RESEARCH ARTICLE

Androgen receptor signaling in the lungs mitigates inflammation and improves the outcome of influenza in mice

Landon G. vom Steeg1, Santosh Dhakal1, Yishak A. Woldetsadik1, Han-Sol Park1, Kathleen R. Mulka2, Emma C. Reilly3, David J. Topham3, Sabra L. Klein1,4*

1 W. Harry Feinstone Department of Molecular Microbiology and Immunology, The Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland, United States of America, 2 Department of Molecular and Comparative Pathobiology, The Johns Hopkins School of Medicine, Baltimore, Maryland, United States of America, 3 Department of Microbiology and Immunology, University of Rochester Medical Center, School of Medicine and Dentistry, Rochester, New York, United States of America, 4 Department of Biochemistry and Molecular Biology, The Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland, United States of America

* sklein2@jhu.edu

Abstract

Circulating androgens can modulate immune cell activity, but the impact of androgens on viral pathogenesis remains unclear. Previous data demonstrate that testosterone reduces the severity of influenza A virus (IAV) infection in male mice by mitigating pulmonary inflammation rather than by affecting viral replication. To examine the immune responses mediated by testosterone to mitigate IAV-induced inflammation, adult male mice remained gonadally intact or were gonadectomized and treated with either placebo or androgen-filled (i.e., testosterone or dihydrotestosterone) capsules prior to sublethal IAV infection. Like intact males, treatment of gonadectomized males with androgens improved the outcome of IAV infection, which was not mediated by changes in the control of virus replication or pulmonary cytokine activity. Instead, androgens accelerated pulmonary leukocyte contraction to limit inflammation. To identify which immune cells were contracting in response to androgens, the composition of pulmonary cellular infiltrates was analyzed and revealed that androgens specifically accelerated the contraction of total pulmonary inflammatory monocytes during peak disease, as well as CD8+ T cells, IAV-specific CD8+ T numbers, cytokine production and degranulation by IAV-specific CD8+ T cells, and the influx of eosinophils into the lungs following clearance of IAV. Neither depletion of eosinophils nor adoptive transfer of CD8+ T cells could reverse the ability of testosterone to protect males against IAV suggesting these were secondary immunologic effects. The effects of testosterone on the contraction of immune cell numbers and activity were blocked by co-administration of the androgen receptor antagonist flutamide and mimicked by treatment with dihydrotestosterone, which was also able to reduce the severity of IAV in female mice. These data suggest that androgen receptor signaling creates a local pulmonary environment that promotes downregulation of detrimental inflammatory immune responses to protect against prolonged influenza disease.
Author summary

In the United States alone, it is estimated that over 2 million men are taking testosterone replacement therapy caused by congenital, acquired, or age-associated reductions in circulating testosterone, with known immunomodulatory effects. Despite the increasing popularity of testosterone replacement therapy, the influence of testosterone deficiency and treatment on clinical outcomes of infectious disease has not been adequately considered. Disease following influenza A virus (IAV) infection is largely immune-mediated, with severe disease often associated with excessive or aberrant immune responses (i.e., a cytokine storm) to the virus. We have made the novel observation that administration of testosterone to male mice improves the outcome of IAV infection not by mitigating global pulmonary cytokine production, but by promoting the specific contraction of pulmonary inflammatory monocytes during peak disease and the frequencies of virus-specific pulmonary CD8\(^+\) T cells and eosinophils in the lungs following control of viral replication. The protective effects of testosterone on IAV pathogenesis are dependent on androgen receptor signaling, which creates a pulmonary environment conducive to reduced pulmonary inflammation. Rather than acting directly on a single cell population, androgen receptor signaling has multicellular effects and creates a local environment that promotes accelerated contraction of inflammatory immune cells. Activation of androgen receptor signaling confers protection during IAV infection by modulating the immune response, which may have therapeutic potential in both male and female patients.

Introduction

Testosterone is a sex steroid hormone produced and released primarily by Leydig cells in the testes of males, which has significant effects on health and disease [1]. In men, low testosterone, whether congenital, acquired, or age-related, is associated with an increased risk of all-cause and cardiovascular-related mortality [2–4]. Additionally, low testosterone in males has been linked to metabolic dysfunction, osteoporosis, muscle weakness, fatigue, cognitive impairment, and sexual dysfunction; while in hypogonadal men, testosterone replacement therapy has been shown to improve cardiovascular disease outcomes, increase quality of life perceptions, and improve age-associated anemia [4–9]. Although safety concerns exist, the perceived benefits of testosterone replacement therapies have resulted in a dramatic increase in its therapeutic use over the last two decades, with an estimated 2.3 million men undergoing testosterone replacement therapy in the United States alone in 2013 [10, 11]. Included in these numbers is a 4-fold increase in testosterone replacement therapy use in reproductively aged males (i.e. 18 to 45 years of age), a demographic often overlooked in studies of the implications of low testosterone [12]. Despite the increasing popularity of testosterone replacement therapy, the influence of testosterone deficiency and treatment on clinical outcomes of infectious disease has not been adequately considered.

The biological effects of testosterone are typically mediated through androgen receptor (AR) signaling [2, 13]. Intracellular ARs are present in cells throughout the body, with testosterone modulating the activities of a variety of tissue and cell types [2]. Notably, ARs are widely expressed in cells of both the innate and adaptive immune system, including macrophages, neutrophils, and T cells [2, 13]. In humans and nonhuman animals, testosterone and its physiologically active metabolite, dihydrotestosterone (DHT), are broadly immunomodulatory and capable of altering the number, function, and differentiation of most immune cell populations.
For example, in the presence of testosterone, murine macrophages increase IL-10 and decrease TNFα synthesis, while T cell numbers and activity (e.g., IL-4 and IL-12 production) are reduced [14, 15]. In adult human males, clinical depletion of testosterone decreases regulatory T cell numbers (Tregs), reduces mitogen-induced IFNγ expression in CD8+ T cells, and suppresses the ability of natural killer cells to proliferate [16]. In contrast, production of cytokines, including TNFα and IL-1β, by monocytes, is enhanced in human males compared with females suggesting a possible immunostimulatory role for testosterone in some cell populations [17] and that sex differences and the immunomodulatory effects of sex steroids can vary based on cell type [18]. Although, the immunomodulatory properties of testosterone are established, the impact of low testosterone on the severity of viral infection remains incompletely characterized. If testosterone is capable of broadly regulating the immune system, then in viral infections where pathogenesis is driven by the immune response rather than viral replication, testosterone is likely to reduce the severity of infection.

Disease following influenza A virus (IAV) infection is largely immune-mediated, with severe disease often associated with excessive or aberrant immune responses (i.e., a cytokine storm) to the virus [19, 20]. We have previously shown that low testosterone in males, whether age-related or surgically-induced, increases the severity of IAV infection [21–23]. Furthermore, these changes are associated with delayed resolution of pulmonary inflammation (i.e., following control of viral replication), independent of either changes in viral replication or induction of growth factors (e.g., amphiregulin), suggesting that the protective effects of testosterone are mediated through downregulation of the inflammatory response during infection [22, 23]. In the current study, we sought to characterize the effects of testosterone on the immune response to IAV using a murine model of IAV infection. We show that testosterone improves the outcomes of IAV infection not by mitigating the cytokine storm, but by promoting the contraction of pulmonary inflammatory monocytes during peak disease and virus-specific pulmonary CD8+ T cells and eosinophils in the lungs following control of viral replication. Neither depletion of eosinophils nor adoptive transfer of CD8+ T cells could mitigate the protective effects of androgens on IAV pathogenesis, suggesting these were secondary effects that were a consequence and not the cause of androgen receptor signaling-mediated protection. The protective effects of androgen receptor signaling in the lungs during IAV pathogenesis were present in both males and females and may have broad therapeutic potential.

Results

Testosterone reduces the severity of IAV infection in male mice

To reproduce and confirm previous reported effects of testosterone on the severity of IAV infection [21, 22], adult male mice underwent sham surgeries or were gonadectomized and received either testosterone or placebo capsules. Gonadectomized males had significantly lower concentrations of circulating testosterone and seminal vesicle mass (i.e., androgen responsive tissue that can be used as a biomarker of circulating concentrations) than either gonad-intact or gonadectomized males that received testosterone (Fig 1A and 1B; \(p < 0.05\)). Following intranasal inoculation with a sub-lethal dose of mouse-adapted (ma)2009 H1N1, mice were monitored for 21 days post inoculation (dpi) for changes in body mass, body temperature, and clinical disease severity. Similar to previous studies [21, 22], testosterone-depleted mice experienced greater body mass loss, hypothermia, and clinical disease severity than either gonad-intact or gonadectomized males that received testosterone (Fig 1C–1E; \(p < 0.05\)). Despite these differences in the severity of IAV infection between testosterone-depleted and testosterone-replete mice, neither peak virus titers (i.e., the highest measured) at 7 dpi nor the clearance of detectable infectious virus from the lungs by 14 dpi was affected by
Fig 1. Testosterone depletion increases the severity of influenza A virus (IAV) infection. Adult male mice were gonadectomized and implanted with either testosterone (gdx + T) or placebo (gdx) containing capsules or received sham surgeries (intact) prior to inoculation with...
Testosterone concentrations in males (Fig 1F), suggesting that testosterone does not alter resistance to IAV infection. An alternate strategy that can be employed by hosts to mitigate the detrimental effects of infection is disease tolerance, which reduces the fitness costs of infection independent of changes in pathogen survival [24, 25]. To test whether testosterone made males more tolerant to IAV infection, we analyzed the interaction between hypothermia and pulmonary viral load at 7 and 14 dpi. Testosterone-replete males experienced less body mass loss relative to viral load than placebo-treated males during both peak viral titers (7 dpi) and following control of viral replication (14 dpi; Fig 1G and 1H; \( p < 0.05 \) in each case). These data suggest that testosterone reduces the severity of IAV infection by making males more tolerant, rather resistant, to IAV infection.

Pulmonary cytokine and chemokine concentrations are not altered by testosterone in males

The severity of IAV infection is associated with induction of pulmonary cytokine and chemokine responses, which can lead to excessive cellular infiltration, pulmonary inflammation, and tissue damage, if improperly regulated [26, 27]. To test whether testosterone altered the kinetics or magnitude of the cytokine and chemokine response during IAV infection, pulmonary concentrations of 24 cytokines and chemokines were measured at selected time-points. Pulmonary concentrations of pro-inflammatory cytokines and chemokines (e.g. IL-6, IFN\( \gamma \), and CCL2) broadly increased in response to infection at 3 and 9 dpi, and then declined following control of viral replication (14 dpi; Fig 2A–2E and S1 Table). The only chemokine that was significantly altered by testosterone treatment was CXCL1, which was found in greater concentrations in the lungs of testosterone-treated than testosterone-depleted male mice at 3 and 14, but not 9, dpi (Fig 2E; \( p < 0.05 \)). Despite the known anti-inflammatory effects of testosterone [28–30], testosterone treatment did not alter pulmonary concentrations of either IL-10 or TGF\( \beta \) during IAV infection (Fig 2F and S1 Table). Taken together, these data suggest that the improved outcome of IAV associated with testosterone is independent of substantial changes in the ‘cytokine storm’ during IAV infection, at least at the time-points selected.

Testosterone alters the influx and contraction of pulmonary immune cells during the resolution of IAV infection

Differences in the numbers and kinetics of immune cells that influx into the lungs during infection can greatly impact IAV pathogenesis [31–33]. To test the hypothesis that testosterone affected immune cell recruitment into the lungs during IAV infection, total numbers of innate and adaptive immune cells were enumerated in the lungs of testosterone or placebo-treated gonadectomized male mice. The total number of leukocytes (i.e., CD45\(^+\) cells) in the lungs peaked at 7 dpi (i.e., during peak virus replication) and was followed by a greater decline in cell numbers in the lungs of testosterone-treated relative to placebo-treated males at 14 and 21 dpi (i.e., after control of virus replication) (Fig 3A; \( p < 0.05 \)). Thus, the largest effect of testosterone on immune cell infiltrates was observed after control of virus replication.
Fig 2. Testosterone does not alter pulmonary cytokine or chemokine concentration during influenza A virus (IAV) infection. Adult male mice were gonadectomized and implanted with either testosterone (gdx + T) or placebo (gdx) containing capsules, and then inoculated with a sub-lethal dose of ma2009 H1N1 IAV or were mock infected. At 0, 3, 9, or 14 dpi (n = 8-11/treatment/time-point), lung tissue was collected and homogenized, and cell free supernatants were used to quantify pulmonary concentrations of IL-6 (A), TNFα (B), CCL2 (C), IFNγ (D), CXCL1 (E), and IL-10 (F). Data represent means +/- SEM from two independent replications and significant differences between treatment groups are denoted by asterisks (*p < 0.05).

https://doi.org/10.1371/journal.ppat.1008506.g002
To identify which immune cell types persisted in the lungs of testosterone-depleted males, we characterized the composition of pulmonary cellular infiltrates. During IAV infection, the numbers of interstitial macrophages, neutrophils, inflammatory monocytes, plasmacytoid dendritic cells, and conventional dendritic cells increased in both testosterone- and placebo-treated gonadectomized males, with the greatest numbers of innate immune cells in the lungs occurring at 7 dpi (Table 1). In contrast, the number of alveolar macrophages declined over the course of infection in all male mice (Table 1).

The only innate immune cell populations affected by testosterone treatment in males were neutrophils, inflammatory monocytes, and eosinophils. The number of pulmonary neutrophils was transiently greater at baseline (0 dpi) and 3pi, but not at other dpi, in gonadectomized males that were treated with testosterone as compared with those treated with placebo (Table 1; \( p < 0.05 \)). Following IAV infection, the contraction of inflammatory monocyte numbers in the lungs was accelerated in testosterone-treated males compared with placebo-treated males during peak disease (i.e., at 7 dpi; Fig 3B; \( p < 0.05 \)). In contrast, a significant influx of eosinophils into lungs occurred in gonadectomized males treated with placebo, but not testosterone, after clearance of detectable virus from the lungs (i.e., 14 and 21 dpi) (Fig 3C; \( p < 0.05 \)).

The influx of eosinophils into the lungs of testosterone-depleted males during the resolution phase of infection may be indicative of eosinophilic pneumonia [34], which could contribute to the greater pulmonary inflammation analyzed previously by histology in testosterone-depleted male mice [22]. To test this hypothesis, histopathological scoring for specific markers

https://doi.org/10.1371/journal.ppat.1008506.g003
of eosinophilic inflammation was performed on H&E stained lung sections. Fourteen dpi, histologic evidence of eosinophilic pulmonary inflammation was greater in placebo-treated compared with testosterone-treated males, which was characterized by the accumulation of eosinophils within the interstitium and alveolar spaces (Fig 3D and Fig 3E; \( p < 0.05 \)).

To determine whether the inhibition of pulmonary eosinophil accumulation was mediating testosterone-dependent protection against severe IAV, mice were gonadectomized, treated with either testosterone or placebo, infected with IAV, and treated daily with either IL-5 neutralizing antibodies or isotype control antibodies between 7 and 12 dpi. Fourteen dpi, the numbers of pulmonary eosinophils were significantly reduced in testosterone-treated compared with placebo-treated males, with IL-5 neutralizing antibody treatment significantly reducing the numbers of pulmonary eosinophils in all males, regardless of testosterone status (Fig 3F; \( p < 0.05 \)). Reduction of pulmonary eosinophils with anti-IL-5 antibody treatment did not protect testosterone-depleted males against IAV (Fig 3G; \( p < 0.05 \)). These data suggest that the inhibition of pulmonary eosinophil accumulation does not directly mediate the protective effects of testosterone on IAV pathogenesis and is likely caused by other testosterone-mediated changes in pulmonary inflammation.

In addition to affecting innate immune cells, testosterone is associated with shifts in the numbers, activities, and differentiation of CD4\(^+\) T cells in experimental models of allergy and autoimmune disease [28, 35–37]. To test the hypothesis that testosterone improved the outcome of IAV infection by shifting populations of CD4\(^+\) T cells, helper T cell type 1 (Th1), type 2 (Th2), type 17 (Th17), and regulatory T (Treg) cells were quantified at several time points before and during IAV infection. Peak numbers of total CD4\(^+\) T cells, Th1, Th2, Th17, and Treg cells occurred in both testosterone-depleted and -replete males at 9 dpi, followed by a reduction of cell numbers at 14 dpi (Table 2). The contraction of total CD4\(^+\) T cell numbers in the lungs was significantly slower in testosterone-depleted as compared with replete males at 14 and 21 dpi (Table 2; \( p < 0.05 \)). There was no effect of testosterone treatment on the numbers of IAV-specific Th1, Th2, or Th17 cells at any time point examined (Table 2). In contrast with previous reports of testosterone-induced expansion of Treg cell numbers [37], Treg cell numbers were greater in the lungs of placebo-treated relative to testosterone-treated gonadectomized males, but only at 21 dpi (Table 2; \( p < 0.05 \)). These data suggest that virus-specific CD4\(^+\) T cells are not the primary cell type mediating the protective effects of testosterone during IAV infection of male mice.

### Table 1. Total numbers of pulmonary myeloid cells following IAV infection in gonadectomized mice treated with placebo (Gdx) or testosterone (Gdx + T).

| Total numbers of cells (x10\(^3\)) | 0dpi | 3dpi | 7dpi | 14dpi |
|-----------------------------------|------|------|------|-------|
| **Alveolar macrophages**          |      |      |      |       |
| Gdx                              | 84.4±16.2 | 63.2±8.6 | 72.1±14.7 | 79.4±16.2 |
| Gdx + T                          | 97.4±17.5 | 91.2±7.4 | 64.2±15.1 | 72.8±10.3 |
| **Interstitial macrophages**      |      |      |      |       |
| Gdx                              | 46.3±6.0 | 188.2±32.1 | 896.0±97.6 | 295.1±38.0 |
| Gdx + T                          | 56.7±11.9 | 106.6±9.8 | 812.7±54.2 | 170.3±18.1 |
| **Neutrophils**                  |      |      |      |       |
| Gdx                              | 75.5±13.7 | 260.4±33.4 | 487.0±45.3 | 208.4±45.8 |
| Gdx + T                          | 243.8±60.7\(^*\) | 407.7±60.2\(^*\) | 487.9±36.6 | 203.4±37.7 |
| **Plasmacytoid dendritic cells** |      |      |      |       |
| Gdx                              | 41.4±8.1 | 170.3±28.9 | 792.5±79.3 | 282.9±34.7 |
| Gdx + T                          | 53.7±12.8 | 101.6±12.8 | 672.5±66.3 | 145.7±14.4 |
| **Dendritic cells**              |      |      |      |       |
| Gdx                              | 86.6±15.2 | 77.3±8.6 | 241.0±23.5 | 142.2±9.2 |
| Gdx + T                          | 90.6±26.1 | 89.4±5.5 | 211.8±13.3 | 105.9±11.8 |

Data are presented as the mean +/- SEM from 2 independent experiments (n = 8-10/treatment/timepoint) and significant differences between treatment groups are bolded and denoted by asterisks (\(^*\) \( p < 0.05 \)).

https://doi.org/10.1371/journal.ppat.1008506.t001
Table 2. Total numbers of pulmonary CD4+ T cells following IAV infection in gonadectomized mice treated with placebo (Gdx) or testosterone (Gdx + T).

|                          | 0dpi       | 5dpi       | 9dpi       | 14dpi      | 21dpi      |
|--------------------------|------------|------------|------------|------------|------------|
| Total CD4+ T cells       | Gdx        | 367.1±37.6 | 538.7±61.7 | 1740.5±248.2 | 838.5±45.9*| 611.7±61.7*|
|                         | Gdx + T    | 289.8±47.3 | 498.8±69.3 | 1487.7±135.3 | 434.6±39.0 | 281.1±40.8 |
| Th1 cells                | Gdx        | 3.3±1.2    | 1.3±0.5    | 14.8±3.0   | 7.1±2.7    | 10.4±1.5   |
|                         | Gdx + T    | 1.7±0.5    | 3.0±1.8    | 19.1±5.8   | 7.6±1.3    | 7.1±1.4    |
| Th2 cells                | Gdx        | 6.4±2.0    | 5.3±1.3    | 33.4±5.3   | 24.5±1.3   | 17.3±3.4   |
|                         | Gdx + T    | 8.3±1.7    | 6.0±1.7    | 36.3±7.4   | 19.3±2.0   | 8.5±0.9    |
| Th17 cells               | Gdx        | 4.5±1.5    | 10.2±4.1   | 24.4±2.8   | 7.5±1.3    | 6.0±0.9    |
|                         | Gdx + T    | 6.0±1.2    | 8.3±2.4    | 23.0±3.1   | 7.7±1.3    | 3.7±1.0    |
| Regulatory T Cells       | Gdx        | 5.3±1.2    | 17.5±2.1   | 28.2±9.6   | 13.8±2.3   | 16.3±1.4*  |
|                         | Gdx + T    | 3.4±0.8    | 12.5±1.4   | 30.5±7.9   | 12.7±3.6   | 7.3±0.8    |

Helper T cell type 1 (Th1), Th2, and Th17 cells were classified as expressing IFNγ, IL-4, or IL-17A respectively in response to virus-specific peptide stimulation. Regulatory T cells were classified as CD4+ CD25+ and FoxP3+ cells. Data represent means +/- SEM from 2 independent experiments (n = 6-12/treatment/timepoint) and significant differences between treatment groups are bolded and denoted by asterisks (*P < 0.05).

https://doi.org/10.1371/journal.ppat.1008506.t002

Virus-specific CD8+ T cells are beneficial for the killing of IAV-infected cells but can also be detrimental to the host by causing immunopathology [38, 39]. Total CD8+ as well as IAV-specific CD8+ T cells influxed into the lungs at 9 dpi (Fig 4A and 4B), which corresponded with peak virus titers (Fig 1F). Testosterone had no effect on either the induction or peak magnitude of total or virus-specific CD8+ T cells measured in the lungs; the contraction of these cells, however, following the clearance of detectable virus (14 and 21 dpi) was significantly improved in testosterone-treated as compared with placebo-treated gonadectomized males (Fig 4A and 4B; P < 0.05 in each case). To determine if testosterone suppressed the activity of virus-specific CD8+ T cells, we assessed cytokine production following ex vivo stimulation with ma2009 H1N1-specific peptide and observed that the number of CD8+ T cells producing either IFNγ or TNFα was significantly reduced in testosterone-treated males relative to testosterone-depleted males after virus had been cleared (14 and 21 dpi) but not during peak virus titers (9 dpi; Fig 4C and Fig 4D; P < 0.05). To further assess whether testosterone affected the functionality of IAV-specific CD8+ T cell we used CD107a as a marker for degranulation in response to ex vivo ma2009 H1N1-specific peptide stimulation. After virus had been cleared from the lungs, the contraction in the numbers of CD8+ T cells staining positive for both surface expression of CD107a and production of IFNγ was delayed in the lungs of testosterone-depleted relative to testosterone-replete male mice (Fig 4E; P < 0.05). The effects of testosterone on the contraction of IAV-specific CD8+ T cells after virus had been cleared from the lungs, was specific to the site of virus replication and not observed in either the spleen or the mediastinal lymph nodes (i.e. pulmonary draining lymph nodes; Table 3). Taken together, these data suggest that testosterone may improve the outcome of primary IAV infection by dampening immunopathology caused by pulmonary virus-specific CD8+ T cell populations.

Testosterone creates a local environment to promote the contraction of CD8+ T cells following control of IAV replication

Testosterone can act both directly and indirectly to alter the biological activities of T cells [28, 40, 41]. For testosterone to have direct effects on the contraction of CD8+ T cells during IAV infection, AR expression would need to occur within these cells. Consistent with previous reports [28, 42–44], Ar mRNA was expressed in enriched splenic CD8+ T cells from both testosterone-depleted and testosterone-replete male mice, with no effect of testosterone treatment on Ar mRNA expression (Fig 5A).
Fig 4. Testosterone treatment reduces numbers and activity of virus-specific CD8$^+$ T cells following control of viral replication. Adult male mice were gonadectomized and implanted with either testosterone (gdx + T) or placebo (gdx) containing capsules, and then inoculated with a sub-lethal dose of ma2009 H1N1 IAV or mock infected. At select days post inoculation (dpi), mice were euthanized, and pulmonary immune cells were quantified by flow cytometry (n = 7-14/treatment/time-point). Surface marker and intracellular staining was used to identify numbers of total CD8$^+$ T cells (A), ma2009 H1N1-specific CD8$^+$ T cells (B), CD8$^+$ T cells producing IFN$\gamma$ (C) or TNF$\alpha$ (D) in response to ex vivo H1N1-specific peptide stimulation, and poly-functional CD8$^+$ T cells expressing both CD107a and IFN$\gamma$ (E) following ex vivo H1N1-specific peptide stimulation. Data represent means +/- SEM from two independent replications and significant differences between treatment groups are denoted by asterisks (* $p < 0.05$).

Table 3. Total numbers of CD8$^+$ T cells in the mediastinal lymph nodes and spleens of gonadectomized mice treated with placebo (Gdx) or testosterone (Gdx + T) following IAV infection.

|                        | Mediastinal Lymph Nodes | Spleen       |
|------------------------|-------------------------|--------------|
|                        | 14dpi  | 21dpi | 14dpi  | 21dpi       |
| **Total CD8$^+$ T cells** |        |        |        |             |
| Gdx                    | 1687.8±128.6 | 1425.7±150.8 | 8030.1±957.2 | 6413.7±405.8 |
| Gdx + T                | 1442.9±166.5 | 1388.1±263.8 | 7949.4±599.9 | 7943.4±614.6 |
| **Tetramer$^+$ CD8$^+$ T cells** |        |        |        |             |
| Gdx                    | 22.0±6.5   | 12.1±1.4 | 75.1±10.2 | 77.6±10.9   |
| Gdx + T                | 13.3±2.4   | 9.5±1.2  | 94.3±17.9 | 77.2±10.9   |
| **IFN$\gamma^+$ CD8$^+$ T cells** |        |        |        |             |
| Gdx                    | 7.9±1.2    | 6.8±2.0  | 69.2±18.0 | 35.2±8.0    |
| Gdx + T                | 6.7±1.5    | 3.7±1.0  | 70.8±13.0 | 36.2±12.5   |
| **TNF$\alpha^+$ CD8$^+$ T cells** |        |        |        |             |
| Gdx                    | 55.6±3.1   | 20.6±6.7 | 256.7±16.8 | 93.7±41.5   |
| Gdx + T                | 47.8±5.2   | 18.0±6.8 | 308.7±43.0 | 103.7±39.4  |

Data are presented as the mean +/- SEM from 2 independent experiments (n = 5/treatment/timepoint).

https://doi.org/10.1371/journal.ppat.1008506.t003
Fig 5. Secondary effects of testosterone on the contraction of CD8+ T cell populations following control of viral replication. Adult male TCR-Ova mice were gonadectomized and implanted with capsules containing testosterone or placebo prior to infection with a sub-lethal dose of WSN-Ova, H1N1 IAV (n = 5/treatment group). At 14 days post inoculation (dpi), mice were euthanized, CD8+ T cells were isolated by negative selection, and splenic mRNA was measured and normalized to GAPDH using the ΔΔCT method (A). Adoptive transfer experiments were performed, and adult male TCR-Ova and CD90.1 mice were gonadectomized and implanted with capsules containing either placebo (gdx) or testosterone (gdx + T). Splenic CD8+ T cells were isolated from placebo- or testosterone-treated donor TCR-Ova mice by negative selection purification, and adoptively transferred by tail vein injection into either placebo- or testosterone-treated male CD90.1 recipient mice. Mice were then infected by intranasal inoculation with a sub-lethal dose of WSN-Ova, H1N1 IAV, and seminal vesicle mass was quantified as the percentage of total body mass (B; n = 6-7/treatment). Mice were monitored daily for changes in body temperature (C). At 14 dpi, mice were euthanized, and lung tissue was collected to quantify the numbers of CD45+ T cells (D), eosinophils (E), total CD8+ T cells (F), Ova-specific CD8+ T cells (G), and adoptively transferred CD90.2+ CD8+ T cells producing IFNγ in responses to OVA-specific peptide stimulation (H) were quantified by flow cytometry (n = 6-7/treatment). Data represent means +/- SEM from two independent replications and significant differences between treatment groups are denoted by asterisks (*p < 0.05). https://doi.org/10.1371/journal.ppat.1008506.g005

Because Ar mRNA was expressed by splenic CD8+ T cells, we hypothesized that testosterone could condition these cells to induce contraction following control of viral replication. To test this hypothesis, TCR-Ova donor mice and CD90.1 recipient mice were gonadectomized and implanted with capsules containing either testosterone or placebo. Enriched splenic CD8+ T cells from naïve TCR-Ova mice were then adoptively transferred into either placebo- or testosterone-treated CD90.1 recipient mice prior to infection with WSN-Ova, H1N1 IAV, and seminal vesicle mass was quantified as the ratio of total body mass (B; n = 6-7/treatment). Mice were monitored daily for changes in body temperature (C). At 14 dpi, mice were euthanized, and lung tissue was collected to quantify the numbers of CD45+ T cells (D), eosinophils (E), total CD8+ T cells (F), Ova-specific CD8+ T cells (G), and adoptively transferred CD90.2+ CD8+ T cells producing IFNγ in responses to OVA-specific peptide stimulation (H) were quantified by flow cytometry (n = 6-7/treatment). Data represent means +/- SEM from two independent replications and significant differences between treatment groups are denoted by asterisks (*p < 0.05).

The protective effects of testosterone during IAV infection are dependent on androgen receptor signaling

Testosterone can be metabolized in tissues, converted into estradiol, and signal through estrogen receptors (ERs) [45]. Estradiol signaling through ERα can dampen inflammation and improve the outcome of IAV infection, at least in female mice [21, 46, 47]. To determine whether the protective effects of testosterone during IAV infection in male mice were caused by signaling through AR or ER, male mice were gonadectomized and implanted with capsules containing either testosterone, placebo, or a combination of testosterone and the AR antagonist, flutamide [48]. Seminal vesicle mass was used as a biomarker to confirm AR inhibition by flutamide and was significantly reduced in males that received testosterone + flutamide treatment as compared with males that received testosterone alone (Fig 6A; p < 0.05). During IAV infection, flutamide treatment inhibited the protective effects of testosterone on morbidity (Fig 6B and 6C; p < 0.05). To assess whether the testosterone-induced changes in CD8+ T cell numbers and activity were also AR-dependent, we evaluated the effects of flutamide on the
contraction of CD8\(^+\) T cells during the resolution phase of infection. Co-treatment of flutamide and testosterone, similar to placebo treatment, resulted in significantly greater numbers of total CD8\(^+\) T cells, 2009 H1N1-specific CD8\(^+\) T cell, and virus-specific CD8\(^+\) T cells producing IFN\(\gamma\) in response to H1N1 specific peptide stimulation at 14 and 21 dpi as compared with testosterone treatment alone (Fig 6D–6F; \(p<0.05\)).

Because flutamide can alter T cell function through off-target GABA-A receptor signaling [49], we sought to confirm the effects of AR signaling on IAV pathogenesis by using the non-aromatizable androgen, dihydrotestosterone (DHT) (i.e., an androgen that cannot be converted into estradiol). Treatment of gonadectomized males with DHT significantly increased seminal vesicle mass relative to placebo-treated males, to a mass consistent with testosterone-treated males (Fig 7A; \(p<0.05\)). Males that were gonadectomized and treated with DHT prior to IAV infection were protected against IAV and experienced a similar level of morbidity (i.e., body mass loss) as testosterone-treated males, which was collectively better than placebo-treated mice (Fig 7B; \(p<0.05\)). Consistent with testosterone, DHT inhibited the influx of eosinophils into lungs, and accelerated the contraction of total numbers of leukocytes (i.e., CD45\(^+\) cells), total numbers of CD8\(^+\) T cells, 2009 H1N1-specific CD8\(^+\) T cell numbers (E), and the number of CD8\(^+\) T cells producing IFN\(\gamma\) in response to ex vivo H1N1-specific peptide stimulation (F) were quantified by flow cytometry (n = 8-10/treatment/time-point). Data represent means +/- SEM from two independent replications and significant differences between treatment groups are denoted by asterisks (\(^*P<0.05\)).

Female mice have lower testosterone concentrations, and like hypogonadal males, experience more severe disease following IAV infection relative to age-matched young adult males [21, 23]. To determine if the protective effects of AR signaling on IAV pathogenesis were sex-dependent, female mice were implanted with capsules containing either DHT or placebo prior to IAV infection. Consistent with previous data [21, 23], gonadally intact female mice treated with placebo experienced increased IAV morbidity (i.e., body mass loss) relative to gonadally intact males (Fig 7H; \(p<0.05\)). Female mice treated with DHT were, however, protected against IAV and experienced similar levels of morbidity (i.e. body mass loss) to gonadally intact males (Fig 7H). These data demonstrate that the protective effects of AR signaling on the severity of IAV infection are not limited to males.

**Discussion**

Inflammatory immune responses, including cytokine production and the cell-mediated activities of both myeloid and lymphoid cells, are required to control IAV infection, but if improperly regulated can contribute to tissue damage and severe outcomes [19, 33, 50–52]. In the current and previous studies [21, 22], androgens, including testosterone and DHT, in male mice reduce the severity of IAV infection by promoting the resolution of pulmonary inflammation rather than by affecting viral replication. The improved resolution of IAV-induced inflammation [22] in androgen-treated males was not caused by suppression of the cytokine storm, but rather by accelerated contraction of pulmonary Ly6C\(^+\) monocytes during peak

---

**Fig 6. The androgen receptor antagonist, flutamide, inhibits the protective effects of testosterone treatment on influenza A virus (IAV) pathogenesis.** Adult male mice were gonadectomized and implanted with capsules containing placebo (gdx), testosterone (gdx + T), or flutamide + testosterone (flutamide + T), and seminal vesicle mass was quantified as the percentage of total body mass (A; n = 9/treatment). Following intranasal inoculation with a sub-lethal dose of ma2009 H1N1 IAV, mice were monitored daily for changes in body mass (B) and body temperature (C) for 21 days post inoculation (dpi; n = 12-15/treatment). At 14- and 21-dpi, the total numbers of CD8\(^+\) T cells (D), H1N1-specific CD8\(^+\) T cell numbers (E), and the number of CD8\(^+\) T cells producing IFN\(\gamma\) in response to ex vivo H1N1-specific peptide stimulation (F) were quantified by flow cytometry (n = 8-10/treatment/time-point). Data represent means +/- SEM from two independent replications and significant differences between treatment groups are denoted by asterisks (\(^*P<0.05\)).
inflammation and the mitigation of pulmonary CD8+ T cells and eosinophils after virus was cleared. The effect of androgens on pulmonary leukocyte activity was dependent on AR signaling in the lungs, which created a pulmonary environment that reduced the numbers and activities of these cells in the lungs following IAV infection.

We and others [21, 23] have shown that males experience less severe disease and recover faster from IAV than females. Data from the current study and others [22, 23] illustrate that androgens limit pulmonary inflammation during IAV infection, thus maintaining greater tolerance during infection in males and even females. In addition to reduced inflammation, males also repair damaged tissue faster than females which is mediated by greater production of epidermal growth factor amphiregulin in males than females [23]. Testosterone does not regulate production of amphiregulin; therefore, elevated levels of both testosterone and amphiregulin contribute to improved IAV outcomes in males than females.

In the current study, the depletion of testosterone resulted in the accumulation of inflammatory monocytes during peak virus replication and eosinophils in the lungs following control of viral replication, which was unexpected given the lack of observed changes in pulmonary concentrations of IL-5, IL-13, and exotoxin. Eosinophils are androgen responsive despite the absence of AR expression [53–55], with testosterone-mediated differences in eosinophilic airway responses instead being attributed to the actions of Type II innate lymphoid cells (ILC2s) [56, 57]. Though not evaluated in this study, androgens have been shown to inhibit the maturation of ILC2s, while decreasing IL-5 production and eosinophilic responses in murine models of airway inflammation [56, 57]. Although the precise role of eosinophils in the immune response to IAV is unclear, previous studies in mice show accumulation of eosinophils in the lungs following control of viral replication [58, 59]. In the current study, depletion of eosinophils during the later stages of infection did not reduce morbidity in testosterone-depleted males, suggesting that the protective effects of testosterone on IAV pathogenesis are not directly mediated by effects on eosinophils. Whether the accumulation of eosinophils during the resolution phase of infection represents the activation of type 2 tissue repair responses [60], or a pathological response contributing to immunopathology warrants further study.

Monocytes play a critical role in the control of respiratory virus infection through the coordination of the immune response (i.e., cytokine and chemokine secretion) in addition to functioning as tissue progenitor cells for several monocyte-derived DC and macrophage populations [53]. Despite this beneficial role, exaggerated or improperly regulated inflammatory monocyte responses to IAV infection contribute to pulmonary damage and adverse clinical outcomes [61, 62]. Consistent with these observations, in the present study depletion of testosterone delayed the contraction of pulmonary Ly6C+ inflammatory monocytes early during IAV infection. Conversely, depletion of testosterone did not alter pulmonary concentrations of Ly6C+ monocyte-associated cytokines or chemokines (e.g., CCL2 and TNFα) [63]. Future studies will need to define the mechanism by which testosterone depletion promotes
the persistence of these cells and determine if they are the primary mediator of testosterone-dependent differences in IAV outcomes.

In response to other inflammatory diseases, including experimental autoimmune encephalomyelitis, testosterone is associated with an expansion of Th2 and Th17 cell populations and suppression of Th1 activity [37, 64, 65]. During IAV pathogenesis, testosterone treatment of males accelerated the contraction of total pulmonary CD4+ numbers but did not lead to shifts in the differentiation of virus-specific CD4+ T helper cell populations. Diversification of the peptide pool used to assess IAV-specific CD4+ T cells may yield differential effects of testosterone on CD4+ T cell subsets as in the current study a single peptide was used for stimulation. Furthermore, although testosterone treatment can promote the expansion in numbers and activation of Treg cells in murine models of inflammation [37, 66, 67], there was only a transient effect of testosterone on Treg cells during IAV infection. Whether this represents differences in the polarizing effects of viral infection versus other inflammatory states should be considered.

During IAV infection, CD8+ T cells also play a critical role in the control of IAV infection through the production of cytokines and the killing of virus-infected cells [68]. Improper regulation or prolonged activation of virus-specific CD8+ T cell responses, however, can also cause immunopathology and severe pulmonary tissue damage [38, 39]. Both in humans and mice, testosterone alters the numbers, cytokine production, and proliferative potential of CD8+ T cells [16, 41]. Consistent with these observations, androgens, including testosterone, accelerated the contraction of virus-specific CD8+ T cells in the lungs, but not in the spleen or mediastinal lymph nodes. The significance of this tissue specific effect is unknown, but whether these effects of testosterone on virus-specific CD8+ T cells involve activation-induced cell death, inhibitory pathways, interactions with tissue-specific cell types, including TNF/iNOS-producing DCs [69], or changes in the establishment of tissue resident memory cell populations warrants future study.

The expression of Ar in enriched splenic CD8+ T cell populations suggested that testosterone might be acting directly on these CD8+ T cell to mitigate IAV pathogenesis. Adoptive transfer studies were conducted and revealed that the presence of testosterone in the recipient mouse was a better predictor of IAV outcome and contraction of virus-specific CD8+ T cells than the presence of testosterone in the donor mice. These data suggest that testosterone does not condition virus-specific CD8+ T cells to induce intrinsic changes in these cells via AR signaling. Instead, testosterone induces changes in these cells that are conditional on the presence of testosterone in the local environment in which they reside. Whether testosterone treatment results in functional AR signaling in CD8+ T cells was not assessed in the current study. As previous reports are inconsistent with regards to the degree and nature of AR signaling in mature CD8+ T cells [40, 44, 70], in vitro co-culture experiments may prove useful in elucidating whether testosterone acts directly on these cells to improve the outcome of IAV infection.

Given the widespread expression of AR both in immune cells and epithelial cells in the lung [53, 71], testosterone may be acting indirectly on virus-specific CD8+ T cells, through interactions with other cells to promote their contraction. One such cell type may be the Ly6C+ inflammatory monocytes which persisted in the lungs of testosterone depleted males, and have recently been shown to promote the persistence of lung resident memory CD8+ T cells following respiratory virus infection [72]. Similarly, suppression of Ly6C+ inflammatory monocyte derived TNF/iNOS-producing DCs reduces tissue specific proliferation and survival of antigen-specific CD8+ effector T cells [69]. Whether testosterone acts through inflammatory monocytes to alter the generation and persistence of virus-specific CD8+ T cell populations warrants future study.
Testosterone can be metabolized by aromatase into estradiol to signal through ERα, which can dampen inflammation and improve the outcome of IAV infection in females [21, 45–47]. Moreover, in male mice gonadectomized prior to the onset of puberty, castration-mediated protection against lethal IAV infection is reversed by testosterone treatment and subsequent conversion to estradiol, but not by treatment with non-aromatizable DHT [73]. In the present study, the protective effects of testosterone on IAV pathogenesis were dependent on AR signaling in the lungs. The discordant findings regarding the impact of androgens on IAV pathogenesis in prepubertal versus adult mice likely represent differences in the developmental effects of sex steroid signaling.

We have previously demonstrated that low concentrations of testosterone, whether age-associated, surgically-induced, or driven by biological sex, causes delayed resolution of pulmonary inflammation following, but not prior to, the control of viral replication [22, 23]. In the present study, delayed resolution of disease was associated with the persistence of total leukocytes and pulmonary CD8⁺ T cells following control of viral replication and an influx of eosinophils into the lungs at 14 dpi. Despite this temporal correlation, neither the depletion of eosinophils nor adoptive transfer of CD8⁺ T cells could reverse the ability of testosterone to protect males against IAV, suggesting that the effect of testosterone on these cell types is secondary to effects on other immune mediators. Testosterone promoted the early contraction of Ly6C⁺ inflammatory monocytes which correlated with the early divergence in morbidity (i.e., clinical severity and body mass) between testosterone-depleted and testosterone replete males during peak pulmonary inflammation. From these data, we hypothesize that androgen-induced changes in inflammatory monocytes maybe the cause of testosterone-mediated protection during IAV and the downstream reduction of eosinophilic inflammation and pulmonary CD8⁺ T cell persistence is a secondary consequence to changes in inflammatory monocyte numbers.

The impact of testosterone on infectious disease outcomes involves many cell types and responses. While our work has shown androgen-induced changes in immune function to be protective, these same immunological changes can be detrimental in other instances, including with amoebic infection, in which treatment with testosterone increases the severity of infection at least in part through inhibition of IFNγ production by natural killer T cells [74–76]. When disease following infection is caused by the inability to control the pathogen, then androgens, like testosterone, are detrimental. Conversely, when disease following infection is largely attributable to immunopathology [19, 53], the immunomodulatory effects of testosterone, and more specifically, AR signaling, are likely protective. These data suggest that testosterone confers protection during IAV infection by modulating the immune response and suggest that androgens may have therapeutic potential in female and hypogonadal male populations.

**Materials and methods**

**Ethical statement**

All experiments were performed in compliance with the standards outlined in the National Research Council’s Guide to the Care and Use of Laboratory Animals. All animal procedures were approved by the Johns Hopkins Animal Care and Use Committee (MO18H262). All efforts were made to minimize animal suffering.

**Animals**

Adult (7–8 weeks old) male and female C57BL/6 mice were purchased from Charles River. For adoptive transfer experiments, male and female TCR-Ova (C57BL/6-Tg(TcraTcrb) 1100Mjb/J) and CD90.1 (B6.PL-Thy1 +/CyJ) mice were purchased from The Jackson Laboratory as
breeding pairs and bred in house to obtain male offspring. All mice were housed at 3–5 animals per microisolator cage under standard BSL-2 housing conditions and given food and water ad libitum.

**Gonadectomy and hormone manipulation**

Adult (8 week old) male mice were anesthetized by intra-peritoneal (IP) inoculation with a ketamine (80 mg/kg) and xylazine (8 mg/kg) cocktail and the testes were removed bilaterally as described previously [21]. Following two weeks recovery, silastic tubing capsules (inner diameter-0.04”, outer diameter- 0.085”; HelixMark) containing crystalline testosterone propionate (7.5 mm; Sigma), crystalline 4,5α-Dihydrotestosterone (5.0 mm; Sigma), or nothing were implanted subcutaneously [77]. For flutamide studies, capsules were prepared as above (2 x 15.0 mm; Sigma) but were implanted at the time of gonadectomy. Adult (8 week old) female mice were left gonadally intact prior to capsule implantation. All capsules were sealed with 2.5 mm of medical adhesive (Factor II, A-100) and incubated at 37˚C overnight in sterile saline solution prior to implantation.

**Virus infection and quantification**

Mouse-adapted A/California/4/09 (ma2009; H1N1; generated by Dr. Andrew Pekosz using a published sequence) [78] or recombinant A/WSN/33 virus containing OVA257-264 (SIINFEKL) peptide in the neuraminidase protein (H1N1; WSN-Ova) [79], were used in all experiments. Mice were anesthetized and infected by intranasal inoculation with ma2009 or WSN-Ova, H1N1 virus (ma2009 = 0.1 MLD$_{50}$; WSN-OVA = 0.4 MLD$_{50}$) diluted in 30μl of DMEM or mock infected with 30μl DMEM. For virus quantification, log$_{10}$ dilutions of lung homogenate were plated onto Madin-Darby canine kidney (MDCK) cell monolayers in replicates of 6 for 5 days at 32˚C. Cells were stained with naphthol blue black (Sigma Aldrich) and scored for cytopathic effect. The 50% tissue culture infectious dose (TCID$_{50}$) was calculated using the Reed-Muench method and was used to back titer all viral inoculums.

**Sample collection and testosterone quantification**

Following infection, rectal temperature and body mass were recorded daily out to 21 days post inoculation (dpi), and clinical disease scores were recorded at select time-points as described previously [22]. For terminal studies, mice were euthanized at select time-points and plasma, whole lungs, spleen, and mediastinal lymph nodes (MLN) were collected. Seminal vesicles were also collected, and mass was recorded as a bio-marker for androgen activity. Total testosterone concentration was quantified in plasma collected at 21 dpi by commercial ELISA kit according to the manufacturer’s instructions (IBL America). To prevent sample degradation, care was taken to limit light and thermal exposure of plasma samples prior to testosterone quantification.

**Pulmonary cytokine and chemokine quantification**

Snap-frozen lung tissue was homogenized in DMEM supplemented with 1% L-glutamine (Gibco), and 1% penicillin-streptomycin (Gibco) and centrifuged to remove cellular debris. Supernatants were collected and Eotaxin, G-CSF, GM-CSF, IFNγ, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p40, IL-12p70, IL-13, IL-17A, CXCL-1, MCP-1, MIP-1α, MIP-1β, RANTES, and TNFα were quantified using the Bio-Plex Pro Mouse Cytokine 23-Plex Assay (Biorad) according to the manufacturer’s instructions. Pulmonary TGFβ concentration was quantified by commercial ELISA kit (R&D Systems). For analyses, IL-9 and IL-17A concentrations remained below the limit of detection at all time-points and were excluded.
Flow cytometry

Lung, spleen, and MLN tissues were harvested, and single cells suspensions were generated by homogenizing tissue through a 100μm nylon filter (Falcon) followed by ACK lysis of red blood cells (Quality biologicals). The total numbers of live cells were determined using a hemocytometer and trypan blue (Invitrogen) exclusion, and cells were resuspended at 1 × 10^6 cells/ml in RPMI 1640 (Cellgro) supplemented with 10% fetal bovine serum (Fisher Scientific), 1% L-glutamine (Gibco), and 1% penicillin-streptomycin (Gibco). For the enumeration of H1N1-specific CD4^+ and CD8^+ T cells, isolated cells were cultured for 5 hrs at 37˚C in media containing IAV specific peptide (2009ma; CD8: NP_{366-374}, or CD4: NP_{311-325}, and WSN-OVA_{1}; CD8: OVA_{257-264}) in the presence of GolgiPlug (BD) and GolgiStop (BD). Following incubation, T cell viability was determined by fixable live/dead far red viability stain (Invitrogen). For all leukocyte populations, Fc receptors were blocked using anti-CD16/32 (BD Biosciences) and panel specific surface markers were stained with the following antibodies: CD4-PerCPCy5.5 (Clone RM1-5; BD), CD8-PerCPCy5.5 (Clone 53–6.7; BD), CD11b-FITC (Clone M1/70; BD), CD11C-APC (Clone HL3; BD), CD25-FITC (Clone 7D4; BD) CD45-PerCPCy5.5 (Clone 30-F11; BD), CD90.1-FITC (Clone OX-7; BD), CD90.2-PE (Clone 53–2.1; BD), CD107a-PE (Clone 1D4B; BD), Ly-6C-PE-Cy7 (Clone AL21; BD), Ly-6G-APC (Clone 1A8; BD), Ly-6G-FITC (Clone 1A8; BD), I-A/I-E (Clone M5/114.15.2), Siglec-F (Clone E50-2440), PE-conjugated tetramer for ma2009 (ASNENVETM; NIH Tetramer Core Facility), and PE-conjugated pentamer for WSN-OVA_{1} (SIINFEKL; Proimmune). Cells were then permeabilized and fixed (BD Cytofix/Cytoperm) prior to intracellular staining with IFNγ-FITC (Clone XMG1.2; BD), IL4-PE (BD), IL17A-PE (BD), and TNFα-PE (Clone MP6-XT22; BD). Intracellular staining with Foxp3 (Clone MF23; BD) was performed following fixation and nuclear permeabilization with the FoxP3/Transcription Factor Staining Buffer Set (eBioscience). Data were acquired using a FACSCalibur flow cytometer (BD) running Cell Quest Pro or an LSRII flow cytometer (BD) running FACSDiva (BD), and analyzed using FlowJo (v.10) software (Tree Star, Inc.). Total live cell counts were determined based on the total live cells counts acquired by trypan blue exclusion staining multiplied by the total live cell percentages for each corresponding gate.

Lung inflation and histologic analysis

Lungs were inflated with zinc-buffered formalin (Z-Fix, Anatech) under constant pressure (25 cm H2), dissected free, and placed in fixative for 48 hrs as previously described [80]. Fixed lung tissues were then embedded in paraffin, cut into 5μm sections, and mounted on glass slides. Consecutive tissue sections were subsequently stained with hematoxylin and eosin (H&E) and used to assess eosinophilic inflammation on a 0–3 scale (0, no inflammation; 1, mild inflammation; 2, moderate inflammation; and 3, severe inflammation) for perivascular, peribronchial, and alveolar areas. The cumulative eosinophilic score represents the sum of each individual inflammation parameter with scoring performed by a single blinded observer in consultation with a board-certified veterinary pathologist. Representative images were taken at 20x and 200x magnification using a Nikon Eclipse E400 camera.

In vivo eosinophil depletion

Mice were given 75 μg of anti-IL-5 antibody (clone TRFK5; eBioscience) or control IgG1 antibody (clone eBRG1; eBioscience) in 75 μl sterile PBS by IP injection daily from 7 through 12 dpi. Efficacy of pulmonary eosinophil depletion was assessed at 14 dpi by flow cytometry.
Real time reverse transcription PCR
Pulmonary single cells suspensions were generated by homogenizing lung tissue through a 100μm nylon filter (Falcon) and CD8⁺ T cells were isolated by negative selection (StemCell Technologies). Total RNA was then isolated from purified CD8⁺ T cells using a commercial kit (Invitrogen) per the manufacturer’s instructions and RNA concentration and purity were measured using a NanoDrop (ThermoFisher Scientific). Pre-designed androgen receptor (Ar) (Mm.PT.58.12425400) and Gapdh (Mm.PT.39a.1) PrimeTime Primers were purchased from Integrated DNA Technologies. Semi-quantitative RT-PCR was performed in 96-well optical reaction plates using SsoFast EvaGreen Supermix (Biorad) on the StepOnePlus RT-PCR system (Applied Biosystems). Gene expression was normalized to Gapdh using the ΔCt method.

Adoptive transfer of CD8⁺ T cells
Splenic tissue was harvested from unprimed gonadectomized male TCR-Ova mice treated with either empty capsules (gdx) or testosterone capsules (gdx + T). CD8⁺ T cells were isolated by tissue homogenization through a 100μm nylon filter (Falcon), followed by negative selection purification (StemCell Technologies). One-hundred thousand purified CD8⁺ T cells were then transferred into gdx and gdx + T treated naive male CD90.1 recipient mice via intravenous inoculation. Recipient mice were infected with WSN-Ova virus 48 hours post transfer.

Statistical analysis
Discrete measures were analyzed by one or two-way ANOVA with significant interactions further analyzed using the Tukey method for pairwise multiple comparisons. Repeated measures were analyzed by mixed-effect model with Bonferroni’s post-test for multiple comparisons. Statistical analyses were performed using GraphPad Prism 8.02 software and mean differences were considered significant at \( P < 0.05 \).

Supporting information
S1 Table. Adult male mice were gonadectomized and implanted with either testosterone (gdx + T) or placebo (gdx) containing capsules, and then inoculated with a sub-lethal dose of ma2009 H1N1 IAV or were mock infected. At 0, 3, 9, or 14 dpi (n = 8-11/treatment/timepoint), lung tissue was collected and homogenized, and cell free supernatants were used to quantify pulmonary concentrations of 24 cytokines and chemokines. Data are presented as the mean +/- SEM from 2 independent experiments (n = 8-11/treatment/timepoint) and significant differences between treatment groups for each timepoint are bolded and denoted by asterisks (* \( P < 0.05 \)).

Acknowledgments
The authors would like to thank the Klein, Pekosz, and Davis laboratories for discussions about these data, Rebecca Ursin for technical assistance with PCR, and Dr. Nicola Heller for technical assistance with the inflammatory monocyte antibody panel. We also thank the Bloomberg Flow Cytometry and Immunology Core and the Sidney Kimmel Comprehensive Cancer Center Immune Monitoring Core for their technical assistance.

Author Contributions
Conceptualization: Landon G. vom Steeg, Sabra L. Klein.
Data curation: Landon G. vom Steeg, Santosh Dhakal, Han-Sol Park, Sabra L. Klein.

Formal analysis: Landon G. vom Steeg, Kathleen R. Mulka.

Funding acquisition: Sabra L. Klein.

Investigation: Landon G. vom Steeg, Santosh Dhakal, Yishak A. Woldetsadik, Han-Sol Park.

Methodology: Emma C. Reilly, David J. Topham.

Supervision: David J. Topham.

Validation: Landon G. vom Steeg, Santosh Dhakal, Yishak A. Woldetsadik, Han-Sol Park.

Visualization: Santosh Dhakal, Yishak A. Woldetsadik, Han-Sol Park, Kathleen R. Mulka.

Writing – original draft: Landon G. vom Steeg, Santosh Dhakal, Sabra L. Klein.

Writing – review & editing: Kathleen R. Mulka, Emma C. Reilly, David J. Topham, Sabra L. Klein.

References

1. Dufau ML, Winters CA, Hattori M, Aquilano D, Baranao JL, Nozu K, et al. Hormonal regulation of androgen production by the Leydig cell. J Steroid Biochem. 1984; 20(1):161–73. Epub 1984/01/01. https://doi.org/10.1016/0022-4731(84)90203-6 PMID: 6323862.

2. Rana K, Davey RA, Zajac JD. Human androgen deficiency: insights gained from androgen receptor knockout mouse models. Asian journal of andrology. 2014; 16(2):169–77. Epub 2014/02/01. https://doi.org/10.4103/1008-682X.122590 PMID: 24480924; PubMed Central PMCID: PMC3955325.

3. Bojesen A, Juul S, Birkebaek N, Gravholt CH. Increased mortality in Klinefelter syndrome. J Clin Endocrinol Metab. 2004; 89(8):3830–4. Epub 2004/08/05. https://doi.org/10.1210/jc.2004-0777 PMID: 15292313.

4. Muraleedharan V, Jones TH. Testosterone and mortality. Clin Endocrinol (Oxf). 2014; 81(4):477–87. Epub 2014/07/22. https://doi.org/10.1111/cen.12503 PMID: 25041142.

5. Cheetham TC, An J, Jacobsen SJ, Niu F, Sidney S, Queensberry CP, et al. Association of Testosterone Replacement With Cardiovascular Outcomes Among Men With Androgen Deficiency. JAMA Intern Med. 2017; 177(4):491–9. Epub 2017/02/28. https://doi.org/10.1001/jamainternmed.2016.9546 PMID: 28241244.

6. Rosen RC, Wu F, Behre HM, Porst H, Meuleman EJH, Maggi M, et al. Quality of Life and Sexual Function Benefits of Long-Term Testosterone Treatment: Longitudinal Results From the Registry of Hypogonadism in Men (RHYME). J Sex Med. 2017; 14(9):1104–15. Epub 2017/08/07. https://doi.org/10.1016/j.jsxm.2017.07.004 PMID: 28781213.

7. Roy CN, Snyder PJ, Stephens-Shields AJ, Artz AS, Bhasin S, Cohen HJ, et al. Association of Testosterone Levels With Anemia in Older Men: A Controlled Clinical Trial. JAMA Intern Med. 2017; 177(4):480–90. Epub 2017/02/28. https://doi.org/10.1001/jamainternmed.2016.9540 PMID: 28241237; PubMed Central PMCID: PMC5433757.

8. Rao PM, Kelly DM, Jones TH. Testosterone and insulin resistance in the metabolic syndrome and T2DM in men. Nat Rev Endocrinol. 2013; 9(8):479–93. Epub 2013/06/26. https://doi.org/10.1038/nrendo.2013.122 PMID: 23797822.

9. Chang S, Skakkebaek A, Gravholt CH. Klinefelter Syndrome and medical treatment: hypogonadism and beyond. Hormones (Athens). 2015; 14(4):531–48. Epub 2016/01/07. https://doi.org/10.14310/horm.2002.1622 PMID: 26732150.

10. Petering RC, Brooks NA. Testosterone Therapy: Review of Clinical Applications. Am Fam Physician. 2017; 96(7):441–9. Epub 2017/11/03. PMID: 29094914.

11. Garnick MB. Testosterone replacement therapy faces FDA scrutiny. JAMA. 2015; 313(6):563–4. Epub 2014/12/17. https://doi.org/10.1001/jama.2014.17334 PMID: 25502018.

12. Rao PK, Boulet SL, Mehta A, Hotaling J, Eisenberg ML, Honig SC, et al. Trends in Testosterone Replacement Therapy Use from 2003 to 2013 among Reproductive-Age Men in the United States. J Urol. 2017; 197(4):1121–6. Epub 2016/10/30. https://doi.org/10.1016/j.juro.2016.10.063 PMID: 27789218.
13. Gubbels Bupp MR, Jørgensen TN. Androgen-Induced Immunosuppression. Front Immunol. 2018; 9:794. Epub 2018/05/15. https://doi.org/10.3389/fimmu.2018.00794 PMID: 29755457; PubMed Central PMCID: PMC5932344.

14. Lin AA, Wojciechowski SE, Hildeman DA. Androgens suppress antigen-specific T cell responses and IFN-γ production during intracranial LCMV infection. J Neuroimmunol. 2010; 226(1–2):8–19. Epub 2010/07/14. https://doi.org/10.1016/j.jneuroim.2010.05.026 PMID: 20619904; PubMed Central PMCID: PMC4222683.

15. D’Agostino P, Milano S, Barbera C, Di Bella G, La Rosa M, Ferlazzo V, et al. Sex hormones modulate inflammatory mediators produced by macrophages. Ann N Y Acad Sci. 1999; 876:426–9. Epub 1999/07/23. https://doi.org/10.1111/j.1749-6632.1999.tb07667.x PMID: 10415638.

16. Page ST, Plymate SR, Bremner WJ, Matsumoto AM, Hess DL, Lin DW, et al. Effect of medical castration on CD4+ CD25+ T cells, CD8+ T cell IFN-γ expression, and NK cells: a physiological role for testosterone and/or its metabolites. Am J Physiol Endocrinol Metab. 2006; 290(5):E856–63. Epub 2005/12/15. https://doi.org/10.1152/ajpendo.00484.2005 PMID: 16352669.

17. Bouman A, Schipper M, Heineeman MJ, Faas MM. Gender difference in the non-specific and specific immune response in humans. Am J Reprod Immunol. 2004; 52(1):19–26. Epub 2004/06/25. https://doi.org/10.1111/j.1600-0897.2004.00177.x PMID: 15214938.

18. Fish EN. The X-files in immunity: sex-based differences predispose immune responses. Nat Rev Immunol. 2008; 8(9):737–44. https://doi.org/10.1038/nri2394 PMID: 18726636.

19. Damjanovic D, Small CL, Jeyanathan M, McCormick S, Xing Z. Immunopathology in influenza virus infection: uncoupling the friend from foe. Clin Immunol. 2012; 144(1):57–69. Epub 2012/07/14. https://doi.org/10.1007/s00281-012-0050-0 PMID: 22673491.

20. Newton AH, Cardani A, Braciale TJ. The host immune response in respiratory virus infection: balancing virus clearance and immunopathology. Semin Immunopathol. 2016; 38(4):57–69. Epub 2016/06/08. https://doi.org/10.1007/s00281-015-0558-0 PMID: 27412205; PubMed Central PMCID: PMC4896975.

21. Robinson DP, Lorenzo ME, Jian W, Klein SL. Elevated 17beta-estradiol protects females from influenza A virus pathogenesis by suppressing inflammatory responses. PLoS Pathog. 2011; 7(7):e1002149. Epub 2011/08/11. https://doi.org/10.1371/journal.ppat.1002149 PMID: 21829352; PubMed Central PMCID: PMC3145801.

22. Vom Steeg LG, Vermillion MS, Hall OJ, Alam O, McFarland R, Chen H, et al. Androgens and influenza pathogenesis in male mice. Am J Physiol Lung Cell Mol Physiol. 2016; 311(6):L1234–L144. Epub 2016/11/07. https://doi.org/10.1152/ajplung.00352.2016 PMID: 27815260; PubMed Central PMCID: PMC5268399.

23. Vermillion MS, Ursin RL, Kuok DIT, Vom Steeg LG, Wohlgemuth N, Hall OJ, et al. Production of amphiregulin and recovery from influenza is greater in males than females. Biol Sex Differ. 2018; 9(1):24. Epub 2018/07/18. https://doi.org/10.1186/s13293-018-0184-8 PMID: 30022205; PubMed Central PMCID: PMC56048771.

24. Medzhitov R, Schneider DS, Soares MP. Disease Tolerance as a Defense Strategy. Science. 2012; 335(6071):936–41. https://doi.org/10.1126/science.1214935 WOS:000300931800037 PMID: 22363001.

25. McCanville JL, Ayres JS. Disease tolerance: concept and mechanisms. Curr Opin Immunol. 2018; 50:88–93. Epub 2017/12/19. https://doi.org/10.1016/j.coi.2017.12.003 PMID: 29253642; PubMed Central PMCID: PMC5848632.

26. Guo XJ, Thomas PG. New fronts emerge in the influenza cytokine storm. Semin Immunopathol. 2017; 39(5):541–50. Epub 2017/05/31. https://doi.org/10.1007/s00281-017-0636-9 PMID: 28553383; PubMed Central PMCID: PMC5580809.

27. Liu Q, Zhou YH, Yang ZQ. The cytokine storm of severe influenza and development of immunomodulatory therapy. Cell Mol Immunol. 2016; 13(1):3–10. Epub 2015/07/21. https://doi.org/10.1038/cmi.2015.74 PMID: 26189369; PubMed Central PMCID: PMC4711683.

28. Liva SM, Voskuhl RR. Testosterone Acts Directly on CD4+ T Lymphocytes to Increase IL-10 Production. The Journal of Immunology. 2001; 167(4):2060–7. https://doi.org/10.4049/jimmunol.167.4.2060 PMID: 11489988.

29. Patil CN, Wallace K, LaMarca BD, Moulana M, Lopez-Ruiz A, Soljancic A, et al. Low-dose testosterone protects against renal ischemia-reperfusion injury by increasing renal IL-10-to-TNF-alpha ratio and attenuating T-cell infiltration. Am J Physiol Renal Physiol. 2016; 311(2):F395–403. Epub 2016/06/03. https://doi.org/10.1152/ajprenal.00454.2015 PMID: 27252490; PubMed Central PMCID: PMC5008676.

30. Olsen NJ, Zhou P, Ong H, Kovacs WJ. Testosterone induces expression of transforming growth factor-beta 1 in the murine thymus. J Steroid Biochem Mol Biol. 1993; 45(5):327–32. Epub 1993/05/01. https://doi.org/10.1016/0960-7660(93)90001-d PMID: 8499342.
31. Lv J, Hua YH, Wang D, Liu AF, An J, Li AM, et al. Kinetics of pulmonary immune cells, antibody responses and their correlations with the viral clearance of influenza A fatal infection in mice. Virology Journal. 2014; 11(57). ArtN 57 https://doi.org/10.1186/1743-422x-11-57 PMID: 24666970

32. Toapanta FR, Ross TM. Impaired immune responses in the lungs of aged mice following influenza infection. Respir Res. 2009; 10:112. Epub 2009/11/20. https://doi.org/10.1186/1465-9921-10-112 PMID: 19218453; PubMed Central PMCID: PMC2642681.

33. Baskin CR, Bielefeldt-Ohmann H, Tumpey TM, Sabourin PJ, Long JP, Garcia-Sastre A, et al. Early and sustained innate immune response defines pathology and death in nonhuman primates infected by highly pathogenic influenza virus. Proc Natl Acad Sci U S A. 2009; 106(9):3455–60. Epub 2009/02/17. https://doi.org/10.1073/pnas.0813234106 PMID: 19218453; PubMed Central PMCID: PMC2642681.

34. Jeon EJ, Kim KH, Min KH. Acute eosinophilic pneumonia associated with 2009 influenza A (H1N1). Thorax. 2010; 65(3):268–70. Epub 2010/03/26. https://doi.org/10.1136/thx.2009.133025 PMID: 20335299.

35. Fuseini H, Yung JA, Cephus JY, Zhang J, Goleniewska K, Polosukhin VV, et al. Testosterone Decreases House Dust Mite-Induced Type 2 and IL-17A-Mediated Airway Inflammation. J Immunol. 2018; 201(7):1843–54. Epub 2018/08/22. https://doi.org/10.4049/jimmunol.1800293 PMID: 30127088; PubMed Central PMCID: PMC6143420.

36. Kissick HT, Sanda MG, Dunn LK, Pellegrini KL, On ST, Noel JK, et al. Androgens alter T-cell immunity by inhibiting T-helper 1 differentiation. Proc Natl Acad Sci U S A. 2009; 106(9):3455–60. Epub 2009/02/17. https://doi.org/10.1073/pnas.0813234106 PMID: 19218453; PubMed Central PMCID: PMC2642681.

37. Fijak M, Schneider E, Klug J, Bhushan S, Hackstein H, Schuler G, et al. Testosterone replacement effectively inhibits the development of experimental autoimmune orchitis in rats: evidence for a direct role of testosterone on regulatory T cell expansion. J Immunol. 2011; 186(9):5162–72. Epub 2011/03/29. https://doi.org/10.4049/jimmunol.1001958 PMID: 21441459.

38. Duan S, Thomas PG. Balancing Immune Protection and Immune Pathology by CD8(+) T-Cell Responses to Influenza Infection. Front Immunol. 2016; 7:25. Epub 2016/02/24. https://doi.org/10.3389/fimmu.2016.00025 PMID: 26904022; PubMed Central PMCID: PMC4742794.

39. van de Sandt CE, Barcena M, Koster AJ, Kasper J, Kirkpatrick CJ, Scott DP, et al. Human CD8(+) T Cells Damage Noninfected Epithelial Cells During Influenza Virus Infection In Vitro. Am J Respir Cell Mol Biol. 2017; 57(5):536–46. Epub 2017/06/15. https://doi.org/10.1165/rcmb.2016-0377OC PMID: 28613916.

40. Dulos DJ, Bagchus WM. Androgens indirectly accelerate thymocyte apoptosis. International Immunopharmacology. 2001; 1:321–8. https://doi.org/10.1016/s1567-5769(00)00029-1 PMID: 11360932.

41. Roden AC, Moser MT, Tri SD, Mercader M, Kuntz SM, Dong H, et al. Augmentation of T Cell Levels and Responses Induced by Androgen Deprivation. The Journal of Immunology. 2004; 173(10):6098–108. https://doi.org/10.4049/jimmunol.173.10.6098 PMID: 15528346.

42. Cohen JH, Daniel L, Cordier G, Saez S, Revillard JP. Sex steroid receptors in peripheral T cells: absence of androgen receptors and restriction of estrogen receptors to OKT8-positive cells. J Immunol. 1983; 131(6):2767–71. Epub 1983/12/01. PMID: 6605988.

43. Olsen NJ, Kovacs WJ. Effects of androgens on T and B lymphocyte development. Immunol Res. 2001; 23(2–3):281–8. Epub 2001/07/11. https://doi.org/10.1385/IIR:23:2-3:281 PMID: 11444393.

44. Kovacs WJ, Olsen NJ. Androgen receptors in human thymocytes. J Immunol. 1987; 139(2):490–3. Epub 1987/07/15. PMID: 3496383.

45. Gooren LJ, Bunck MC. Androgen replacement therapy: present and future. Drugs. 2004; 64(17):1861–91. Epub 2004/08/27. https://doi.org/10.2165/00003495-200464170-00002 PMID: 15329035.

46. Robinson DP, Hall OJ, Nilles TL, Beam JH, Klein SL. 17beta-estradiol protects females against influenza by recruiting neutrophils and increasing virus-specific CD8 T cell responses in the lungs. J Virol. 2014; 88(9):4711–20. Epub 2014/02/14. https://doi.org/10.1128/JVI.02081-13 PMID: 24522912; PubMed Central PMCID: PMC3993800.

47. Vermillion MS, Ursin RL, Attreed SE, Klein SL. Estriol Reduces Pulmonary Immune Cell Recruitment and Inflammation to Protect Female Mice From Severe Influenza. Endocrinology. 2018; 159(9):3306–20. Epub 2018/07/23. https://doi.org/10.1210/ed.2018-00486 PMID: 30032246; PubMed Central PMCID: PMC6109301.

48. Labrie F. Mechanism of action and pure antiandrogenic properties of flutamide. Cancer. 1993; 72(12 Suppl):3816–27. Epub 1993/12/15. https://doi.org/10.1002/1097-0142(19931215)72:12<3816::aid-cncr2820721711>3.0.co;2-3 PMID: 8252497.
49. Pu Y, Xu M, Liang Y, Yang K, Guo Y, Yang X, et al. Androgen receptor antagonists compromise T cell response against prostate cancer leading to early tumor relapse. Sci Transl Med. 2016; 8(333):333ra47. Epub 2016/04/08. https://doi.org/10.1126/scitranslmed.aad5659 PMID: 27053771.

50. Fukuyama S, Kawaoka Y. The pathogenesis of influenza virus infections: the contributions of virus and host factors. Curr Opin Immunol. 2011; 23(4):481–6. Epub 2011/08/16. https://doi.org/10.1016/j.coi.2011.07.016 PMID: 21840185; PubMed Central PMCID: PMC3163725.

51. Kuiken T, Riteau B, Fouchier RA, Rimmelzaan GF. Pathogenesis of influenza virus infections: the good, the bad and the ugly. Curr Opin Virol. 2012; 2(3):276–86. Epub 2012/06/20. https://doi.org/10.1016/j.coiv.2012.02.013 PMID: 22709515.

52. Duan M, Hibbs ML, Chen W. The contributions of lung macrophage and monocyte heterogeneity to influenza pathogenesis. Immunol Cell Biol. 2017; 95(3):225–35. Epub 2016/10/19. https://doi.org/10.1007/icb.2016.97 PMID: 27670791.

53. Kadel S, Kovats S. Sex Hormones Regulate Innate Immune Cells and Promote Differential Response in Respiratory Virus Infection. Front Immunol. 2018; 9:1653. Epub 2018/08/07. https://doi.org/10.3389/fimmu.2018.01653 PMID: 30079065; PubMed Central PMCID: PMC6062604.

54. Mantalaris A, Panoskaltsis N, Sakai Y, Bourne P, Chang C, Messing EM, et al. Localization of androgen receptor expression in human bone marrow. J Pathol. 2001; 193(3):361–6. Epub 2001/03/10. https://doi.org/10.1002/1096-9896(200103)193:3<361::AID-PATH1105>3.0.CO;2-W PMID: 11301417.

55. Gilliver SC. Sex steroids as inflammatory regulators. J Steroid Biochem Mol Biol. 2010; 120(2–3):105–15. Epub 2010/01/05. https://doi.org/10.1016/j.jsbmb.2009.12.015 PMID: 20045727.

56. Laffont S, Blanquart E, Savignac M, Cenac C, Laverny G, Metzger D, et al. Androgen signaling negatively controls group 2 innate lymphoid cells. J Exp Med. 2017; 214(6):1581–92. Epub 2017/05/10. https://doi.org/10.1084/jem.20161807 PMID: 28484078; PubMed Central PMCID: PMC5461006.

57. Cephus JY, Stier MT, Fuseini H, Yung JA, Toki S, Bloodworth MH, et al. Testosterone Attenuates Inflammation, Enhances Lung Regeneration, and Promotes Healing After Bacterial Pneumonia. J Immunol. 2014; 200(7):2391–404. Epub 2014/02/16. https://doi.org/10.4049/jimmunol.1701543 PMID: 24945006; PubMed Central PMCID: PMC5680989.

58. Wareing MD, Lyon AB, Lu B, Gerard C, Sarawar SR. Chemokine expression during the development and resolution of a pulmonary leukocyte response to influenza A virus infection in mice. J Leukoc Biol. 2004; 76(4):886–95. Epub 2004/07/09. https://doi.org/10.1189/jl.1203644 PMID: 15240757.

59. Gorski SA, Hahn YS, Braciale TJ. Group 2 innate lymphoid cell production of IL-5 is regulated by NKT cell-derived IL-12. PLoS Pathog. 2013; 9(9):e1003615. Epub 2013/09/27. https://doi.org/10.1371/journal.ppat.1003615 PMID: 24068930; PubMed Central PMCID: PMC3777868.

60. Montecilli LA, Sonnenberg GF, Abt MC, Alenghat T, Ziegler CGK, Doering TA, et al. Innate lymphoid cells promote lung-tissue homeostasis after infection with influenza virus. Nature immunology. 2011; 12(11):1045–54. https://doi.org/10.1038/ni.2131 PMID: 21946417.

61. Coates BM, Staricha KL, Koch CM, Cheng Y, Shumaker DK, Budinger GRS, et al. Innate Monocytes Drive Influenza A Virus-Mediated Lung Injury in Juvenile Mice. J Immunol. 2018; 200(7):2391–404. Epub 2018/02/16. https://doi.org/10.4049/jimmunol.1701543 PMID: 29445006; PubMed Central PMCID: PMC5680989.

62. Cole SL, Dunning J, Kok WL, Benham KH, Benlahrech A, Repapi E, et al. M1-like monocytes are a major immunological determinant of severity in previously healthy adults with life-threatening influenza. JCI Insight. 2017; 2(7):e91868. Epub 2017/04/14. https://doi.org/10.1172/jci.insight.91868 PMID: 28405622; PubMed Central PMCID: PMC5374077 exists.

63. Lavuva G, Chorro L, Spaulding E, Soudja SM. Inflammatory monocyte effector mechanisms. Cell Immunol. 2014; 291(1–2):32–40. Epub 2014/09/11. https://doi.org/10.1016/j.cellimm.2014.07.007 PMID: 25205002; PubMed Central PMCID: PMC4457438.

64. Dalal M, Kim S, Voskuhl RR. Testosterone therapy ameliorates experimental autoimmune encephalomyelitis and induces a Thelper 2 bias in the autoantigen-specific T lymphocyte response. J Immunol. 1997; 159(1):3–6. Epub 1997/07/01. PMID: 9200430.

65. Zhang MA, Rego D, Moshkova M, Kebir H, Chruscinski A, Nguyen H, et al. Peroxisome proliferator-activated receptor (PPAR) alpha and gamma regulate IFNgamma and IL-17A production by human T cells in a sex-specific way. Proc Natl Acad Sci U S A. 2012; 109(24):9505–10. Epub 2012/06/01. https://doi.org/10.1073/pnas.1118458109 PMID: 22647601; PubMed Central PMCID: PMC3386070.

66. Fijak M, Damm LJ, Wenzel JP, Aslani F, Walecki M, Wahle E, et al. Influence of Testosterone on Inflammatory Response in Testicular Cells and Expression of Transcription Factor Foxp3 in T Cells. Am J Reprod Immunol. 2015; 74(1):12–25. Epub 2015/01/20. https://doi.org/10.1111/aji.12363 PMID: 25598450.

67. Walecki M, Eisel F, Klug J, Baal N, Paradowska-Dogan A, Wahle E, et al. Androgen receptor modulates Foxp3 expression in CD4+CD25+Foxp3+ regulatory T-cells. Mol Biol Cell. 2015; 26(15):2845–57. Epub 2015/08/05. https://doi.org/10.1121/2015.015295.
68. Grant EJ, Quiñones-Parra SM, Clemens EB, Kedzierska K. Human influenza viruses and CD8+ T cell responses. Current Opinion in Virology. 2016; 16:132–42. https://doi.org/10.1016/j.coviro.2016.01.016 PMID: 26974887

69. Aldridge JR Jr, Moseley CE, Boltz DA, Negovetich NJ, Reynolds C, Franks J, et al. TNF/iNOS-producing dendritic cells are the necessary evil of lethal influenza virus infection. Proc Natl Acad Sci U S A. 2009; 106(13):5306–11. Epub 2009/03/13. https://doi.org/10.1073/pnas.0900655106 PMID: 19279209; PubMed Central PMCID: PMC2664048.

70. Benten WP, Lieberherr M, Giese G, Wrehike C, Stamm O, Sekeris CE, et al. Functional testosterone receptors in plasma membranes of T cells. Faseb j. 1999; 13(1):123–33. Epub 1999/01/05. https://doi.org/10.1096/fasebj.13.1.123 PMID: 9872937.

71. Mikkonen L, Pihlajaamaa P, Sahu B, Zhang F-P, Jänne OA. Androgen receptor and androgen-dependent gene expression in lung. Molecular and Cellular Endocrinology. 2010; 317(1):14–24. https://doi.org/10.1016/j.mce.2009.12.022

72. Desai P, Tahiliani V, Stanfield J, Abboud G, Salek-Ardakani S. Inflammatory monocytes contribute to the persistence of CXCR3(hi) CX3CR1(lo) circulating and lung-resident memory CD8(+) T cells following respiratory virus infection. Immunol Cell Biol. 2018; 96(4):370–8. Epub 2018/01/25. https://doi.org/10.1111/imcb.12006 PMID: 29363162; PubMed Central PMCID: PMC3570563.

73. Suber F, Kcobz L. Childhood tolerance of severe influenza: a mortality analysis in mice. Am J Physiol Lung Cell Mol Physiol. 2017; 313(6):L1087–L95. Epub 2017/09/09. https://doi.org/10.1152/ajplung.00364.2017 PMID: 28882815; PubMed Central PMCID: PMC5814705.

74. Lotter H, Heik E, Berrin H, Jacobs T, Prehn C, Adamski J, et al. Testosterone increases susceptibility to amebic liver abscess in mice and mediates inhibition of IFNgamma secretion in natural killer T cells. PLoS One. 2013; 8(2):e55694. Epub 2013/02/21. https://doi.org/10.1371/journal.pone.0055694 PMID: 23424637; PubMed Central PMCID: PMC3570563.

75. Berrin H, Lotter H. Sex Bias in the Outcome of Human Tropical Infectious Diseases: Influence of Steroid Hormones. The Journal of Infectious Diseases. 2014; 209(suppl_3):S107–S13. https://doi.org/10.1093/infdis/jit610 PMID: 24966190

76. Cervantes-Rebolledo C, Moreno-Mendoza N, Morales-Montor J, De La Torre P, Laclette JP, Carrero JC. Gonadectomy inhibits development of experimental amoebic liver abscess in hamsters through downregulation of the inflammatory immune response. Parasite Immunol. 2009; 31(8):447–56. Epub 2009/08/04. https://doi.org/10.1111/j.1365-3024.2009.01120.x PMID: 19646209.

77. Vom Steeg LG, Vermillion MS, Hall OJ, Alam O, McFarland R, Chen H, et al. Age and testosterone mediate influenza pathogenesis in male mice. American Journal of Physiology-Lung Cellular and Molecular Physiology. 2016; 311(6):L1234–L44. https://doi.org/10.1152/ajplung.00352.2016 PMID: 27815620

78. Ye J, Sorrell EM, Cai Y, Shao H, Xu K, Pena L, et al. Variations in the hemagglutinin of the 2009 H1N1 pandemic virus: potential for strains with altered virulence phenotype? PLoS Pathog. 2010; 6(10):e1001145. Epub 2010/10/27. https://doi.org/10.1371/journal.ppat.1001145 PMID: 20976194; PubMed Central PMCID: PMC2954835.

79. Topham DJ, Castrucci MR, Wingo FS, Belz GT, Doherty PC. The Role of Antigen in the Localization of Naive, Acutely Activated, and Memory CD8+ T Cells to the Lung During Influenza Pneumonia. The Journal of Immunology. 2001; 167(12):6983–90. https://doi.org/10.4049/jimmunol.167.12.6983 PMID: 11739518

80. Vermillion MS, Nelson A, Vom Steeg L, Loube J, Mitzner W, Klein SL. Pregnancy preserves pulmonary function following influenza virus infection in C57BL/6 mice. Am J Physiol Lung Cell Mol Physiol. 2018; 315(4):L517–L25. Epub 2018/06/01. https://doi.org/10.1152/ajplung.00066.2018 PMID: 29847990; PubMed Central PMCID: PMC6230880.