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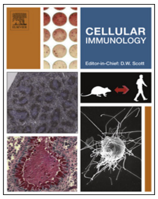

Research paper

Testosterone treatment of aged male mice improves some but not all aspects of age-associated increases in influenza severity

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ABSTRACT

The severity of influenza increases with age, with worse disease in aged males than females. Testosterone concentrations decline with age in males, which may impact influenza pathogenesis. Aged male mice were treated with testosterone or placebo and outcomes during influenza A virus (IAV) infection were compared with adult male mice. Aged males experienced greater morbidity and mortality than adult males, which was partially improved by testosterone treatment of aged males. Aged males cleared IAV from lungs slower than adult males, regardless of testosterone treatment. As compared with adult males, aged males experienced pulmonary, but not systemic, cytokine dysregulation, and delayed influx and contraction of IAV-specific $CD8^+$ T cells in the lungs. Testosterone treatment in aged males partially restored pulmonary cytokine responses to levels consistent with adult males but did not alter the age-associated changes in IAV-specific $CD8^+$ T cells. Testosterone only modestly improves outcomes of influenza in aged males.

1. Introduction

Aged individuals are at a greater risk of severe disease following influenza virus infection. Although infection rates are generally higher in infants and children [1], individuals over the age of 65 account for 54–70% of all influenza-related hospitalizations and 70–90% of all influenza-related deaths [2,3]. Moreover, while females experience more severe disease than males among reproductively aged cohorts [4,5], among individuals 65 years or older, males are more likely than females to be hospitalized and succumb to infection with both seasonal and pandemic influenza viruses [5,6]. The factors that protect males against influenza in early adulthood and contribute to their vulnerability to influenza in later life are currently not known.

Aging in both males and females is associated with elevated systemic inflammation (e.g., elevated serum concentrations of IL-6 and TNFα), as well as a decreased ability to respond to specific immunological challenges [7–9]. The effects of aging are often most pronounced in the context of T cell-mediated responses, with significant reductions in the numbers of naïve T cells, the accumulation of memory T cells, restrictions in T cell receptor repertoire, limited expansion of antigen-specific T cell populations, resistance to activation-induced apoptosis, and changes in effector activity (e.g., altered cytokine production and decreased cytotoxicity) [10–12]. Moreover, defects in the induction of the innate immune response also have been reported and result in dysregulated cytokine and chemokine production in response to infection [13,14], thus demonstrating the breadth of age-related immune dysfunction.

In the context of influenza pathogenesis, animal models provide further evidence for aging-related perturbation. Murine models of primary influenza A virus (IAV) infection, which have used primarily if not exclusively female mice, generally demonstrate that aged mice experience greater morbidity and mortality, are slower to control viral replication, and suffer more extensive pulmonary damage than younger mice [15–18]. These changes in infection outcome are often associated with a slower activation of the innate immune response, delayed induction of the pulmonary cytokine and chemokine response, and reduced innate immune cell functional activity including impairments in alveolar macrophage phagocytosis [15,16]. Similarly, the induction of virus-specific $CD8^+$ T cell responses, which play a critical role in the killing of virally infected cells, are delayed and of reduced peak magnitude in murine models of aging [15,18].

In addition to immunological changes, aging is associated with hormonal changes that alter physiology. For males, aging results in a gradual decline in both total and bioavailable testosterone, with 19% of

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men in their sixties and 49% of men in their eighties meeting the clinical criteria for low testosterone [19–21]. Testosterone has profound effects on systemic health and disease by altering metabolic function, cardiovascular function, and immune function [22–24]. Testosterone, primarily through activation of androgen receptors in immune cells, can alter the numbers and functions of both innate and adaptive immune cells to generally protect against a variety of inflammation-mediated diseases [25–27]. For example, treatment with testosterone reduces proinflammatory cytokine production (e.g., IL-1β, IL-6, and TNFα) by dendritic cells [28], whereas depletion of testosterone increases $CD8^+$ T cell numbers and suppresses their proliferative potential in response to stimulation [29]. Despite these observations, few studies have considered the effects of age-related declines in testosterone on the outcomes of infectious or inflammatory diseases.

We have previously shown that the age-related decline in testosterone concentrations is associated with increased mortality and disease severity following IAV infection [17]. Moreover, the rescue of testosterone in aged males decreases mortality and reduces clinical disease severity independent of changes in viral replication or pulmonary inflammation [17]. In the current study, we sought to characterize the effects of testosterone replacement therapy on the immune response to IAV infection in aged males. We show that testosterone replacement in aged males promotes greater tolerance of IAV infection (i.e., reduced morbidity relative to detectable pulmonary virus titers) and improves pulmonary cytokine responses but has no effect on the age-associated delayed clearance of virus or dysregulation of virus-specific $CD8^+$ T cells in the lungs of male mice. We interpret these data to suggest that testosterone replacement therapy in aged male mice improves some, but not all, age-associated changes in immune responses and susceptibility to IAVs.

2. Materials and methods

2.1. Animals

All animal procedures were approved by the Johns Hopkins University Animal Care and Use Committee (MO18H262). Adult (2–3 months of age) and aged (17–18 months of age) male C57BL/6CR mice were purchased from Charles River or the National Institute of Aging, respectively. All mice were housed with 2–5 animals per microisolator cage under standard BSL-2 housing conditions and given food and water *ad libitum*. Experiments were conducted as a series of replicates, and animal numbers are provided in the legends.

2.2. Testosterone administration and quantification

Testosterone was administered by subcutaneously implanting a silicone tubing capsule (inner diameter-0.04″, outer diameter- 0.085″; HelixMark) containing crystalline testosterone propionate (7.5 mm; Sigma) as described previously [17]. 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. Total testosterone concentrations in plasma were quantified 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. All infections occurred one week following the initiation of testosterone treatment in aged male mice.

2.3. Virus infection and quantification

Mouse-adapted A/California/4/09 (ma2009; H1N1; generated by Dr. Andrew Pekosz using a published sequence [30]) was used in all experiments. Mice were anesthetized and infected by intranasal inoculation with ma2009 (0.1 MLD_{50} for young males) diluted in 30 µl of DMEM or mock infected with 30 µl DMEM. For virus quantification, $\log_{10}$ dilutions of lung homogenate were plated onto Madin-Darby canine kidney (MDCK) cell monolayers (kindly provided by Dr. Andrew Pekosz) 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.

2.4. Sample collection

Following infection, rectal temperature, body mass, and mortality were recorded daily out to 21 days post inoculation (dpi), and clinical disease scores were recorded at select time-points as described previously [17]. For terminal studies, mice were euthanized at select time-points and plasma and whole lungs were collected.

2.5. Pulmonary cytokine and chemokine quantification

Snap-frozen lung tissue was homogenized in DMEM supplemented with 1% L-glutamine (Gibco), and 1% penicillin-streptomycin (Gibco) followed by centrifugation to remove cellular debris. Supernatants were collected and 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, Eotaxin, G-CSF, GM-CSF, IFNγ, TNFα, CXCL1, CCL2, CCL3, CCL4, and CCL5 were quantified using the Bio-Plex Pro Mouse Cytokine 23-Plex Assay (Biorad) according to the manufacturer's instructions. For analyses, the concentration of Eotaxin and IL-9 remained below the limit of detection at all time-points and was excluded.

2.6. Plasma cytokine and chemokine quantification

Plasma was collected and 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, Eotaxin, G-CSF, GM-CSF, IFNγ, TNFα, CXCL1, CCL2, CCL3, CCL4, and CCL5 were quantified using the Bio-Plex Pro Mouse Cytokine 23-Plex Assay (Biorad) on a Curiox DA-Bead Droparray plate at a sample volume of 10 µl, as previously described [31]. For analyses, the concentration of Eotaxin remained below the limit of detection at all time-points and was excluded.

2.7. $CD8^+$ T cell quantification

Lung tissue was harvested, and single cells suspensions were generated by homogenizing tissue through a 100 µm 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 ma2009-specific $CD8^+$ T cells, isolated cells were cultured for 5 h at 37 °C in media containing ma2009-specific peptide ($NP_{366-374}$,) in the presence of GolgiPlug (BD) and GolgiStop (BD). Following incubation, $CD8^+$ T cell viability was determined by fixable live/dead far red viability stain (Invitrogen), Fc receptors were blocked using anti-CD16/32 (BD Biosciences), and cells were stained for CD8-PerCPCy5.5 (Clone 53–6.7; BD) and PE-conjugated tetramer for ma2009 (ASNENVETM; NIH Tetramer Core Facility). Cells were then permeabilized and fixed (BD Cytofix/Cytoperm) prior to intracellular staining with IFNγ-FITC (Clone XMG1.2; BD) and TNFα-PE (Clone MP6-XT22; BD). Data were acquired using a FACSCailubr flow cytometer (BD) running Cell Quest Pro and analyzed using FlowJo (v.10) software (Tree Star, Inc.). Total 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.

2.8. 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. Survival was analyzed using a Kaplan Meyer survival curve followed by log-rank testing. Disease trajectory curves were analyzed by piecewise linear regression. Statistical analyses were performed using GraphPad Prism 8.1.1 software and mean differences were considered significant at $p < 0.05$.

3. Results

3.1. Testosterone improves clinical disease severity tolerance to IAV infection in aged males

To assess whether testosterone replacement in aged males reduces the severity of IAV infection, aged male mice were implanted with either testosterone or placebo containing capsules and compared with gonad-intact adult male mice that received no exogenous hormone treatment. Aged males treated with placebo had significantly lower concentrations of circulating testosterone than adult males; whereas treatment of aged males with testosterone brought concentrations of testosterone to within the physiological range of adult males (Fig. 1A; $p < 0.05$, one-way ANOVA). Following intranasal inoculation with a low dose (i.e., sub-lethal in adult males) of ma2009 H1N1 IAV, mice were monitored for 21 days post inoculation (dpi) for changes in morbidity, clinical disease severity, and mortality. Similar to previously published results [17], aged male mice experienced greater body mass loss, hypothermia, and clinical disease severity than adult male mice, and were significantly more likely to die following IAV infection (Fig. 1B–E; $p < 0.05$ in each case, mixed-effect model or log-rank test). Exogenous testosterone treatment in aged male mice reduced clinical disease severity, but did not significantly improve body mass loss, hypothermia, or mortality following IAV infection (Fig. 1B–E; $p < 0.05$, mixed-effect model or log-rank test). Aged, both testosterone and placebo treated, and adult males had similar peak virus titers at 7 dpi (Fig. 1F). The control of viral replication was delayed in aged males, regardless of testosterone treatment, as compared with adult males, resulting in greater virus titers in aged than adult males at 11 dpi (Fig. 1F; $p < 0.05$, two-way ANOVA), with complete clearance of detectable virus in all animals by 14 dpi.

We hypothesized that aging may increase influenza severity in males by altering one of two host defense strategies against infection: resistance (i.e., the ability to control virus replication) or tolerance (i.e., the ability to limit disease caused by replicating virus) [32,33]. To determine if age or testosterone treatment altered host resistance or tolerance to IAV infection, disease trajectory curves were generated by plotting markers of host health (i.e., hypothermia and body mass loss) relative to viral titers over time [34]. Replicating virus resulted in similar disease trajectories (i.e., slope of the lines) at 3 and 7 dpi for all males. By 11 dpi, the impact of virus replication on disease severity was greater in aged males, regardless of testosterone treatment, relative to adult males (Fig. 1G–H, $p < 0.05$, piecewise regression). The improvement in host health as detectable virus was being cleared from the lungs (14 dpi) resulted in a steeper line for aged males treated with testosterone than for aged males treated with placebo, suggesting that testosterone treatment improved tolerance of IAV after virus clearance (Fig. 1G–H; $p < 0.05$, piecewise regression). Taken together, these data suggest that age impacts both resistance (i.e., control of virus replication) and tolerance (i.e., disease relative to viral load), whereas testosterone affects tolerance, but not resistance, to IAV in aged males.

3.2. Age and testosterone treatment alter the cytokine and chemokine responses to IAV infection in the lungs and blood

Severe influenza is often associated with an excessive induction of pulmonary cytokine and chemokine responses (i.e., the cytokine storm), which can lead to uncontrolled cellular infiltration, pulmonary inflammation, and tissue damage [35,36]. Moreover, advanced age is associated with dysregulation of the cytokine and chemokine response in murine models of pulmonary infections [13]. To determine whether age and testosterone treatment altered the cytokine and chemokine response to IAV infection, pulmonary concentrations of 23 cytokines and chemokines were measured at select dpi. To control for age-related differences in baseline pulmonary concentrations of cytokines and chemokines (Supplemental Table 1), fold changes relative to mock infection were determined for each treatment group. Following IAV infection, the pulmonary concentrations of most cytokines and chemokines increased in response to infection at 3 and 7 dpi, and then declined at 11 dpi for all treatment groups (Fig. 2 and Supplemental Table 1; $p < 0.05$ in each case, two-way ANOVA). Relative to aged males, the induction of IL-3, IL-6, IL-10, IL-13, IFNγ, TNFα, CCL2, and CCL5 was greater in the lungs of adult than aged males, with these differences being most pronounced at 7 dpi (Fig. 2B, E, F, H, I, K and L; $p < 0.05$ in each case, two-way ANOVA). In contrast, the induction of IL-5 was greater in the lungs of aged than adult males at 3 and 7 dpi (Fig. 2D; $p < 0.05$, two-way ANOVA). The only pulmonary cytokines altered by testosterone treatment of aged males were IL-1β, IL-3, IL-4, and CCL2, with the induction of IL-3, IL-4, and CCL2 being increased and the induction of IL-1β being suppressed by the presence as compared with the absence of testosterone in aged males (Fig. 2A–C and K; $p < 0.05$ in each case, two-way ANOVA).

Excessive or dysregulated systemic cytokine and chemokine concentrations have also been correlated with severe outcomes following IAV infection [37,38]. As advanced age is associated with increases in serum proinflammatory cytokine concentrations [7,8], we next evaluated age- and testosterone-associated changes in the induction of peripheral blood cytokine and chemokine concentrations in response to IAV infection. Broadly, the concentrations of cytokines and chemokines were lower in blood than in the lungs during IAV (Fig. 3 and Supplemental Table 2). Overall, aging (in the absence of testosterone treatment) reduced the induction of cytokines and chemokines in the blood (Fig. 3). Among aged males that were not treated with testosterone, the induction of plasma IL-3, IL-4, CXCL1, and CCL2 was suppressed as compared with adult males, with the age-associated changes being most pronounced over time for IL-4 (Fig. 3B, C, J, and K; $p < 0.05$ in each case, two-way ANOVA). Testosterone treatment of aged males increased the induction of IL-3, IL-6, IL-13, and CCL2 relative to aged males treated with placebo (Fig. 3B, D, G and K; $p < 0.05$ in each case, two-way ANOVA). Testosterone treatment of aged males also increased the induction of systemic IL-10 relative to either adult males or aged males treated with placebo (Fig. 3F; $p < 0.05$, two-way ANOVA). Taken together, these data suggest that testosterone treatment of aged males partially rescues age-related dysregulation of the cytokine and chemokine responses to IAV infection, with these effects being more pronounced locally (i.e., in the lungs) than systemically (i.e., in the blood).

3.3. Aging prolongs the number and activity of pulmonary $CD8^+$ T cells during IAV infection in males

Virus-specific $CD8^+$ T cells can contribute to both the control of IAV infection and pathology during infection, especially when improperly regulated [39,40]. Because aging is associated with dysregulation of the $CD8^+$ T cell response to IAV infection [15,18,41], we analyzed the effects of testosterone treatment on $CD8^+$ T cell responses during infection. In adult males, the greatest numbers of total $CD8^+$ T cells, IAV-specific $CD8^+$ T cells, and $CD8^+$ T cells producing IFNγ in response to

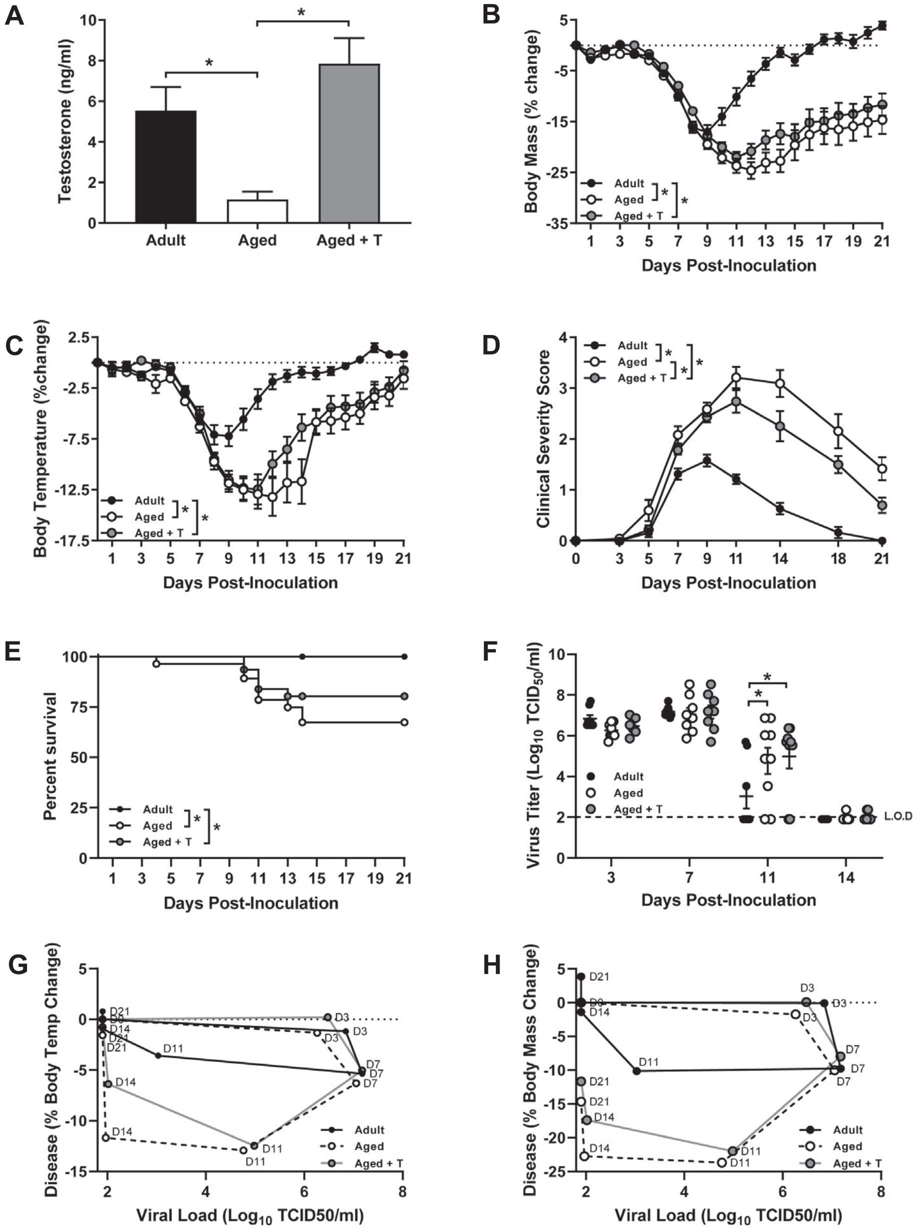

Fig. 1. Effects of testosterone replacement on the outcome of influenza virus infection in aged male mice. Adult male mice (adult) that received no hormone and aged male mice that were either implanted with placebo (aged) or testosterone (aged + T), were inoculated with ma2009 H1N1 IAV or mock infected. Plasma was collected from mock infected mice and testosterone concentrations (A) were analyzed by ELISA (n = 8–10/group). Following IAV infection, mice (n = 19–23/group) were monitored daily for changes in body mass (B), body temperature (C), clinical disease severity (D), and survival (E). Infectious virus was measured in the lungs by $TCID_{50}$ at 3, 7, 11, or 14 days post inoculation (dpi; F; n = 6–9/group/time-point). Disease trajectory curves were generated by plotting pulmonary virus load relative to either body temperature (F) or body mass (G) changes during IAV infection. Data represent means ± SEM from two independent replications and significant differences between treatment groups are denoted by asterisks ($*p < 0.05$).

ex vivo stimulation with ma2009 H1N1-specific peptide (Fig. 4A–C), corresponded with the presence of infectious virus in the lungs at 7 and 11 dpi (Fig. 1F), with cell numbers declining after clearance of detectable virus at 14 and 21 dpi. In contrast, among aged males not treated with testosterone, frequencies of total $CD8^+$ T cells, IAV-specific $CD8^+$ T cells, and $CD8^+$ T cells producing IFNγ peaked as virus was being cleared from the lungs (11 dpi) and remained elevated after detectable virus clearance (14 and 21 dpi) (Fig. 4A–C). As a result, adult males had greater numbers of IAV-specific $CD8^+$ T cells than aged males at 7 dpi, and aged males had greater numbers of total $CD8^+$ T cells, IAV-specific $CD8^+$ T cells, and $CD8^+$ T cells producing IFNγ than adult males at 14 and 21 dpi (Fig. 4A–C; $p < 0.05$ in each case, two-way ANOVA). Treatment of aged males with testosterone did not affect the influx or activity of $CD8^+$ T cells when infectious virus was present in the lungs (7 and 11 dpi), but did partially reduce the total number of $CD8^+$ T cells, number IAV-specific $CD8^+$ T cells, and activity of IAV-specific $CD8^+$ T cells in the lungs after detectable virus had been cleared, resulting in no significance differences in $CD8^+$ T cell numbers or activity in the lungs of aged males treated with testosterone and adult males at either 14 or 21 dpi (Fig. 4A–C). Taken together, aging is associated with dysregulation of $CD8^+$ T cells resulting in prolonged presence of these cells in the lungs after virus clearance. Treatment with testosterone does not significantly improve age-related dysregulation of virus-specific $CD8^+$ T cell responses to IAV infection.

4. Discussion

Aging is associated with broad dysregulation of the immune system leading to an increased risk of severe disease following IAV infection [15–18]. For males, circulating testosterone concentrations also decline with age [19–21], with low testosterone, whether age-related or surgically induced in young males, increasing the severity of influenza independent of changes in viral replication [17,42]. In the present study, we tested the hypothesis that testosterone treatment of aged males would reduce age-related dysfunction of the immune response to IAV infection. Aged males that were not treated with testosterone had

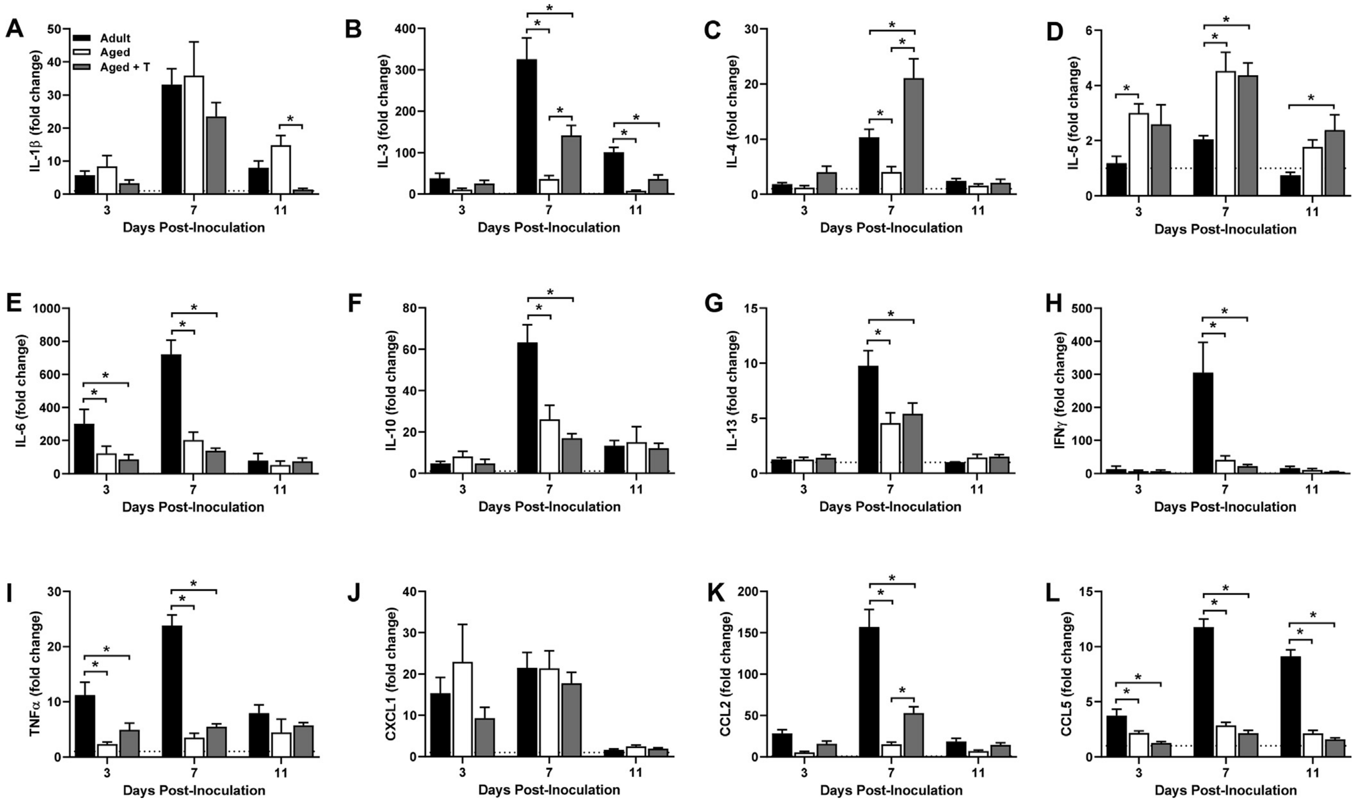

Fig. 2. Effects of testosterone replacement on pulmonary cytokine or chemokine induction during influenza A virus (IAV) infection in aged male mice. Adult male mice (adult) that received no hormone and aged male mice that were either implanted with placebo (aged) or testosterone (aged + T), were inoculated with ma2009 H1N1 IAV or mock infected. At 0, 3, 7, or 11 days post inoculation (dpi; n = 7–12/group/time-point), lung tissue was harvested and homogenized, and cell free supernatants were used to quantify the fold induction of IL-1β (A), IL-3 (B), IL-4 (C), IL-5 (D), IL-6 (E), IL-10 (F), IL-13 (G), IFNγ (H), TNFα (I), CXCL1 (J), CCL2 (K), and CCL5 (L) in the lungs. Data represent fold change ± SEM relative to mock infected mice (0 dpi) from two independent replications and significant differences between treatment groups are denoted by asterisks ($*p < 0.05$).

greater morbidity, clinical disease, and mortality than adult males. Aged males also cleared virus from their lungs more slowly, had reduced cytokine and chemokine induction after IAV infection, and had prolonged virus-specific CD8$^+$ T cell numbers and activity as compared with adult males. Testosterone treatment of aged males reduced the clinical disease severity of IAV, improved tolerance to infection, and reduced pulmonary cytokine dysregulation, but had only modest effects on pulmonary CD8$^+$ T cell numbers or activities. Among aged males not treated with testosterone, the observed delay in the control of viral replication and in the resolution of disease suggests that aging affects resistance and tolerance to IAV infection. In the current and previous studies [17,43], testosterone treatment of either aged (this study) or adult [17] males did not alter control of viral replication, but rather affected the severity of disease by promoting tolerance to IAV (i.e., reducing the impact of IAV infection on host health and recovery from infection).

Although aging was associated with delayed clearance of detectable virus from the lungs, detectable virus was cleared from the lungs of all mice, regardless of age or testosterone concentrations by 14 dpi. Immediately after virus clearance, young, but not aged, males rapidly returned to baseline health suggesting that virus replication was not the only variable contributing to delayed recovery in aged males. Resolution of CD8+ T cell frequencies and activity was delayed in aged compared with adult males and may contribute to immunopathology and delated recovery from IAV in aged males. Previous studies further show that reduced pulmonary repair of damaged tissue caused by inflammation, including production of amphiregulin, contributes to delayed recovery following IAV infection in females compared with males, which is independent of testosterone [43]. Whether aging causes reduced amphiregulin signaling in the lungs to contribute to slower recovery in aged as compared with adult males requires consideration.

Cytokines and chemokines are local mediators of both the innate and adaptive immune response to IAV infection [44]. In our study, the early induction of pulmonary cytokine and chemokine responses to IAV infection in adult males was broadly suppressed in aged males not treated with testosterone, suggesting an age-related defect in the activation of the immune response to infection. Testosterone treatment of aged males increased the induction of some cytokines and chemokines, including IL-3, IL-4, and CCL2, in the lungs of aged males. The concentrations and induction of cytokines and chemokines was greater in the lungs than in the blood during IAV infection. These data suggest that local as opposed to systemic immunity is critical for age- and to a lesser extent testosterone-related changes in immune responses and recovery from IAV.

During IAV infection, virus-specific CD8$^+$ T cells are beneficial for the control of viral replication, but improper regulation or prolonged activation of these cells can contribute to severe disease [39,40]. Consistent with previous reports in females [18,45–47], aging in males was associated with broad dysregulation of the CD8$^+$ T cell response to IAV infection, with both the induction and contraction of virus-specific CD8$^+$ T cells delayed with advanced age. In contrast with observations from peripheral blood collected from aged humans [48], testosterone treatment of aged male mice failed to significantly alter the induction, contraction, or activity of IAV-specific CD8$^+$ T cells. Whether aging renders CD8$^+$ T cells refractory to testosterone treatment has not yet been considered and warrants further study.

Antibody-mediate immunity also is induced following IAV infection. Previous data illustrate that aged males have lower IAV-specific antibody responses than adult males following either IAV infection [17] or vaccination [49]. Following IAV infection, there is no effect of

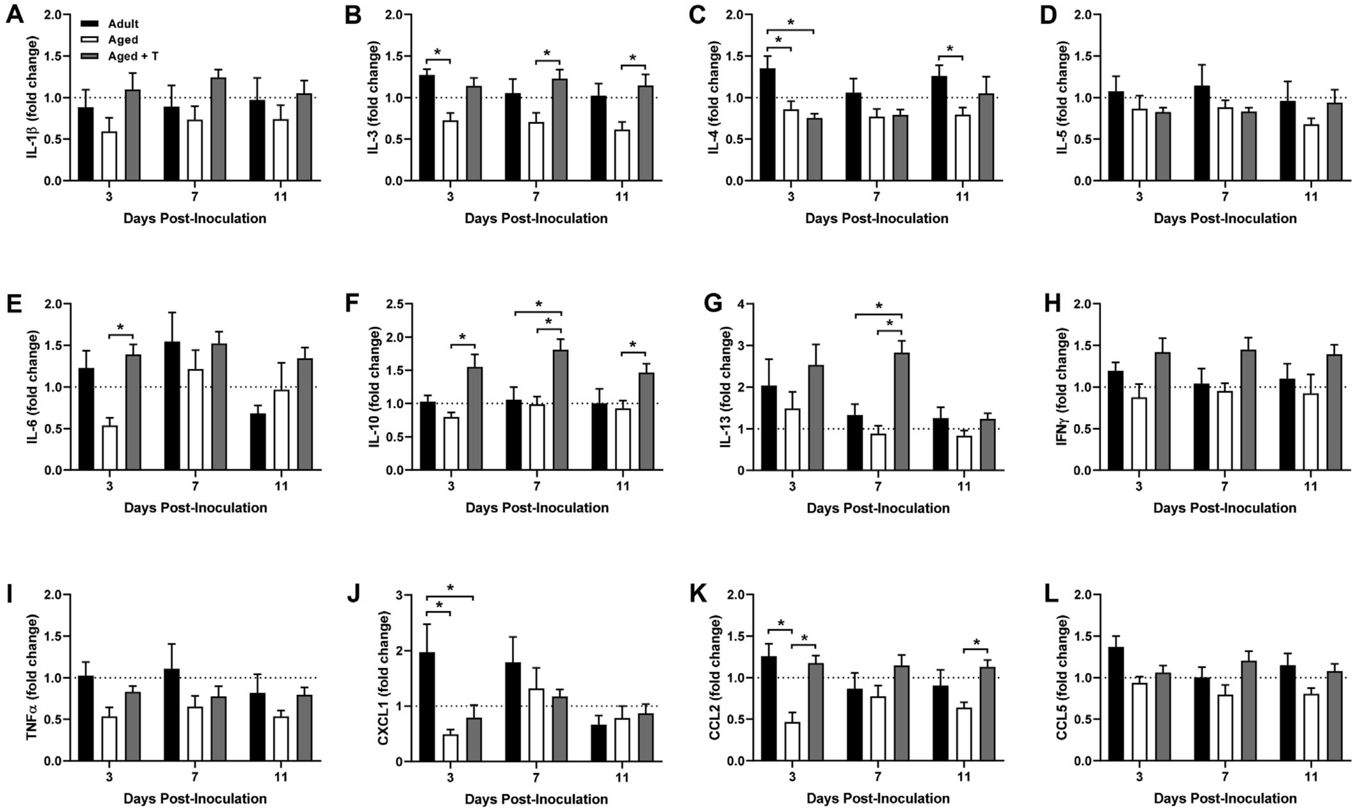

Fig. 3. Effects of testosterone replacement on plasma cytokine or chemokine induction following influenza A virus (IAV) infection in aged male mice. Adult male mice (adult) that received no hormone and aged male mice that were either implanted with placebo (aged) or testosterone (aged + T), were inoculated with ma2009 H1N1 IAV or mock infected. At 0, 3, 7, or 11 days post inoculation (dpi; n = 5–7/group/time-point), plasma was collected and used to quantify the fold induction of IL-1β (A), IL-3 (B), IL-4 (C), IL-5 (D), IL-6 (E), IL-10 (F), IL-13 (G), IFNγ (H), TNFα (I), CXCL1 (J), CCL2 (K), and CCL5 (L) in plasma relative to mock infected mice. Data represent fold change ± SEM relative to mock infected mice (0 dpi) from two independent replications and significant differences between treatment groups are denoted by asterisks ($^*p < 0.05$).

testosterone on influenza-specific antibody responses [17]. In contrast, testosterone appears to suppress antibody responses to an inactivated IAV vaccine, at least in adult males [49]. The mechanisms mediating how aging and sex steroids affect antibody responses during either IAV infection or vaccination remain to be determined.

In the current study, short duration treatment with exogenous testosterone only partially rescued some aspects of age-related immune dysregulation during IAV infection. These findings are consistent with previous studies [17,50] showing incomplete rescue of immune function following testosterone treatment in aged males. For example, androgen replacement in aged male macaques fails to increase $CD8^+$ T cell numbers and cytokine production following IAV vaccination [50]. Furthermore, aged male mice are unresponsive to the protective effects of testosterone treatment observed among adult males in murine models of experimental autoimmune encephalomyelitis [51]. Given the breadth and complexity of age-related changes in immune function [9], these data suggest that many age-related changes in immune function are not caused by low circulating testosterone. Moreover, as the effects of testosterone are largely immunosuppressive [52], in instances where aging results in defects in the ability to productively mount an immune response to immunological challenge, testosterone is unlikely to rescue function. In contrast, where age-related increases in disease severity result from excessive inflammation, testosterone is likely to be protective.

The limited responsiveness of aged male mice to testosterone treatment may be due to age-related changes in androgen receptor (AR) signaling potential. Studies in mice demonstrate that AR nuclear translocation is reduced with advanced age [53], and studies in humans show that overall AR expression is reduced in the elderly [54]. As, testosterone classically alters cellular function through AR-mediated gene transcription changes [55], age-related reductions in either AR expression or translocation may offset the impact of testosterone replacement therapy on immune cell function.

There also may be age-related changes in the tissue-specific availability of circulating testosterone. For example, in humans, the expression of sex hormone binding globulin, which binds to testosterone reducing its ability to interact with the AR, and the expression of aromatase, which can enzymatical convert testosterone in the tissues to 17β-estradiol, both increase with advanced aged [56,57]. Whether age-related changes in either the bioavailability of testosterone or in androgen signaling potential are reducing the efficacy to testosterone replacement therapy on IAV pathogenesis warrants future consideration.

In summary, our data suggest that testosterone treatment of aged males moderately reduces the severity of influenza but is insufficient to fully recover age-related immune system dysfunction during IAV infection. To date, studies on the effects of aging on IAV severity have used exclusively female mice or have not consider the sex of the animals used [58]. The results of this study illustrate profound effects of aging on immune responses and outcomes following IAV infection in male mice and suggest that age-related reductions in testosterone is only one factor contributing to the greater severity of influenza in aged males.

Data availability statement

There are no restrictions on data availability. Please contact the corresponding author for access to data.

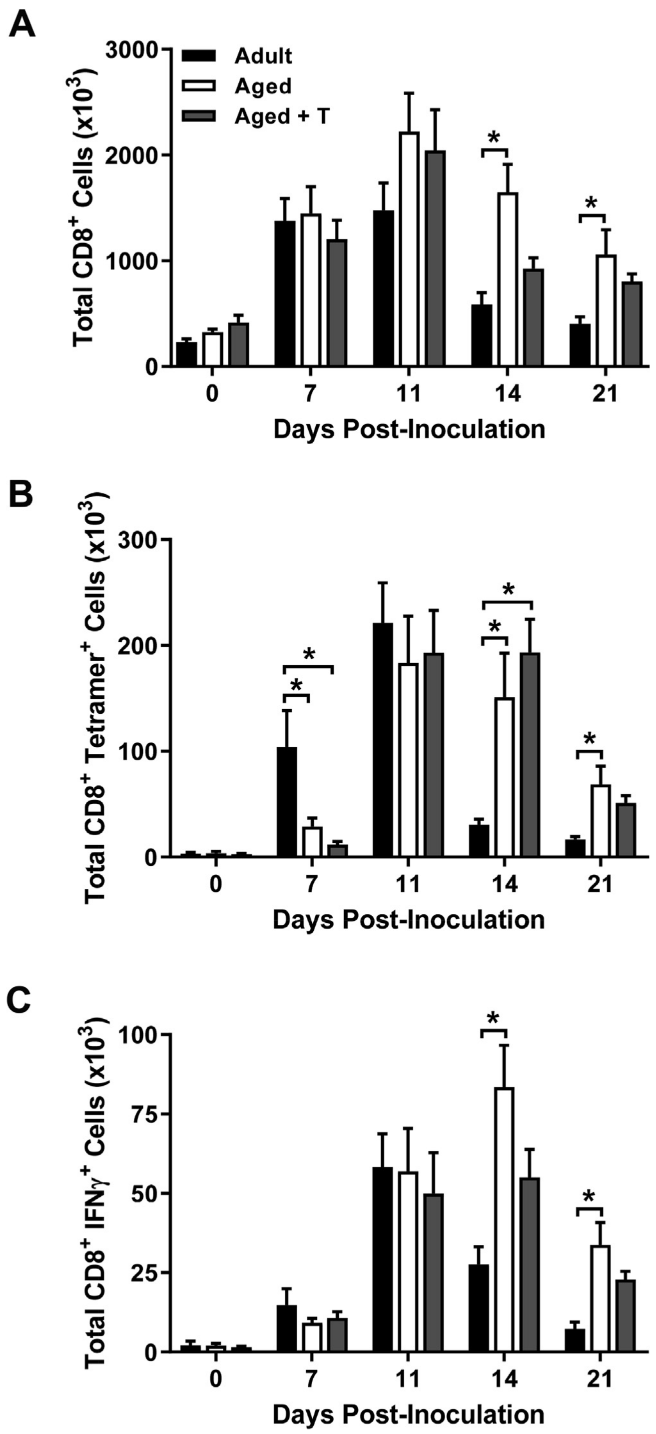

Fig. 4. Effects of testosterone replacement on the numbers and activity of virus-specific CD8$^+$ T cells during influenza virus infection in aged male mice. Adult male mice (adult) that received no hormone and aged male mice that were either implanted with placebo (aged) or testosterone (aged + T), were inoculated with 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 = 5–12/group/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 IFNγ (C) in response to *ex vivo* H1N1-specific peptide stimulation, 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 (*$p < 0.05$).

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Contributions

S.K., B.Z. and L.G.vS. conceived the studies. L.G.vS. and S.A. performed experiments, data analyses, and visualization. L.G.vS and S.K. wrote the manuscript. All authors reviewed and edited the final manuscript draft.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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