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Antibody responses and cross protection against lethal influenza A viruses differ between the sexes in C57BL/6 Mice

Maria E. Lorenzo^{1,3,*}, Andrea Hodgson^{1,*}, Dionne P. Robinson¹, Jenifer B. Kaplan¹, Andrew Pekosz¹, and Sabra L. Klein^{1,2}

¹The W. Harry Feinstone Department of Molecular Microbiology and Immunology, The Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland ²Department of Biochemistry and Molecular Biology, The Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland ³Department of Molecular and Comparative Pathobiology, The Johns Hopkins School of Medicine, Baltimore, Maryland

Abstract

A mouse model was used to determine if protective immunity to influenza A virus infection differs between the sexes. The median lethal dose of H1N1 or H3N2 was lower for naïve females than males. After a sublethal, primary infection with H1N1 or H3N2, females and males showed a similar transient morbidity, but females generated more neutralizing and total anti-influenza A virus antibodies. Immunized males and females showed similar protection against secondary challenge with a homologous virus, but males experienced greater morbidity and had higher lung viral titers after infection with a lethal dose of heterologous virus. Females develop stronger humoral immune responses and greater cross protection against heterosubtypic virus challenge.

Keywords

humoral immunity; influenza vaccine; sex difference

1. Introduction

Males and females of species ranging from humans to rodents differ in their responses to viruses, including human immunodeficiency virus (HIV), herpes simplex viruses, hantaviruses, influenza viruses, measles virus, coxsackievirus, and West Nile virus [1]. Although behavioral factors can influence exposure to viruses, genetic as well as physiological differences between the sexes can contribute to differential immune responses to infection [2]. Increased immune responses can lead to more efficient control of virus replication but also can result in increased disease caused by development of

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Address correspondence to: Sabra Klein, Department of Molecular Microbiology and Immunology, The Johns Hopkins Bloomberg School of Public Health, 615 North Wolfe Street, Baltimore, Maryland 21205, Phone: (410) 955-8898, Fax: (410) 955-0105, saklein@jhsph.edu.

*joint first authorship

Current address: Jenifer Kaplan, Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

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immunopathology [3]. Sex differences in immune function are partly mediated by circulating sex steroid hormones [1, 4].

Sex differences in the incidence of influenza have been documented in humans [5–7]. For example, disease and case fatality rates are higher among women than men following exposure to highly pathogenic H5N1 avian influenza [8–11]. More recently, sex differences were reported in hospitalizations associated with exposure to pandemic 2009 H1N1 infection, in which women appear to experience worse disease outcome than men [12–16]. Pregnancy and differences in the presentation of various risk factors contribute to worse outcome following 2009 H1N1 infection in women than men [17]. Whether immune responses to influenza virus infection differ between men and women has not been reported.

Rates of immunization against influenza are reportedly either similar between the sexes or lower in women [18–22] and may be influenced by greater negative beliefs about the risks of vaccination [23] and lower acceptance of vaccines [24, 25] among women. Antibody responses to the seasonal trivalent inactivated vaccines (TIV) are higher in women than men [26–29]. Whether antibody responses to the live attenuated influenza vaccine differ between the sexes has not been reported, to date. Women also report more frequent and severe local and systemic reactions to the seasonal TIV than men [28, 30, 31]. The mechanisms mediating sex differences in antibody responses and adverse side effects following vaccination have not been thoroughly investigated. It also is not clear if stronger antibody responses in women confer greater protection from influenza.

Small animal models have been instrumental in defining protective immunity against influenza A virus infection [32]. Immunization with inactivated influenza vaccines primarily results in subtype-specific immunity that is mediated by antibodies generated against the hemagglutinin (HA) and neuraminidase (NA) proteins that neutralize and assist with clearance of virus [33, 34]. In contrast, recovery from natural infection or from inoculation with live influenza viruses induces cross protective immunity against different subtypes of influenza viruses (i.e., heterosubtypic immunity) that is thought to be primarily mediated by T cells that recognize conserved epitopes of internal proteins, including nucleoprotein (NP) and matrix (M) [35–37]. The importance of heterosubtypic immunity in human populations is debated, but evidence suggests that cross protective immune responses, including neutralizing antibodies and T cells, exist and may be important for protection against new circulating strains of influenza A viruses, such as pandemic 2009 H1N1 [38–40] and avian H5N1 influenza virus [41, 42]. In rodent models, primary infection with a sublethal dose of influenza A virus induces cross-protective immunity against lethal infection with a heterosubtypic virus strain [43–48]. To date, a majority of research into the mechanisms of cross protective immunity against lethal influenza A viruses has been conducted using female BALB/c or C57BL/6 mice [32, 33, 43–45, 49–53]. There are few studies detailing heterosubtypic immunity in males [54], but many studies that either do not report the sex of the mice or combine the responses of males and females [55–60]. We report that females mount higher immune responses to primary influenza A virus infection and are better protected against a subsequent heterosubtypic lethal virus challenge than males.

2. Materials and Methods

2.1. Animals

Adult male and female C57BL/6 mice (7–9 weeks of age; 19–24 g starting body mass) were purchased from NCI Frederick. Mice were maintained 5/microisolator cage under standard housing conditions with a 14:10 light:dark cycle and *ad libitum* access to food and water. All experiments were approved by the JHU Animal Care and Use Committee and conducted using BSL-2 practices and procedures.

2.2. Viruses

The mouse-adapted influenza A/Puerto Rico/8/34 (PR8; H1N1; courtesy of Maryna C. Eichelberger) and A/Hong Kong/68 (HK68; H3N2; courtesy of Innocent N. Mbawuike) strains were used for all animal infections.

2.3 Virus Purification

Viruses were grown in Madin-Darby canine kidney (MDCK) cells at 37°C in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 100 U/ml of penicillin, 100 µg/ml of streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.5% BSA and 5 µg/ml of acetyltrypsin (infection media). Viruses were pelleted by centrifugation over a 20% sucrose in PBS solution in a Sorvall TH641 rotor at 118,000 g for 1 h at 4°C. The pelleted virus was resuspended in PBS and stored at -80°C.

2.4. Mouse LD₅₀ Experiments

Male and female mice (n = 10/sex/dose) were anesthetized with ketamine-xylazine (80mg/kg and 8mg/kg respectively, intramuscular) and inoculated intranasally (in) with one of 5 log₁₀ dilutions of H1N1 or H3N2 virus in DMEM. Body mass, rectal temperature, and mortality were monitored for 21 days. The median lethal dose (LD₅₀) was calculated by the Reed-Muench method.

2.5. Immunization and Lethal Challenge

Male and female mice (n = 5–10/sex/experimental group) were anesthetized with ketamine-xylazine and inoculated with a sublethal dose of H1N1 (10¹ TCID₅₀/mouse) or H3N2 (10¹ or 10² TCID₅₀/mouse) in DMEM. Body mass, rectal temperature, and mortality were monitored daily for 21 days. Mice were bled from the retro orbital sinus under inhalant anesthesia (isoflurane, induction dose 3–4%), as specified for each experiment. Serum was heat inactivated at 56°C for 30 min and stored at -80°C. Animals were challenged with a lethal dose of influenza virus (10⁴ TCID₅₀/mouse of H1N1 or 10⁵ TCID₅₀/mouse of H3N2 in DMEM) under ketamine-xylazine anesthesia, as specified for each experiment. Morbidity and mortality data were collected daily for an additional 21 days or animals were euthanized for tissue collection at specified time points post-lethal challenge.

2.6. Antibody Neutralization Assay

Serum was added to infection media and serially diluted (1:2 dilutions). One hundred TCID₅₀ units of virus were added to the diluted samples and incubated at room temperature for 1 h. The diluted samples and virus were added to MDCK cells at 100% confluence in 96 well plates and incubated overnight at 37°C. After 16–18 h of incubation, the inoculums were removed, the cells were washed with PBS, fresh infection media was added, and the cells were incubated for 4 days for H3N2 or 5 days for H1N1. Cytopathic effect (CPE) was scored following staining with naphthol blue-black. Each sample dilution series was run in quadruplicate and the titer was calculated as the highest serum dilution that eliminated virus CPE in 2 out of 4 wells per dilution. A sample was considered negative if there was no neutralization at the 1:4 dilution of serum.

2.7. Anti-influenza total IgG ELISA

ELISA plates (Microtiter 96 well high binding plates; Greiner Bio-One) were coated overnight at 4°C with 100 ng of purified H1N1 or H3N2, 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.

2.8. Tissue Harvest

Mice were euthanized with an intraperitoneal (ip) overdose of ketamine-xylazine. Tissues were collected days 0, 1, 3, or 5 post-challenge for virus titration. Lung tissues were snap frozen, stored at -80°C until processing, and then homogenized in 1 ml of DMEM. Samples were centrifuged and supernatants were collected and stored at -80°C.

2.9. Virus TCID₅₀

MDCK cells were plated in 96-well plates, grown to confluence, and infected with serial 10-fold dilutions of lung homogenates in infection media. The cells were incubated for 4 days (H3N2) or 5 days (H1N1) at 37°C, stained with naphthol blue black, and CPE was scored visually. The Reed-Muench method was used to calculate the tissue culture infectious dose that caused CPE in 50% of a monolayer of MDCK cells (TCID₅₀).

2.10. Statistical Analyses

Kaplan Meier survival curves were compared using log rank analyses. The proportion of animals that survived influenza A virus infection was compared among experimental groups using chi-square analyses. Morbidity data were analyzed with multivariate ANOVAs (MANOVAs) with one within-subjects variable (days) and one between-subjects variable (sex) and significant interactions were further analyzed using planned comparisons. Antibody responses and virus titers were analyzed with t-tests or 2-way ANOVAs with day post-infection/challenge and sex as the independent variables and significant interactions were further analyzed using the Tukey method for pairwise comparisons. Associations between dependent measures were analyzed with Pearson product moment correlations. Mean differences were considered statistically significant if $p < 0.05$.

3. Results

3.1. Sex differences in morbidity and mortality from primary influenza A virus infection

To determine whether the sexes respond differently to influenza A virus infection, male and female C57BL/6 mice were inoculated with H1N1 or H3N2 using 5 log₁₀ dilutions to determine the LD₅₀ for each sex. For H1N1, the LD₅₀ for females (42 TCID₅₀) was 11-fold lower than the LD₅₀ for males (475 TCID₅₀) ($X^2 p < 0.05$; Figure 1A and B). Similarly for H3N2, the female and male LD₅₀ were 616 TCID₅₀ and 2080 TCID₅₀ respectively, corresponding to an almost 4-fold lower lethal dose for females than males, although this did not reach statistical significance (Figure 1C and D).

Sex differences in morbidity following infection with H1N1 or H3N2 viruses were dose-dependent. Sex differences were apparent after infection with median doses (10^2 or 10^3 TCID₅₀) of H1N1, as females experienced a greater drop in body mass and rectal temperature than males (MANOVA sex $\times$ day $p < 0.05$ in each case; Supplemental Figure 1). At low (10^1 TCID₅₀) or high (10^4 or 10^5 TCID₅₀) doses of H1N1, sex differences in morbidity were not present. For H3N2, which is less lethal than H1N1 in C57BL/6 mice, females inoculated with 10^3 or 10^4 TCID₅₀ showed greater morbidity than their male counterparts (MANOVA sex $\times$ day $p < 0.05$ in each case; Supplemental Figure 2), whereas

the sexes did not differ in response to either low (10^1 or 10^2 TCID₅₀) or high (10^5 TCID₅₀) doses of H3N2. In summary, sex differences are observed in response to infection with H1N1 and H3N2 viruses, with naïve females experiencing a greater reduction of body mass and temperature and increased mortality when compared to males, and this effect is dose-dependent.

3.2. Sex differences in response to homologous virus challenge

To establish whether males and females are differentially protected against a secondary, homologous virus challenge, mice were first inoculated with either H1N1 (10^1 TCID₅₀) or H3N2 (10^2 TCID₅₀). Males and females showed a similar, significant transient drop in body mass and temperature after H1N1 (Figure 2A and B) or H3N2 (Figure 3A and B) infection (MANOVA day main effect $p < 0.05$ in each case). To examine whether males and females mount differential humoral immune responses to sublethal influenza infection, neutralizing and total anti-influenza antibody responses were measured. In response to sublethal H1N1 infection, females generated higher neutralizing antibody responses at 21 and 28 days post-infection (2-way ANOVA sex $\times$ day $p < 0.05$; Figure 4A) and greater anti-H1N1 IgG responses at 14 and 21 days post-infection (2-way ANOVA sex $\times$ day $p < 0.05$; Figure 4B) than males. In response to sublethal H3N2 infection, females produced higher neutralizing antibody and anti-H3N2 IgG responses at 28 days post-infection (2-way ANOVA sex $\times$ day $p < 0.05$ in each case; Figure 4C and 4D). Taken together, these data indicate that sex differences in antibody responses to influenza infection are evident at virus doses that do not demonstrate significant sex differences in morbidity or mortality.

At day 35 post-primary infection, mice were challenged with a lethal dose of homologous virus (10^4 TCID₅₀ of H1N1 or 10^5 TCID₅₀ of H3N2). Males and females responded similarly to the homologous challenge, and no significant sex difference in morbidity was observed (Figures 2C, 2D, 3C, and 3D). Following sublethal infection, both sexes showed protection against infection with homologous virus, despite females having significantly higher total and neutralizing antibody levels than males. This indicates that both sexes reached the threshold of immunity needed for protection against a lethal challenge with homologous virus.

3.3. Sex differences in response to heterosubtypic virus challenge

To examine if there are sex differences in heterosubtypic immunity following a sublethal influenza virus infection, mice were inoculated with 10^1 TCID₅₀ of H1N1 (Figure 5A and B) resulting in a significant decrease in body mass and temperatures in both males and females with a return to baseline by day 17 post infection. Females had a greater loss in body mass compared to males (MANOVA sex $\times$ day $p < 0.05$; Figure 5A), but this was not seen consistently at this virus dose (Supplemental Figure 3A). Sex differences were not observed for body temperature (Figure 5B; Supplemental Figure 3B). Females developed higher neutralizing antibody and total anti-H1N1 IgG responses than males 35 days post-infection (t-test $p < 0.05$ in each case; Figure 5C and D), consistent with previous results (Figure 4A and 4B). Mice were challenged at day 42 post-H1N1 infection with H3N2 (10^5 TCID₅₀) and no sex differences in the percentage change in body mass were noted (Figure 5E); females however, experienced significantly less hypothermia than males (MANOVA sex $\times$ day $p < 0.05$; Figure 5F). The sex difference in hypothermia following heterosubtypic challenge was confirmed when mice were followed for a longer period of time post challenge (Supplemental Figure 3C and 3D). Titers of H3N2 in the lungs were significantly lower days 1 and 5 post-challenge in females than males (2-way ANOVA sex $\times$ day $p < 0.05$; Figure 5G). Animals with higher neutralizing antibody titers against H1N1 35 days post-infection had significantly lower H3N2 titers 1 day post-challenge (Pearson product moment $r = -0.65$, $p < 0.05$). Titers of H3N2 were lower in the lungs of immunized than naïve mice

(2-way ANOVA treatment $\times$ day $p < 0.05$; Supplemental Figure 4A and Figure 5G), and titers of H3N2 did not differ between naïve male and female mice (Supplemental Figure 4A).

To determine if there were sex differences after a lethal heterosubtypic challenge with H1N1, mice were first inoculated with 10^1 TCID₅₀ of H3N2 (Figure 6A and B) resulting in females having a more pronounced drop in body mass, but not body temperature, than males (MANOVA sex $\times$ day $p < 0.05$; Figure 6A and B). These results were confirmed in an experiment using 10^2 TCID₅₀ of H3N2 to infect mice (Supplemental Figure 5A and 5B), but were not observed in the initial experiments using these low virus doses (Supplemental Figure 2). At day 42 post-infection, females had significantly higher neutralizing antibody and anti-H3N2 IgG titers than males (t-test $p < 0.05$ in each case; Figure 6C and D). Animals were challenged with a lethal dose of H1N1 (10^4 TCID₅₀) at day 56 post-H3N2 infection. Males had a significant decline in body mass and temperature (MANOVA sex $\times$ day $p < 0.05$ in each case) when compared to females (Figure 6E and F). Similar changes were observed in mice that were administered a slightly higher dose of H3N2 (10^2 TCID₅₀) as a primary infection and followed for morbidity to 21 days post-H1N1 challenge (Supplemental Figure 5C and 5D). Overall, females had significantly lower titers of H1N1 in the lungs than males, regardless of day post-challenge (2-way ANOVA sex main effect $p < 0.05$; Figure 6G). Animals with higher neutralizing and total IgG titers against H3N2 42 days post-infection had significantly lower titers of H1N1 virus in their lungs 3 days post-challenge (Pearson product moment $r = -0.54$ and $r = -0.72$, respectively, $p < 0.05$ in each case). Titers of H1N1 virus were lower in the lungs of immunized than naïve mice (2-way ANOVA treatment $\times$ day, $p < 0.05$; Supplemental Figure 4B and Figure 6G), and H1N1 titers did not differ between naïve male and female mice (Supplemental Figure 4B). In summary, females mount higher humoral immune responses to influenza A viruses and exhibit greater cross protection against heterosubtypic virus challenge, resulting in lower viral loads in the lungs of female than male mice.

3.4 Sex differences in cross-reactive antibody titers

To assess whether cross-reacting antibody titers differed between the sexes, antibody responses were analyzed against heterosubtypic virus. Neutralizing antibodies against heterosubtypic viruses were not detected in either male or female mice (data not shown). Cross-reacting total IgG titers were detected against heterosubtypic viruses. Females immunized against H1N1 exhibiting slightly higher cross-reacting antibodies against H3N2 (t-test $p = 0.090$, Figure 7A) and females immunized against H3N2 showing higher cross-reacting antibodies against H1N1 (t-test $p = 0.052$, Figure 7B) than their male counterparts, although this did not reach statistical significance. These data suggest that cross-reacting antibody titers, as measured by ELISA and NT assays, may not explain the observed enhanced protection of female mice from heterologous virus challenge.

4. Discussion

Despite data illustrating that sex plays an important role in responses to vaccines, many studies do not document sex-specific effects in immune responses to vaccines or vaccine efficacy [61]. The lack of sex-specific analyses can be attributed to i) deficient statistical power to observe meaningful differences between the sexes, ii) no a priori hypothesis that the sexes will differ in their responses to vaccination, iii) limited sample collection and analysis that does not allow for an assessment of the kinetics of immune responses, and iv) the use of vaccine doses which are large enough to mask sex differences in immune responses. Several studies illustrate that females generate higher humoral immune responses and experience more frequent and severe side effects following seasonal TIV than males [26–31].

We have developed an animal model to systematically examine differences between the sexes in cross protective immunity against influenza A viruses, which will serve as an important tool for uncovering the mechanisms mediating sex differences in response to influenza vaccines and infection. Our data support and extend available clinical data by showing that, like women, female mice mount higher neutralizing and total antibody responses against a sublethal primary infection/vaccination with influenza A viruses than males [26–29]. Our data further illustrate that females are better protected against lethal challenge with heterosubtypic strains of influenza A viruses than males. By using an animal model that allows for differentiation of morbidity, we were able to assess both the humoral immune responses that affect disease severity as well as protection from infection. Elevated immunity afforded females greater protection than males against lethal challenge with heterosubtypic viruses, but both sexes were equally protected against lethal challenge with homologous virus. This suggests that the magnitude of the protection provided by immunity to a primary heterologous influenza A virus infection was greater in females than males. Additional studies are necessary to compare immune responses in males and females after vaccination with an inactivated, split virus vaccine to determine if sex differences in humoral immunity, cross-protection, or both are also observed.

Sex differences in disease were not seen at all virus inoculums indicating that the dose of virus is important for assessing sex-specific changes in viral pathogenesis. At lethal doses, naïve males and females experience profound morbidity and death and at sublethal doses both sexes experience transient morbidity, but survived infection. Sex-specific differences in immune responses, however, were detected at doses where morbidity and mortality resulting from infection were indistinguishable between the sexes, illustrating our ability to decouple sex differences in immunity from sex differences in disease outcome. This observation demonstrates the complex role that sex can play in affecting the host response to virus infection and vaccination.

The LD₅₀ for H1N1 and H3N2 viruses were lower for female than male mice. In the present study, male and female mice were administered equivalent intranasal inoculums of virus, regardless of their starting body mass. One hypothesis for the observed sex difference in the LD₅₀ might be that the smaller body mass of female mice contributes to increased morbidity and mortality in naïve mice. Although adult male mice weigh more than females, absolute lung mass is not consistently different between the sexes and relative lung mass is often greater in C57BL/6 females prior to infection (Robinson and Klein, unpublished data). Further, in aged animals, where the starting body mass of females at the time of inoculation is similar to that of young adult males, females still experience greater morbidity and mortality than their age-matched male counterparts [62]. Among females, ovariectomized animals that receive exogenous estradiol have significantly reduced morbidity and mortality from H1N1 infection, despite having a starting body mass that is similar to that of ovariectomized females that do not receive hormone replacement [63]. Differences between the sexes and in response to hormone treatment also do not appear to reflect differences in infectious virus titers, but rather are associated with differences in inflammatory cytokine and chemokine responses during a primary infection in both C57BL/6 and BALB/c mice [63, 64]. Collectively, these data suggest that the biological response, including innate and adaptive immune responses, rather than starting body mass or virus replication, contribute to differences in the outcome of primary infection between the sexes.

The role of serum antibody responses in protection against influenza infection has been demonstrated [65, 66]. In the present study, we conducted a detailed analysis of the kinetics and magnitude of the functional (i.e., neutralizing) and total antibody responses against a primary sublethal infection with influenza A viruses. Our data reveal that females consistently mounted higher neutralizing and total antibody responses against H1N1 and

H3N2 viruses, but that the kinetics of these responses differed depending on the strain of influenza A virus and type of antibody response measured. As compared with H3N2, H1N1 was more lethal and resulted in an earlier divergence of the antibody response between males and females. The kinetics of the neutralizing and total antibody responses to H1N1 also were distinct, in which females generated higher total antibody responses than males 14 days post-infection, whereas their neutralizing antibody responses were not higher than males until 21 days post-infection. In contrast, the kinetics of the sex differences in neutralizing and total antibody responses against H3N2 were remarkably similar. Sex differences in cross-reactive neutralizing antibodies were not observed against either H1N1 or H3N2 viruses. Conversely, cross-reactive total antibodies were present at somewhat higher titers among female than male mice. In humans, women reportedly have a higher frequency of cross-reacting antibodies against pandemic 2009 H1N1 than men, as measured by hemagglutination inhibition [67]. Although hemagglutination inhibition titers correlate well with virus neutralizing titers [68], the strain specificity of the virus neutralization assay may have reduced detection of cross-reacting antibodies against HA in the present study. Alternatively, higher antibody titers in females may result in greater cross-protection via other mechanisms, including activation of complement or T cell responses.

Primary infection with a sublethal dose of influenza A virus provides cross protection against secondary challenge with a heterosubtypic virus primarily through increased activity and numbers of virus-specific CD8⁺ cytotoxic T lymphocytes (CTLs) that recognize shared epitopes of internal proteins [44, 56, 57]. There are, however, data illustrating that CTLs are not the sole mechanism mediating heterosubtypic immunity as mice depleted of CD8⁺ and CD4⁺ T cells, deficient in β 2-microglobulin, or deficient in IFN- γ still have cross-protective immunity against heterosubtypic challenge [49–52, 54]. Further, mice that are depleted of B cells (i.e., μ MT mice) are not protected against heterosubtypic challenge with lethal doses of influenza A viruses [47, 50–52]. Passive transfer of immune serum prior to challenge with lethal influenza A virus also confers protection from heterosubtypic infection in some models [40, 48, 58, 69]. There is growing evidence that both B and T cells contribute to protective immunity against heterosubtypic strains of influenza A viruses, including 2009 H1N1 [70]. The nature of the immune response mediating increased heterosubtypic immunity in females has not been identified, but future studies will evaluate the breadth and magnitude of the T cell responses associated with heterosubtypic immunity as well as the specific T cell subsets that are induced in order to fully evaluate the nature of sex-specific immunity and cross protection against influenza A viruses. Furthermore, broadly cross-reactive antibody responses targeting conserved proteins or epitopes (e.g., M2 or HA2) have been identified in vaccinated and infected individuals [40, 71]. Future studies must examine if the stronger overall antibody response in females leads to higher levels of these types of cross reactive, protective antibodies against conserved epitopes.

Sex differences in innate immunity likely contribute to subsequent differences in adaptive immune responses to influenza A viruses. Innate immune responses, including detection of nucleic acids by pattern recognition receptors (e.g., toll-like receptor 7), are typically higher in females than males [61, 72]. Inflammatory cytokine and chemokine responses are higher in the lungs of female than male mice following primary infection with H1N1 or H3N1 viruses [63, 64]. Efficiency of antigen presentation by innate immune cells also is greater in cells from females than males [73] which likely contributes to higher adaptive immune responses in females [61].

Several studies illustrate that sex and sex hormones are factors that can impact the magnitude and quality of antibody responses. Both basal titers of immunoglobulin [74] as well as antibody responses to viruses and vaccines [1, 27] are higher in females than males. 17 β -estradiol, at physiological concentrations, can stimulate antibody production by B cells

[75–77], including antibody responses to an inactivated influenza vaccine administered in BALB/c mice [78]. Whether sex hormones affect the protective immune responses induced by vaccines and subsequent susceptibility to a lethal virus infection is less well characterized. Evaluation of sex-specific factors, whether genetic or hormonal, that influence how females are better protected against lethal influenza A virus challenge could have important public health implications.

Highlights

- We developed a small animal model to determine if protective immunity against influenza A viruses is higher in females than males.
- We examined humoral immune responses to primary sublethal infection with H1N1 or H3N2 viruses and virus titers and morbidity following secondary lethal challenge with homologous or heterologous virus.
- Females mount higher neutralizing and total anti-influenza A virus antibodies than males.
- Males and females are equally protected against homologous virus challenge, but males experience greater morbidity and have higher viral titers in their lungs after infection with a lethal dose of heterologous virus.
- We conclude that females develop greater cross protection against influenza A viruses than males.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Percentage survival of male and female mice ($n = 10/\text{sex}/\text{dose}$) infected with one of 5 $\log_{10}$ dilutions of influenza A viruses H1N1 or H3N2. The LD₅₀ was 11-fold higher for males (A) than females (B) inoculated with H1N1 ($p < 0.05$) and 4-fold higher for males (C) than females (D) inoculated with H3N2.

Figure 2.

Percentage change ($\pm$ SEM) from baseline (day 0) for body mass and temperature in male and female mice ($n = 10/\text{sex}$) infected with a sublethal dose of H1N1 (10^1 TCID₅₀) (A and B). At 35 days post-infection, mice were challenged with a lethal dose of H1N1 (10^4 TCID₅₀) (C and D). No sex differences were observed.

Figure 3. Percentage change ($\pm$ SEM) from baseline (day 0) for body mass and temperature in male and female mice ($n = 10/\text{sex}$) infected with a sublethal dose of H3N2 (10^2 TCID_{50}) (A and B). At 35 days post-infection, mice were challenged with a lethal homologous dose of H3N2 (10^5 TCID_{50}) (C and D). No sex differences were observed.

Figure 4.

Neutralizing antibody and total anti-influenza antibody titers in serum collected from male and female mice ($n = 10/\text{sex}$) at 14, 21 and 28 days post-infection with a sublethal dose of H1N1 (10^1 TCID_{50}) (**A** and **B**) or sublethal dose of H3N2 (10^2 TCID_{50}) (**C** and **D**). An asterisk (*) indicates significant difference between males and females at that time point, 2-way ANOVA, $p < 0.05$.

Figure 5.

Percentage change ($\pm$ SEM) from baseline (day 0) for body mass and temperature in male and female mice ($n = 50/\text{sex}$) infected with a sublethal dose of H1N1 (10^1 TCID₅₀) (**A** and **B**). At 35 days post-infection, neutralizing (NT) (**C**) and total anti-H1N1 IgG (**D**) antibody responses were measured in serum. At 42 days post-infection, mice were challenged with a lethal dose of H3N2 (10^5 TCID₅₀) and monitored for morbidity (**E** and **F**). At the specified time points, animals were euthanized and H3N2 infectious virus titers were measured in lung homogenates (**G**). An asterisk (*) indicates significant differences between males and females at the specified time point, MANOVA (A–B, E–F), t-test (C and D), or 2-way ANOVA (G), $p < 0.05$.

Figure 6.

Percentage change ($\pm$ SEM) from baseline (day 0) for body mass and temperature in male and female mice ($n = 50/\text{sex}$) infected with a sublethal dose of H3N2 (10^1 TCID₅₀) (**A** and **B**). At 42 days post-infection, neutralizing (NT) (**C**) and total anti-H3N2 IgG (**D**) antibody responses were measured in serum. At 56 days post-infection, mice were challenged with a lethal dose of H1N1 (10^4 TCID₅₀) and monitored for morbidity (**E** and **F**). At the specified time points, animals were euthanized and H1N1 infectious virus titers were measured in lung homogenates (**G**). An asterisk (*) indicates differences between males and females, MANOVA (A–B, E–F), t-test (C and D), or 2-way ANOVA (G), $p < 0.05$.

Figure 7. Mean ($\pm$ SEM) titers of cross-reactive total IgG in male and female mice infected with sublethal doses of H1N1 (10^1 TCID₅₀) (A) or H3N2 (10^1 TCID₅₀) (B).