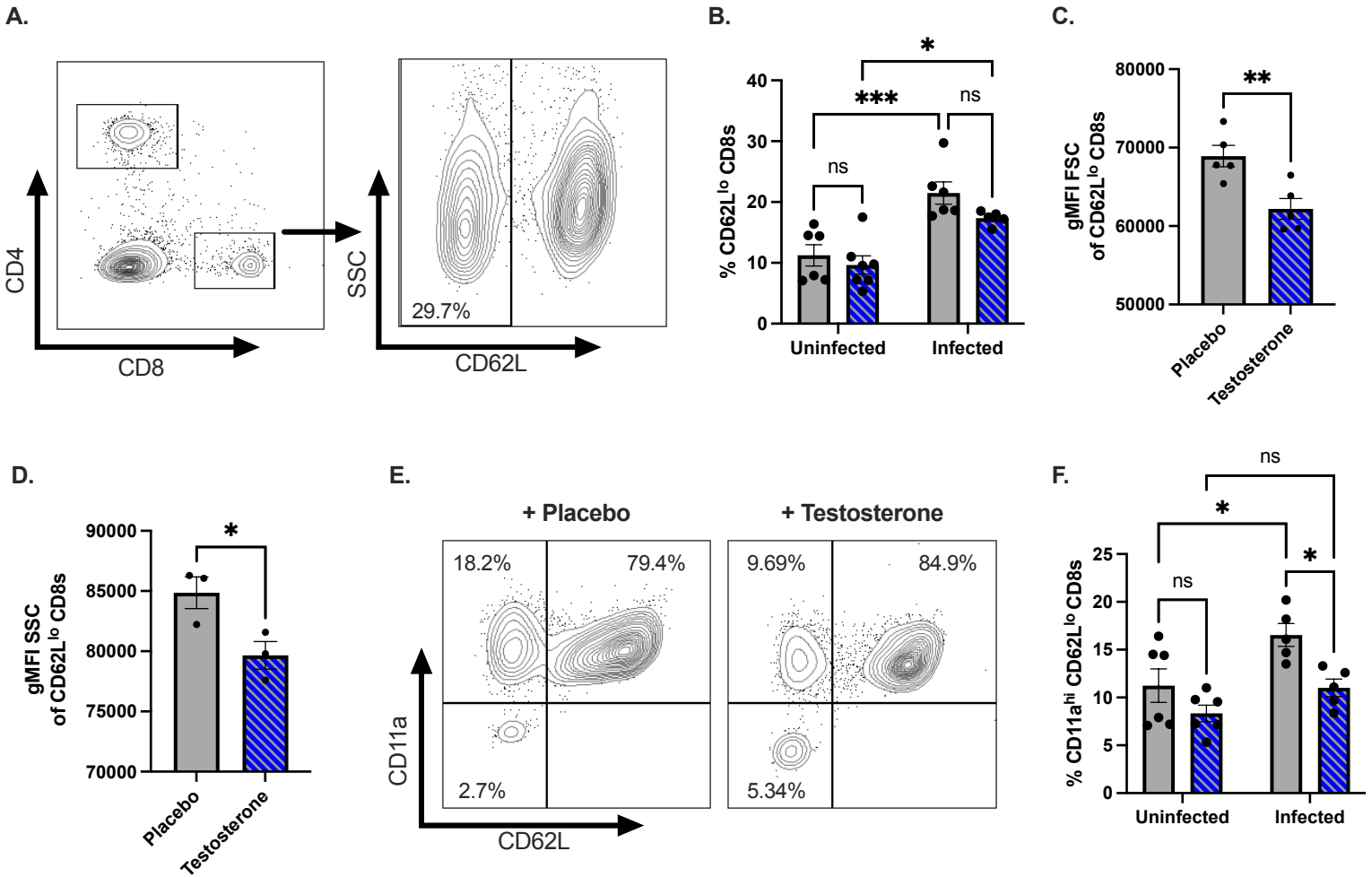
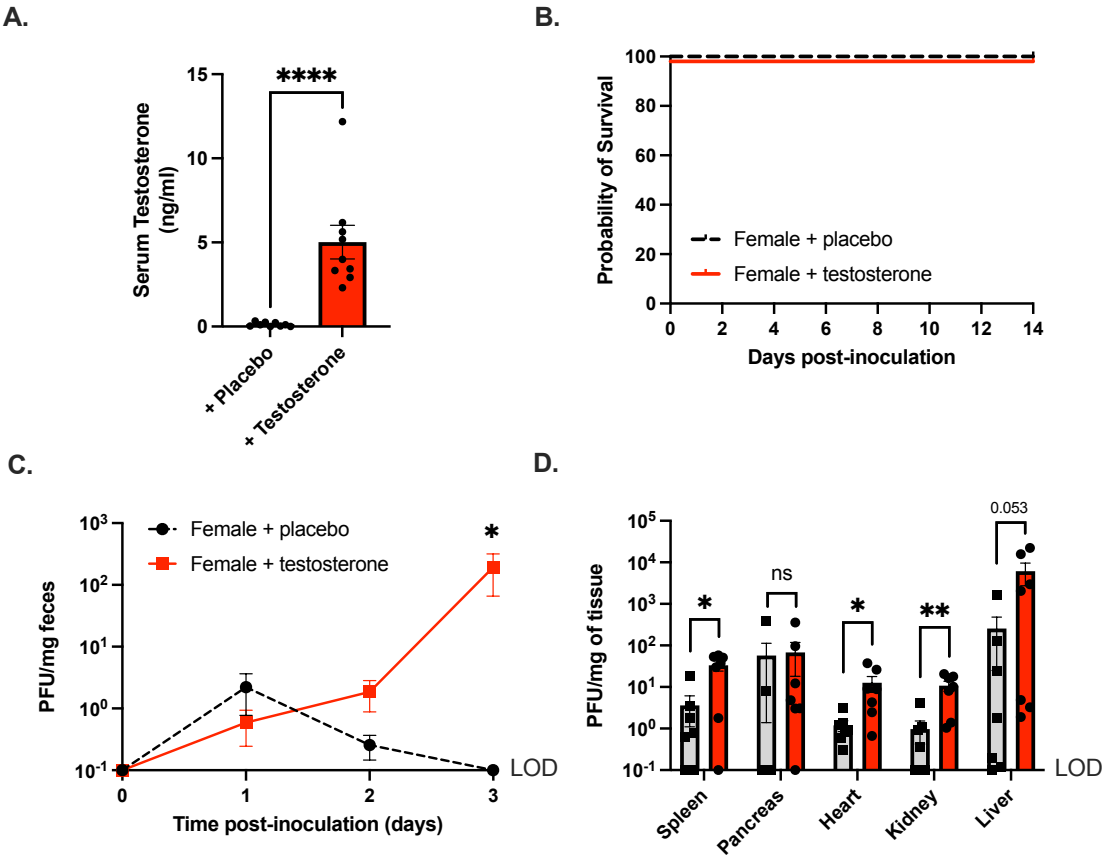

A.
CD4
CD8
SSC
CD62L
29.7%
B.
% CD62L^lo CD8s
40
30
20
10
0
*
***
ns
ns
Uninfected
Infected
C.
gMFI FSC of CD62L^lo CD8s
80000
70000
60000
50000
**
Placebo
Testosterone
D.
gMFI SSC of CD62L^lo CD8s
90000
85000
80000
75000
70000
*
Placebo
Testosterone
E.
+ Placebo
+ Testosterone
18.2%
79.4%
2.7%
9.69%
84.9%
5.34%
CD11a
CD62L
F.
% CD11a^hi CD62L^lo CD8s
25
20
15
10
5
0
ns
*
ns
*
Uninfected
Infected

Figure 7


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
*
LOD
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}
*
ns
*
**
0.053
LOD
Spleen
Pancreas
Heart
Kidney
Liver