The Effects of Elevated Testosterone on the Outcome of ma2009 H1N1 Influenza A Virus infection in Old Male Mice

by

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ABSTRACT

Testosterone (T) has anti-inflammatory properties, and has been shown to play a role in the pathogenesis of infectious and autoimmune diseases. Disease pathology from influenza A virus (IAV) infection is caused by an excessive pro-inflammatory response, and severe disease is especially common among elderly men, who have lower T levels. We tested the hypothesis that T will protect against IAV pathogenesis in young adult (8-10 weeks) and old (17-18 months) male mice. To study the effects of T in young adult male mice, the mice were gonadectomized, treated with T or placebo capsules, infected with a sub-lethal dose of H1N1, and monitored for morbidity and mortality. In old male mice, serum T is naturally low, therefore gonadectomies are not required before the subsequent steps. In addition, we attempted to elevate testosterone levels endogenously in old male mice through steroidogenic drugs called TSPO ligands. However, two different drugs tested did not significantly raise serum testosterone levels. T-treated young adult males experienced lower morbidity, and had lower IgG antibody titers compared to placebo-treated young adult males. In old males, while the same dose of T was not protective against morbidity from IAV infection, treatment with a higher dose of T resulted in improved recovery from influenza disease. Protection from morbidity by testosterone treatment is not reflected in the expression of Ki67, which is a marker for proliferation, in the lungs at 21 days after infection. This suggests that either the improved recovery from disease is not associated with improved repair, or day 21, which is after peak disease and viral clearance, is too late a time point to

detect any difference. We hypothesize that old males require a higher dose of T in order to mitigate the chronic pro-inflammatory state brought about by aging.

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INTRODUCTION

Influenza Pathogenesis

Influenza viruses are an enveloped viruses of the *Orthomyxoviridae* family with a segmented, (-) ssRNA genome (Baigent & McCauley, 2003). Influenza A, B, and C are the three types of *Orthomyxoviridae* currently known to infect humans, with influenza A and B being the most associated with severe disease (Baigent & McCauley, 2003). Influenza disease is characterized by varying severity of febrile and respiratory symptoms (Lagace-Wiens et al., 2010). While illness from seasonal strains generally lasts one to two weeks, influenza can also result in hospitalizations and death. The virus is transmitted through respiratory droplets and primarily infects epithelial cells of the respiratory tract. The disease is considered a worldwide public health problem because of the ability of the virus to change every year, its rapid transmission between humans, and the resulting pandemic potential (Lagace-Wiens et al., 2010).

RNA genomes are especially susceptible to mutations due to the absence of proofreading mechanisms during replication, as opposed to the proofreading and repair mechanisms found in DNA viruses (Lauring et al., 2013). This allows RNA viruses to evade immune systems and anti-viral drugs by increasing the likelihood of a mutation that confers resistance. Single nucleotide mutations that enable influenza viruses to escape host immunity are referred to as antigenic drift (Bouvier & Palese, 2008). In addition, the segmented nature of the influenza virus genome allows reassortment between different strains of each viral species (Bouvier & Palese, 2008). For instance,

two influenza A strains, after coinfecting a host cell, can exchange segments; the resulting recombinant strain can have altered replication rates, thereby contributing to altered virulence (Pappas et al., 2008). This is known as antigenic shift. Influenza viruses infect birds, pigs, and other animals alongside humans, and therefore segments previously not seen in humans can arise through reassortment and human contact with animals, and have unpredictable effects (Bouvier & Palese, 2008).

These mechanisms for genetic variation make influenza seasonal outbreaks a logistical challenge as new vaccines need to be developed every year (Kidd, 2014). Despite these efforts, seasonal influenza strains are still a significant cause of morbidity as well as mortality, having been associated with an average of 23,640 deaths per year in the US between 1976 and 2007 (CDC, 2010). There have also historically been multiple pandemics of influenza as a result of new strains of the virus that arose that were easily transmissible, and that the human population had no preexisting immunity to. The 1918 influenza pandemic killed an estimated 50 million people around the world (Taubenberger et al., 2012).

Populations that experience the greatest disease severity are children, the elderly, pregnant women, and obese individuals which are all, to some degree, immunocompromised populations (Mauskopf et al., 2013). Sex differences in disease outcome have also been observed. Women of reproductive age (16-49) are more susceptible to severe disease from influenza infection, whereas among older individuals (65+) men experience more severe disease (Klein, 2012). Influenza disease pathology is primarily caused by a dysregulated inflammatory response (Damjanovic et al., 2012).

These differences in disease pathology are therefore thought to be a result of different inflammatory environments possibly mediated by hormonal differences between the groups of individuals as well as across the life course (Klein, 2012).

Aging, the Immune System, and the Outcome of Infectious Diseases

Immunosenescence refers to the progressive functional decline of the immune system with age (Castelo-Branco & Soveral, 2014). It is also associated with a chronic low-grade pro-inflammatory state, which is thought to be a result of increased oxidative stress in aging cells and elevated levels of certain pro-inflammatory cytokines (Cannizzo et al., 2011). Both the innate and adaptive arms of the immune system are affected.

Within the innate immune system, a few noteworthy changes are: 1) plasmacytoid DCs (pDCs) undergo a decrease in the number of IFN-α producing cells, even though total numbers remain similar to younger age groups (Jing et al., 2009); 2) monocyte-derived DCs from aged subjects have increased reactivity to self-antigens such as DNA, which is thought to contribute to chronic inflammation during aging (Agrawal et al., 2009); 3) monocytes undergo changes in the numbers of different subpopulations, and also show a cumulative decline in the secretion of IL-6 and TNF-α (Nyugen et al. 2010); and 4) neutrophils exhibit reduced phagocytic ability and decreased bacteriocidal activity in the elderly (Wenisch et al., 2000).

Data on the adaptive immune system from animal models as well as humans show that aging is associated with reduced clonal diversity of naïve CD4+ T cells (Naylor

et al., 2005), increased frequency of central memory, reduced frequency of effector memory CD4+ T cells (Kang et al., 2004), reduced clonal diversity of CD8+ T cells (Messaoudi et al., 2004), and increased frequency of effector memory and effector CD8+ cells (Hong et al., 2004). Decrease in IL-2 production during aging contributes to decreased proliferation of all thymic-derived T cells (Effros & Walford, 1983). The decreased ability to proliferate and preserve T cell receptor (TCR) diversity likely contributes to the reduced immune-surveillance that results in increased susceptibility to infectious diseases among aged individuals. CD4+ and CD8+ T cells from aged individuals generally do not express the co-stimulatory molecule CD28, and this is thought to confer the cells with resistance to apoptosis (Vallejo et al., 2000). Loss of IL-2 also results in increased IFN-γ production from $CD28^{null}$ cells, which potentially contributes to chronic inflammation during aging (Kared et al., 2014). Loss of IL-2 in conjunction with an increase in IL-1β results in an increase in the number of T helper 17 cells (Th17) cells (Lim et al., 2014). This results in an increase in the basal Th17/Regulatory T (Treg) cell ratio (Schmitt et al., 2013), even though the basal levels of Treg cells do not vary significantly with age (Hwang et al., 2009). Dysregulation of Th17 responses possibly favors inflammation and contributes to age-associated autoimmune diseases. In contrast, the Th17/Treg cell ratio decreases with age after stimulation, suggesting an increase in the production of suppressive cells after infection (Schmitt et al., 2013). Increased activity of Treg cells could prevent rejection of tumor cells and contribute to cancer (Fessler et al., 2013).

Aging is also associated with changes in B cell function. Elderly individuals have been shown to have decreased clonal diversity of B cells compared to young individuals (Gibson et al., 2009). In a study conducted with an inactivated seasonal influenza vaccine, antigen-specific plasmablasts, as well as the number of antibodies produced by each cell, were shown to be reduced in elderly individuals (70- to 100- years old) in response to vaccination as compared to younger individuals (18- to 30- years old) (Sasaki et al., 2011). However, antibodies induced by the vaccine in the elderly individuals were shown to react with greater avidity and affinity to the 2009 pandemic H1N1 virus than those in the young individuals. In a study conducted with an activated split 2009 pandemic H1N1 vaccine, antibody levels and avidity were both shown to be higher in elderly individuals (66- to 83- years old) than in young individuals (18- to 65- years old), possibly as a result of preexisting immunity to a related H1N1 strain in elderly populations (Khurana et al., 2012). Receipt of seasonal influenza vaccine by intramuscular injection results in significantly higher antibody titers in elderly (>65 years-old) females than in elderly males (Engler et al., 2008). In addition, elderly (>65 years-old) males have a higher incidence of severe disease from influenza after having received the seasonal influenza vaccine (Wang et al., 2002). Therefore, while there appears to be evidence for the general decline of B cell function with age, some data also suggest that the qualitative antibody response is improved in elderly individuals, and the functional changes vary by gender.

A mouse study on the lungs during aging showed that pulmonary macrophages exist in a highly activated state in older mice (18 months) compared to younger mice (3

months) (Canan et al., 2014). The older mice were also shown to have elevated basal levels of the pro-inflammatory cytokines IFN-γ, TNF-α, and IL-12 in the lungs. This possibly contributes to the chronic inflammatory state associated with aging. In response to *Mycobacterium tuberculosis* infection, pulmonary macrophages in the older mice exhibited greater uptake of bacteria, but lower activation by IFN-γ (Canan et al., 2014). Another study showed that older mice (16-18 months old) showed greater morbidity from a sub-lethal dose of influenza A virus than younger mice (2-3 months) (Yin et al., 2014). The study showed that this was at least partially caused by more damage to alveolar Type I and Type II cells, and delayed epithelial repair. This supports the existence of a chronic pro-inflammatory environment in the lungs in aged mice.

Aging is therefore characterized by a weakened response to infections as a result of a decline in function in the innate and adaptive arms of the immune system, as well as a chronic low-grade pro-inflammatory state possibly brought about by changes in the Th17/Treg balance, and increased basal levels of various pro-inflammatory cytokines.

Testosterone, the Immune System, and Aging

Testosterone is a steroid hormone that has been shown to have anti-inflammatory properties. It has been shown to be protective in a mouse model of rheumatoid arthritis, which is an autoimmune disease, through the downregulation of autoantibodies (Keith et al., 2013). Testosterone also decreased IFN-γ production from natural killer T (NKT) cells in a mouse model of amebic liver abscess, which is caused by

infection with *Entamoeba histolytica* (Lotter et al., 2013). In a study of influenza, gonadectomized male mice showed reduced survival when infected with a lethal dose of the virus, without showing any change in viral titers relative to infected gonadally-intact mice, suggesting that testosterone has a protective effect on disease outcome through modulating the immune response against the virus (Robinson et al., 2011). High testosterone in men is associated with lower antibody titers in response to influenza vaccination (Furman et al., 2014).

Testosterone can act directly on androgen receptors present in CD4+ T cells to increase the production of the anti-inflammatory cytokine IL-10 (Liva et al., 2001). It also suppresses the generation of reactive oxygen species and IL-8 in human granulocytes and monocytes (Boje et al., 2012). Reactive oxygen species are involved in the induction of an inflammatory response, and IL-8 is a chemokine that directs neutrophils and other granulocytes to the site of damage or infection.

Preliminary research from our lab has shown that testosterone has a protective effect on the disease pathology caused by Influenza A virus infection in male mice. Administration of exogenous testosterone to gonadectomized young male mice resulted in less weight loss, which is a measure of disease pathology, in response to influenza infection compared to gonadectomized mice treated with placebo only. Clinical scoring of observable symptoms of disease also showed a protective effect of testosterone on observable morbidity from influenza infection. Influenza disease is mainly caused by immune-mediated pathology in response to the virus (Damjanovic et al., 2012), so the

anti-inflammatory properties of testosterone are thought to be responsible for its protective effects against influenza disease pathology.

Testosterone in males decreases with age in both humans (Maggio et al., 2005) and rodents (Coquelin & Desjardins, 1982). Old male mice infected with influenza exhibit lower survival rates than young male mice, which we hypothesize to suggest that testosterone may have a protective effect against influenza disease in young mice. Low testosterone in old males may lead to immune dysregulation as a result of the loss of the anti-inflammatory effects of testosterone seen in younger males. The hormone leptin also may play a role in the increased inflammation seen in old males. It is required for the proliferation of activated CD+ T cells, which are important mediators of inflammation upon infection (Saucillo et al., 2014). Leptin has been shown to be elevated in populations with low testosterone such as women, older men, (Furman et al., 2014), and men treated with cetrorelix, which reversibly reduced testosterone to castrate levels (Büchter et al., 1999).

We hypothesize that the chronic pro-inflammatory state associated with aging in men is therefore possibly partially linked to a decrease in testosterone levels. This is supported by data showing the interaction of testosterone with the immune system and the outcome of infectious diseases (Liva et al., 2001; Keith et al., 2013). For diseases such as influenza, that are primarily caused a dysregulated inflammatory response (Damjanovic et al., 2012), decreased testosterone may result in more severe disease

pathology in the elderly, despite the general decline of immune function with age (Castelo-Branco & Soveral, 2014).

Models for Studying the Effects of Testosterone in Old Male Mice

The mouse model that we use for studying the effects of testosterone requires the subcutaneous implantation of silastic capsules containing crystalline testosterone propionate (TP) between the scapulae of the mice, which results in the slow release of testosterone into the bloodstream (Hetzler et al., 2008). The length of the capsule determines the rate of release of testosterone. Previous research in our lab has utilized this model to show the protective effects of testosterone against influenza disease from infection with maPR8 influenza in castrated young male mice (introduce in results). In old male mice, studies have been conducted using exogenous administration of testosterone in non-castrated mice to observe the effects of testosterone on androgen receptor expression, anti-anxiety behavior, and cognitive performance (Hill et al., 2004; Frye et al., 2008). This is made possible because old male mice have low endogenous testosterone levels (Coquelin & Desjardins, 1982). Implanting testosterone capsules without castrating the mice could also more accurately model the effects of exogenous testosterone administration in older, hypogonadal men. An alternative way of studying the effects of testosterone involves the use of TSPO (Translocator Protein) ligands, which activate the steroidogenic pathway to upregulate endogenous testosterone.

Steroidogenesis is the process by which cholesterol is converted to biologically active steroid hormones (Stocco & Clark, 1996). The primary sites of steroidogenesis are testicular Leydig cells in males, and ovarian granulosa and theca cells in females. Testosterone synthesis in Leydig cells begins, like in all steroidogenic cells, with the import of cholesterol from the outer mitochondrial membrane (OMM) to the inner mitochondrial membrane (IMM) (Zirkin & Chen, 2000). This is mediated by the OMM proteins Steroidogenic Acute Regulatory Protein (StAR) and Translocator Protein (TSPO), and is the rate-limiting step for steroidogenesis. Cholesterol is metabolized by the enzyme P450 side-chain cleavage enzyme (CYP11A1) to pregnenolone in the mitochondria (Zirkin & Chen, 2000). Pregnenolone is converted to progesterone by mitochondrial or microsomal 3β-hydroxysteroid dehydrogenases (HSD3B). Progesterone in smooth endoplasmic reticulum is converted to androstenedione by 17α-hydroxylase/C17–20-lyase (CYP17A1). Androstenedione is metabolized by HSD3B to testosterone (Zirkin & Chen, 2000). Less direct routes with additional intermediates exist for testosterone synthesis in Leydig cells.

TSPO, previously known as the peripheral benzodiazepine receptor (PBR), is found on the OMM of mitochondria in steroidogenic tissue, organs such as lungs, liver, and kidneys, and in the central nervous system (Austin et al., 2013). In steroidogenic tissue, TSPO releases cholesterol into the IMM. While the exact mechanism is not completely understood, ligand-induced stabilization of TSPO is thought to result in the release of cholesterol (Scarf & Kassiou, 2011). Exogenous administration of synthetic ligands (FGIN-1-27 and Ro5-4864) that bind to TSPO have been shown to upregulate

testosterone synthesis in primary Leydig cells isolated from Brown Norway rats (Chung et al., 2013). FGIN-1-27 was also shown to increase circulating testosterone concentrations when administered to both young and old Brown Norway rats. This has important clinical implications for hypogonadal men because direct administration of testosterone can result in infertility in men (Crosnoe et al., 2013). Testosterone negatively regulates itself by acting on LH-producing gonatroph cells in the anterior pituitary gland. High levels of testosterone in the serum from exogenous administration shuts down LH synthesis in the gonatroph cells. Without stimulation from LH, testosterone synthesis stops in Leydig cells in the testes, and this can result in the production of non-viable sperm cells (Crosnoe et al., 2013). TSPO ligands therefore constitute a promising potential therapy for hypogonadism, as the testosterone is then produced endogenously by the Leydig cells. Alongside its effects on testosterone, TSPO ligands have been shown to have anti-inflammatory effects in non-reproductive tissues (Zhao et al., 2011). If TSPO ligands significantly increase testosterone production in the testes of old male mice, it could possibly serve as an effective model for studying the effects of testosterone on the immune response to influenza, while also studying the therapeutic potential of TSPO ligands.

Using either exogenous or endogenous models for elevating testosterone in old male mice, we sought to test the hypothesis that elevation of circulating testosterone concentrations in old, hypogonadal males may reduce the detrimental effects of influenza infection by decreasing pulmonary pathology, and promoting survival of old male mice following infection.

METHODS

Animals

Young (8-10 weeks of age) and old (17-18 months of age) male C57BL/6 mice were obtained from Charles River and the NIA respectively, and housed up to 5 per microisolator cage under standard BSL-2 housing condition with food and water ad libitum. Young male mice were surgically gonadectomized. All animal procedures were approved by the Johns Hopkins University Animal Care and Use Committee (ACUC) under animal protocol M012H270 and performed in compliance with the National Research Council's guide to Care and Use of Laboratory Animals. Experiments were conducted as a series of replicates and animal numbers varied and are provided in the legends.

Virus Infection and Quantification

Mouse-adapted influenza A virus, A/California/4/09/H1N1 (ma2009;H1N1) was generated, and kindly provided by Dr. Daniel Perez at University of Maryland, College Park (Ye et al., 2010). Mice were anesthetized by intramuscular injection of ketamine/xylazine cocktail, then intranasally inoculated with 30μl of Dulbecco's Modified Eagle Media (DMEM) for the mock-infection, or ma2009 diluted in DMEM ($TCID_{50}$=2) (Lorenzo et al., 2011). For virus quantification, log_{10} dilutions of lung homogenates were plated onto a monolayer of Madin-Darby Canine Kidney (MDCK)

cells in replicates of 6 for five days. Cells were stained with naphthol blue black (Sigma Aldrich) and scored for cytopathic effects (CPE). The 50% tissue culture infectious dose ($TCID_{50}$) was calculated according to the Reed-Muench method (Reed & Muench, 1938), and was used to back titer all inoculums.

TSPO Ligand Administration

Mice were intraperitoneally injected daily, with one of two compounds: PK 11195: 1-(2-chloro-phenyl)-N-methyl-N-(l-methylpropyl)-1-isoquinoline carboxamide (Sigma), or Ro5-4864: 7-chloro-5-(4-chlorophenyl)-1,3-dihydro-1-methyl-2H-1,4-benzodiazepin-2-one (Sigma). Each experimental group was injected with either 3 mg/kg or 0.3 mg/kg of either PK 11195 or Ro5-4864 dissolved in 10% dimethylsulphoxide (DMSO) and 90% PBS with Ca^{+} and Mg^{+}. The control group was injected with the vehicle solution alone.

Testosterone Administration

Testosterone (T) was administered by subcutaneously implanting a silastic capsule (0.040 inch inner diameter id, 0.085 inch outer diameter, 12.5 mm and 20 mm length for 7.5 mm T capsules and 15 mm T capsules respectively) between the scapulae, containing 100% crystalline testosterone propionate (Sigma) as previously described (Hetzler et al., 2008). The capsules were enclosed by 2.5 mm of medical adhesive on both ends, and equilibrated in sterile physiological saline. The 7.5 mm T capsules have been shown to produce serum testosterone levels approximating the higher end of the

physiological range in young male C57BL/6 mice for at least 28 days post implantation (unpublished data). Animals in the untreated group were similarly anesthetized and received implants of blank capsules.

Sample Collection

Body mass was recorded daily for 14 days in the ligand pilot study, and body mass, and body temperature were recorded daily for 21 days and clinical scores were recorded at various time points in the influenza morbidity studies. Clinical scores were adapted from the SHIRPA primary screen, and morbidity from influenza in the mice were assigned a total of scores of 0 or 1 for hyperapnea, piloerection, hunched, and no escape, and 5 for death. In the ligand pilot study, serum was collected on days 3 and 7, and stored at -80°C until they were thawed for serum testosterone measurement by radioimmunoassay as previously described. The mice were euthanized on day 7, and seminal vesicles and testes were collected to weigh, and to weigh and measure testicular testosterone, respectively. In the morbidity studies, mock and influenza-infected males were euthanized at one of several days post-infection (dpi), at which time, serum was collected to measure antibody titers, and whole lungs were either snap-frozen and stored at -80°C to measure viral titers, or inflated with Z-fix to observe inflammation by immunohistochemistry as described below.

Immunohistochemistry and Staining

Lungs were inflated, fixed in Z-fix, embedded in paraffin, cut into 5 μm sections,

and mounted on glass slides. Slides were deparafinized with xylene and rehydrated in graded ethanol. Heat-induced antigen retrieval with citrate buffer was performed and slides were blocked with 10% normal goat serum prior to overnight incubation with the primary antibody in a humidified chamber. The slides were then treated with 3% hydrogen peroxide to block endogenous peroxidase activity. Detection of the primary antibody signal was done using the EXPOSE rabbit specific HRP/DAB detection kit (abcam). Primary antibodies included: rabbit anti-Ki67 (Abcam), anti-IAV NA antibody. Images were taken using a Nikon Eclipse E800 and analyzed using ImageJ (NIH).

Anti-influenza total IgG ELISA

ELISA plates (96 well, company here) were coated overnight at 4°C with 100 ng of purified ma2009 H1N1, after which plates were washed and blocked for 1 h with blocking solution (10% dry skim milk powder in PBS). Plates were washed, duplicate diluted serum samples were added in a 2-fold series starting at 1:1000, and plates were incubated at 37°C for 1 h. Anti-mouse IgG secondary antibody (1:5000; Peroxidase AffiniPure Goat Anti-mouse IgG; Jackson Immunoresearch Laboratories) was added and plates were incubated for 1 h at 37°C. Reactions were developed with 3,3',5,5' tetramethylbenzidine (TMB) and stopped using 1N HCL. Plates were read at 450 nm absorbance on a plate reader. To determine the antibody titer, a cutoff value was determined by multiplying the average ELISA values of serum from naïve animals at each dilution by 3. The sample ELISA titer was the highest serum dilution of that sample series with a value above the cutoff. A sample was considered positive only if the

average OD was 3 times higher than the corresponding dilution value of naïve serum.

Statistical Analyses

Morbidity data were analyzed with a multivariate analysis of variance (MANOVA) with one within-subjects variable (days) and one between-subjects variable (treatment) and significant interactions were further analyzed using planned comparisons. Serum proteins, organ weights, virus titers, and IHC data were analyzed using one-way ANOVA or t tests, significant interactions were further analyzed using the Tukey method for pairwise multiple comparisons. Mean differences were considered statistically significant if $p<0.05$.

RESULTS

Administration of testosterone results in reduced morbidity and antibody titers in young male mice following infection with influenza A virus

Young male C57BL/6 mice were gonadectomized and implanted with placebo capsules or 7.5 mm testosterone propionate capsules, which elevate serum testosterone to the upper limit of the physiological range observed in intact young male mice (Fig. 1A). Following infection with ma2009 H1N1 influenza A virus, testosterone-treated mice showed reduced percentage loss of body mass (Fig. 1B), and had a lower clinical disease score of observable symptoms (Fig. 1C) compared to placebo-treated mice. Antibody titers in serum samples collected at day 21 post infection were lower for the testosterone-treated mice compared to the placebo-treated mice (Fig. 1D). These data suggest that treatment with testosterone affects the outcome of influenza, at least in young male mice.

Administration of the TSPO Ligands Ro5-4864 and PK11195 does not increase testosterone concentrations in old male mice

Testosterone concentrations decline with age, even in mice (Coquelin & Desjardins, 1982). To test the hypothesis that endogenous testosterone levels can be increased in old male mice, old male C57BL/6 mice were treated with high (3 mg/kg) or low (0.3 mg/kg) doses of Ro5-4864 or PK11195, or vehicle. The mice were weighed daily for the seven day duration of the treatment to determine if TSPO ligands cause notable

toxicity. Treatment with TSPO ligand did not change body mass relative to vehicle-treated males (Fig. 2A). Mice treated with the low dose (0.3 mg/kg) of either TSPO ligand (Ro5-4864 and PK11195) showed an increase in seminal vesicle mass relative to those treated with the high doses (3 mg/kg) of TSPO ligands or vehicle for 7 days (Fig. 2B). Despite the observed changes in seminal vesicle mass, which is androgen-dependent, there was no significant increase in serum testosterone concentrations after treatment with either low or high doses of either TSPO ligand after either 3 or 7 days of treatment (Fig. 2C,D). Similarly, testicular production of testosterone was not significantly elevated by treatment with TSPO ligands (Fig. 2E). These data suggest that TSPO ligands, at least at the tested doses, are not sufficient to elevate testosterone in old male mice.

Administration of low dose testosterone does not alter the outcome of infection with ma2009 H1N1 in old male mice

To test the hypothesis that exogenous elevation of testosterone in old males will improve influenza pathogenesis, old male C57BL/6 mice were left intact and implanted with placebo capsules or 7.5 mm (low dose) testosterone propionate capsules, which elevate serum testosterone to the upper limit of the physiological range observed in intact young male mice (Fig. 3A). Following infection with ma2009 H1N1, low dose testosterone-treated mice showed no change in percentage loss of body mass (Fig. 3B) or survival (Fig. 3C) compared to placebo-treated mice. The average day of death after

infection was slightly later for the testosterone-treated (15±3.00) compared with placebo-treated (11.7±1.20) mice. Antibody titers in serum samples collected at day 28 post infection were similarly low between the low dose testosterone-treated mice and the placebo-treated mice (Fig. 3D). These data suggest that old male mice may be less responsive to testosterone treatment than their young male counterparts, at least with regard to influenza virus infection.

Administration of high dose testosterone improves the outcome of infection with ma2009 H1N1 in old male mice

To test the hypothesis that old male mice require higher doses of testosterone to alter the outcome of influenza infection, old male C57BL/6 mice were left intact and implanted with placebo capsules or 15 mm (high dose) testosterone propionate capsules. Serum testosterone concentrations were not significantly increased at 21 days after infection (28 days after implantation) (Fig. 4A). Following infection with ma2009 H1N1 influenza A virus, high dose testosterone-treated mice showed reduced percentage loss of body mass (Fig. 4B), but similar survival (Fig. 4C) compared to placebo-treated mice. The average day of death after infection was slightly later for the testosterone-treated (13.8±2.03) compared with placebo-treated mice (12±0.68). Antibody titers in serum samples collected at day 21 post infection were similarly low between the high dose testosterone-treated mice and the placebo-treated mice (Fig.

4D). These data suggest that testosterone treatment can improve outcome measures of influenza virus infection.

Protection from influenza pathogenesis is not associated with increased proliferation in the lungs at 21 days post infection

Testosterone (T) treatment in young male mice (with 7.5 mm T capsules), and old male mice (with 15 mm T capsules) was protective against morbidity from influenza virus infection. To test the hypothesis that this was due to increased repair in the testosterone-treated mice, we measured the expression of Ki67, which is a marker for cellular proliferation, in the lungs at 21 days after infection. There was no significant difference in Ki67 expression between placebo-treated and testosterone-treated young male mice, and between placebo-treated and high dose testosterone-treated old male mice (Fig. 5B). These data suggest that there is either no effect of testosterone on repair, or the effect can be better detected at an earlier time point.

Figure 1

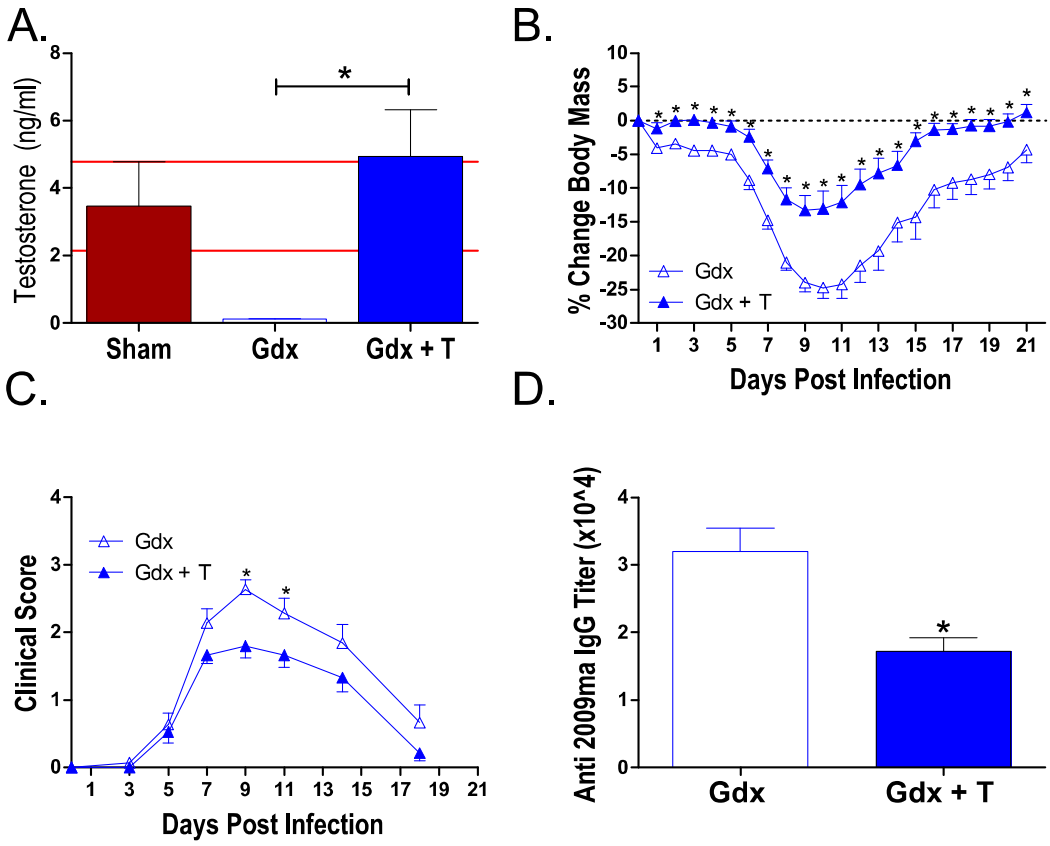


Figure 1: Effects of testosterone (T) on the outcome of ma2009 H1N1 influenza infection in young male mice (2 mo). Young male mice were gonadectomized (gdx) and implanted with 7.5 mm T (n=15) or placebo (n=14) capsules. Serum T was measured by RIA (A), body mass was measured daily (B), and clinical scores were measured on days 0, 3, 5, 7, 9, 11, 14, and 18 post infection (C). Antibody titers were analyzed 21 days post infection by ELISA (D). Data shown are the mean ± SEM. Asterisks (*) denote $p<0.05$. Red lines in panel A represent physiological serum T range in young male mice.

Figure 2

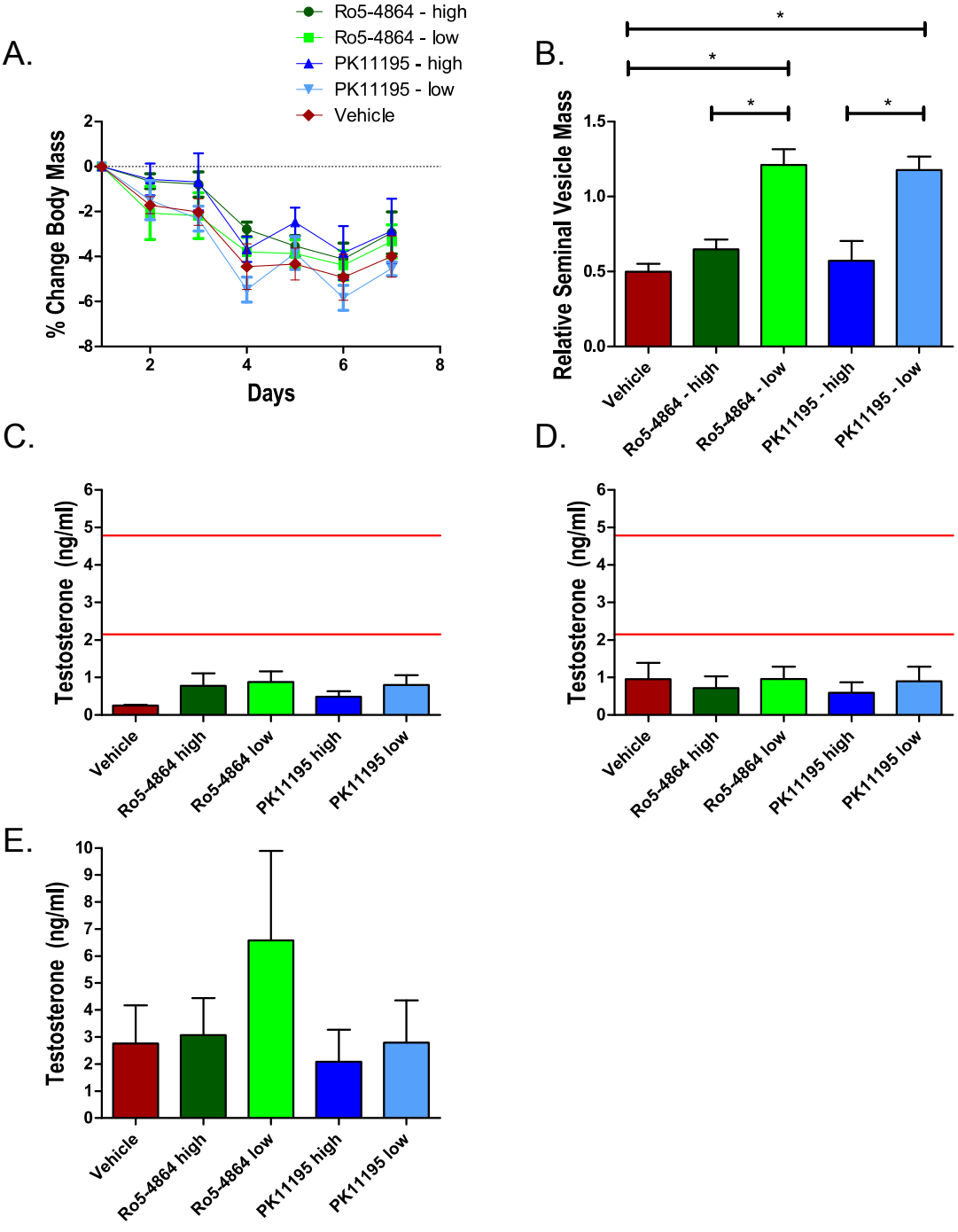

A.
Ro5-4864 - high
Ro5-4864 - low
PK11195 - high
PK11195 - low
Vehicle
% Change Body Mass
Days
B.
Relative Seminal Vesicle Mass
Vehicle
Ro5-4864 - high
Ro5-4864 - low
PK11195 - high
PK11195 - low
C.
Testosterone (ng/ml)
Vehicle
Ro5-4864 high
Ro5-4864 low
PK11195 high
PK11195 low
D.
Testosterone (ng/ml)
Vehicle
Ro5-4864 high
Ro5-4864 low
PK11195 high
PK11195 low
E.
Testosterone (ng/ml)
Vehicle
Ro5-4864 high
Ro5-4864 low
PK11195 high
PK11195 low

Figure 2: Effects of Ro5-4864 and PK11195 on body mass, and serum and testicular testosterone (T) in old male mice. Old male mice received either Ro5-4864 or PK11195 [low (L): 0.3 mg/kg body mass; high (H): 3 mg/kg body mass] or placebo by ip injection for 7 days (n=5 in each group). Body mass was measured daily over the course of the seven day study (A). Seminal vesicle mass was measured after 7 days, and data is presented as a percentage of total body mass of each mouse (B). Serum T at days 3 (C) and 7 (D), and testicular T (D) were measured by RIA. Data shown are the mean ± SEM. *P < 0.05. Red lines in panel C and D represent physiological serum testosterone range in young male mice.

Figure 3

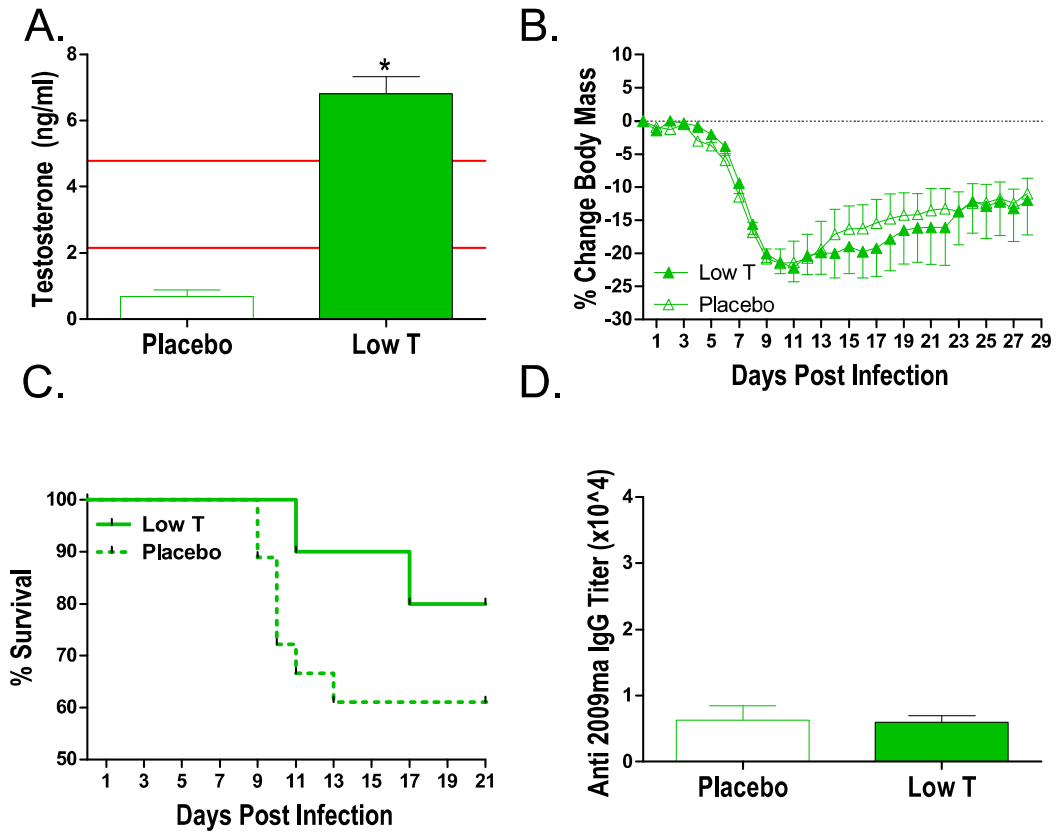


Figure 3: Effects of low dose testosterone (low T) on the outcome of ma2009 H1N1 influenza infection in old male mice (17 mo). Old male mice were implanted with low T (n=10) or placebo capsules (n=9). Serum T was measured by RIA (A), body mass was measured daily (B), and survival was assessed using the Kaplan-Meier method (C). Antibody titers were analyzed 28 days post infection by ELISA (D). Data shown are the

mean ± SEM. Asterisks (*) denote $p<0.05$. Red lines in panel A represent physiological serum T range in young male mice.

Figure 4

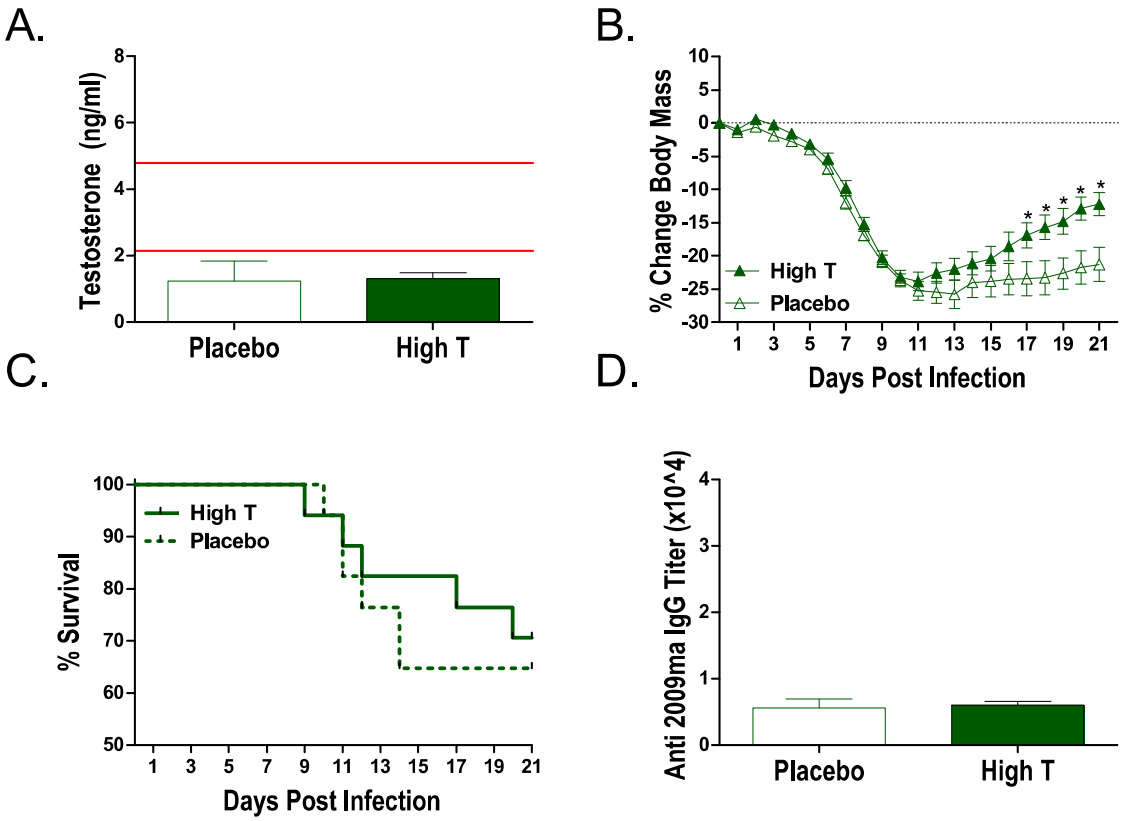
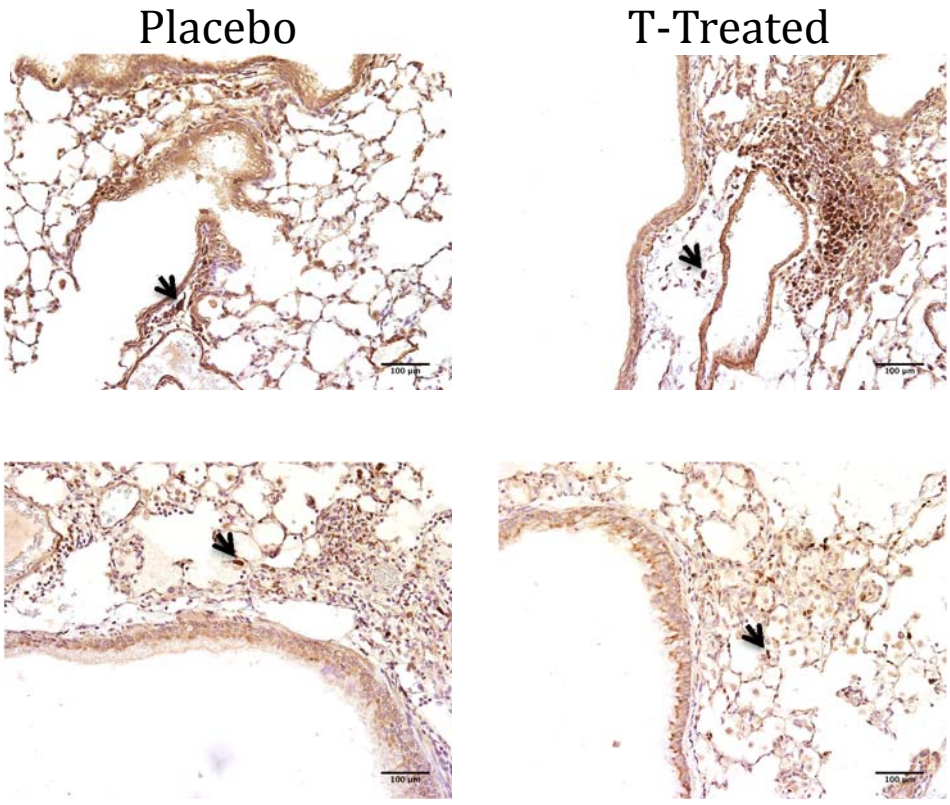


Figure 4: Effects of high dose testosterone (high T) on the outcome of ma2009 H1N1 influenza infection in old male mice (17 mo). Old male mice were implanted with high T or placebo capsules (n=17 in each group). Serum T was measured by RIA (A), body mass was measured daily (B), and survival was assessed using the Kaplan-Meier method (C). Antibody titers were analyzed at 21 days post infection by ELISA (D). Data shown are the mean ± SEM. Asterisks (*) denote $p<0.05$. Red lines in panel A represent physiological serum T range in young male mice.

Figure 5

A.


Placebo
T-Treated
Young
Old

B.

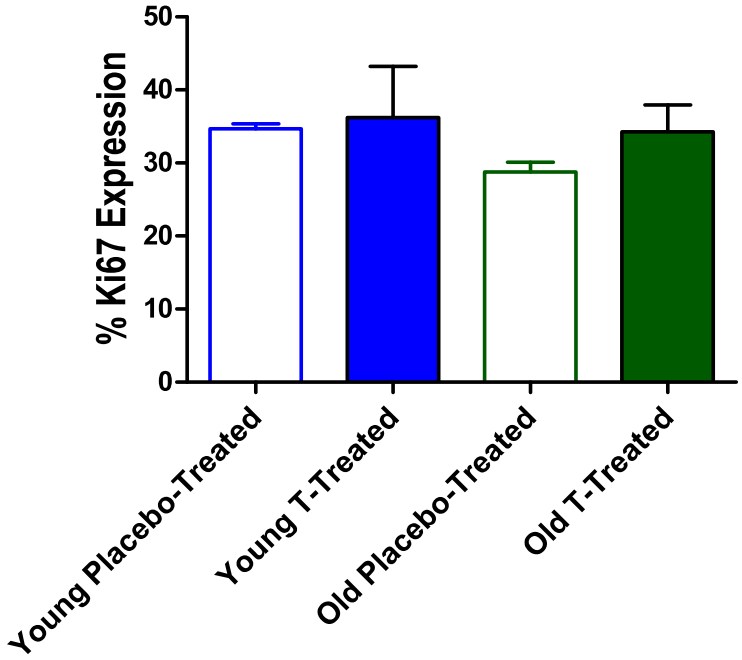

% Ki67 Expression
50
40
30
20
10
0
Young Placebo-Treated
Young T-Treated
Old Placebo-Treated
Old T-Treated

Figure 5: Effects of testosterone treatment on ki67 expression 21 days post infection with ma2009 H1N1 influenza in young and old male mice. Paraffin-embedded lungs were deparafinized and stained with anti-Ki67 antibody and DAB substrate (brown) and counterstained with hematoxylin (purple). Representative images are provided for lungs from young and old placebo and testosterone-treated male mice (A). Percentage of Ki67+ nuclei was calculated in lungs tissue from young and old placebo and testosterone-treated male mice (n=3 in each group) (B). Arrows indicate Ki67 positive staining.

DISCUSSION

Conclusions

Age-related changes in immune function result in more severe disease from influenza infection in the elderly (Parzych et al., 2013). Because influenza disease pathology is primarily mediated by a dysregulated inflammatory response (Damjanovic et al., 2012), this is possibly influenced by the chronic pro-inflammatory state associated with aging, which results in higher basal levels of pro-inflammatory cytokines and may contribute to more severe disease in the elderly (Cannizzo et al., 2011). Compared to elderly females, as well as young males, elderly males are more susceptible to severe disease (Serfling et al., 1967). Testosterone has been shown to have anti-inflammatory properties in various models (Keith et al., 2013; Lotter et al., 2013), and decreasing levels of testosterone with age in men (Maggio et al., 2005) could contribute to the increased severity of disease in elderly men. Therefore, we hypothesized that testosterone administration would be protective against influenza disease in our mouse model of influenza by modulating the inflammatory response to infection.

We assessed the effects of testosterone in young male mice to confirm that testosterone affects influenza pathogenesis. Administration of testosterone (7.5 mm) and restoration of serum testosterone to the upper end of physiological levels was protective against morbidity from infection with ma2009 H1N1 influenza A virus in gonadectomized young male mice. These results are consistent with reports in the

literature of the anti-inflammatory properties of testosterone (Keith et al., 2013; Lotter et al., 2013), as well as previous research from the lab using maPR8 H1N1 influenza virus, which showed that gonadectomized young male mice showed lower survival than intact young male mice in response to a lethal dose of virus without observing any differences in viral titers (Robinson et al., 2011). Antibody titers were significantly lower in the serum collected at day 21 from the testosterone-treated mice, which further points towards an immunosuppressive role for testosterone. This replicates findings in a study conducted with human populations that showed that men with higher testosterone levels produce a lower antibody response in response to a trivalent inactivated influenza vaccine compared to men with lower testosterone (Furman et al. 2014).

Old male mice have lower testosterone levels as compared with younger male mice (Coquelin & Desjardins, 1982). In order to determine whether testosterone is protective in old male mice, we first looked at a means of raising testosterone levels endogenously, as that could be an alternative to directly administering testosterone to elderly males. TSPO ligands have been shown to raise testosterone to physiological levels in old male rats by stimulating steroidogenesis in the testes (Chung et al., 2013). We hypothesized that the same would be true in old male mice. However, administration of two different TSPO ligands, Ro5-4864 and PK11195, in high or low doses did not significantly raise either testicular or serum testosterone levels in old male C57BL/6 mice. While the trending association between low dose TSPO ligand and higher serum testosterone may reach statistical significance with a larger sample, none of the

groups showed testosterone elevated to within the physiological range of testosterone observed in healthy young mice. However, the mice treated with low doses of the ligands showed a significant increase in seminal vesicle mass, providing some evidence that these TSPO ligands have a greater physiological effect at low than high doses. The dose response of the drug does not appear to be linear, therefore it is difficult to determine whether a higher or lower dose of TSPO ligand would increase testosterone to a greater extent. A possible reason for TSPO ligands raising testosterone levels in rats but not in mice may be differences in binding with the TSPO protein. We therefore decided that TSPO ligand would not serve as an effective model to study the effects of testosterone on influenza pathogenesis in old male C57BL/6 mice. For that, we reverted to our model of testosterone replacement using capsules as in young male mice.

Aging results in an overall decline in immune function (Castelo-Branco & Soveral, 2014), as well as an increase in the basal levels of pro-inflammatory cytokines (Cannizzo et al., 2011). Whether age-related changes in immune function could be reversed by treatment with testosterone was tested. In contrast to the data from young male mice showing that lower testosterone is associated with higher antibody titers, older male mice produced lower antibody titers than younger male mice, regardless of whether they were treated with testosterone. This suggests that vaccines would not be as effective in elderly individuals, and this is consistent with studies conducted on influenza vaccine efficacy in humans (Sasaki et al., 2011). Decreased antibody responses resulting from both aging and testosterone administration in young male mice indicate the need for tailoring vaccinations to specific population subsets to ensure sufficient protection.

Administration of low dose testosterone, despite elevating serum testosterone to the upper end of physiological levels seen in young male mice, was not protective against morbidity or survival from infection with ma2009 H1N1 influenza A virus in old male mice. This is possibly a result of the chronic pro-inflammatory state associated with aging (Cannizzo et al., 2011), which may not be counteracted sufficiently by the same dose of testosterone that was protective in young male mice. It is also possible that aging results in a decrease in androgen receptor expression, which would limit the effects of testosterone. Administration of high dose testosterone was protective against morbidity but not survival from infection with ma2009 H1N1 influenza A virus in old male C57BL/6 mice. However, testosterone levels in the high dose testosterone-treated mice had been depleted to levels comparable to placebo-treated mice 28 days after implantation (21 days post infection). We hypothesize that this is because testosterone in the high dose capsules diffused out at a faster rate as a result of an increased surface area compared to that of the low dose capsules. The protection against morbidity from influenza pathogenesis in the high dose testosterone-treated group despite the early depletion of the hormone in the serum suggests that testosterone may be having an early effect on the immune response to the virus, and resulting in downstream protective effects. This is consistent with a study showing that testosterone downregulates the expression of toll-like receptor 4 (TLR-4) in macrophages in mice; TLR-4 is a key trigger for inflammation and innate immunity. Both doses of testosterone resulted in a trend of delay in the average day of death, again suggesting a protective effect.

None of the treatment groups of old male mice fully recovered to baseline body mass after infection, which is consistent with evidence in the literature suggesting that aging results in decreased recovery from influenza infection (Yin et al., 2014). However, high dose testosterone resulted in significantly improved recovery, which suggests that testosterone is one of several factors that can influence the outcome of influenza disease in old male mice, and that the higher dose of testosterone is sufficient in counteracting some of the excessive inflammation that is associated with the disease. Testosterone has been shown to accelerate repair in other models of injury (Hetzler et al., 2008). We therefore hypothesized that decreased morbidity from influenza virus infection in testosterone-treated young male mice and high dose testosterone-treated old male mice might partially be influenced by increased repair in the lungs after infection. However, our data from 21 days after infection does not suggest that is the case. It may possibly be more useful to look at ki67 at earlier time points during and before peak of disease according to the morbidity curves, in order to more accurately assess a role for repair in the protective effect of testosterone against morbidity from influenza virus infection.

Future Directions

The lower dose of testosterone, despite increasing serum testosterone levels in old male mice to the upper levels of the physiological range of testosterone seen in young male mice, did not have the same protective effect against morbidity as in the

young male mice. To test whether this is because of a decrease in androgen receptor (AR) expression with age, we will measure AR expression in young and old male mice with and without testosterone treatment.

The depletion of testosterone at 21 days post infection is a limitation to the interpretation of this data as maintaining a constant environment of elevated testosterone is important to our hypothesis. To have a more concrete understanding of what part of the response to influenza virus infection is being affected by testosterone, we will determine the time after implantation of the high dose testosterone capsules at which serum testosterone peaks and how long it takes to be depleted after that. We will collect serum at various time point post infection, and measure testosterone.

We hypothesized that the protection from influenza pathogenesis in testosterone-treated young male mice and high dose testosterone-treated old male mice is possibly a repair of accelerated repair in the lungs, as testosterone has been shown to upregulate repair in other models of injury (Hetzler et al., 2008). At 21 days after infection, we do not see a difference in expression of Ki67, which is a marker for proliferation, between lungs from placebo-treated and testosterone-treated mice among both young and old age groups. As day 21 is after peak infection and viral clearance (Robinson et al., 2011), it is possible that it is too late to detect a difference at this point. We will therefore measure Ki67 expression in lungs at days 7 and 14 post infection to detect a possible difference in epithelial repair at earlier time points during the infection.

Public Health Significance

Influenza is a global public health burden. Seasonal influenza is a significant cause of mortality every year (Lagace-Wiens et al., 2010). Owing to the mutation-prone nature of its RNA genome (Lauring et al., 2013), new vaccines need to be researched and manufactured every year to combat the spread of the virus in the population. While seasonal strains are not always severe, influenza can result in hospitalizations and death, especially in at-risk populations, including the elderly (Mauskopf et al., 2013). Elderly men, in particular, tend to be more susceptible to severe disease compared to elderly women and young men (Serfling et al., 1967). This, combined with the fact that elderly men produce lower antibody titers than elderly women in response to influenza vaccination makes influenza a serious threat for elderly men (Sasaki et al., 2011).

Low testosterone and its accompanying effects are also a significant burden among elderly men (Maggio et al., 2005). It is possibly associated with the increased severity of influenza in elderly men, as testosterone has been shown to have anti-inflammatory properties (Lotter et al., 2013), and severe pathology from influenza is known to be caused by an excessive inflammatory response to infection (Damjanovic et al., 2012). We therefore hypothesized that testosterone administration may provide some protection for elderly men against influenza pathogenesis.

We utilized old male mice with low testosterone as our model for elderly men in the population. Our results suggest that testosterone could be useful in decreasing morbidity from influenza, but further research is required to elucidate the mechanisms

of this protection, and to determine any possible side effects of immunomodulation by testosterone.

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Undergraduate Research. Dr. David Gilbert's laboratory at Florida State University (January 2012-May 2013). Principal responsibilities included BAC and plasmid purification, molecular cloning, and genetic recombineering

Sc.M. Research. Dr. Sabra Klein's laboratory at Johns Hopkins Bloomberg School of Public Health (September 2014-present). Principal responsibilities include conducting animal studies, including infection, dissection and tissue collection as well as assays including virus titration, ELISAs, virus neutralization, radioimmunoassays, and immunohistochemistry

Volunteer Experience:

UMAR Boxing. Tutored, and organized volunteering trips for children in an after-school program (January 2014-May 2014)

Art With a Heart. Co-instructed a summer art class for middle-school children across five schools in Baltimore (June 2014-August 2014)

Presentations:

2012 Undergraduate Research and Creative Activities Awards Symposium. "Is Late Replication Necessary for G9a-Mediated Methylation to Occur?"

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