Biological sex affects vaccine efficacy and protection against influenza in mice

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Biological sex affects adaptive immune responses, which could impact influenza infection and vaccine efficacy. Infection of mice with 2009 H1N1 induced antibody responses, $CD4^+$ T cell and $CD8^+$ T cell memory responses that were greater in females than males; both sexes, however, were equally protected against secondary challenge with an H1N1 drift variant virus. To test whether greater antibody in females is sufficient for protection against influenza, males and females were immunized with an inactivated H1N1 vaccine that induced predominantly antibody-mediated immunity. Following vaccination, females had greater antibody responses and protection against challenge with an H1N1 drift variant virus than males. Antibody derived from vaccinated females was better at protecting both naïve males and females than antibody from males, and this protection was associated with increased antibody specificity and avidity to the H1N1 virus. The expression of *Tlr7* was greater in B cells from vaccinated females than males and was associated with reduced DNA methylation in the *Tlr7* promoter region, higher neutralizing antibody, class switch recombination, and antibody avidity in females. Deletion of *Tlr7* reduced sex differences in vaccine-induced antibody responses and protection following challenge and had a greater impact on responses in females than males. Taken together, these data illustrate that greater TLR7 activation and antibody production in females improves the efficacy of vaccination against influenza.

antibody secreting cells | DNA methylation | isotope switching | $CD8^+$ T cell memory | toll-like receptor 7

Both sex (i.e., biological differences) and gender (i.e., social or cultural influences) impact vaccine acceptance, responses, and outcomes (1). Adult human females consistently mount higher adaptive immune responses to vaccines than their male counterparts. For example, adult human females have higher antibody responses to influenza, hepatitis B, herpes virus, yellow fever, rabies, and smallpox virus vaccines than males (1). Whether this results in greater vaccine efficacy in females has not been considered.

Influenza is a significant public health threat, with influenza A viruses causing seasonal epidemics, occasional outbreaks, and sporadic pandemics. Available influenza virus vaccines are the best defense against severe disease, but with vaccine effectiveness ranging from 30 to 60%, development of new vaccine formulations, including universal influenza vaccines, is required to improve protection (2, 3). Currently available formulations include versions of both live and inactivated influenza viruses (4). An important benefit of live vaccines that more closely mimic natural influenza exposure is the induction of broad immunity, including antibody and $CD8^+$ T cell memory responses, and greater protection against drift variants, whereas the primary benefit of inactivated influenza vaccines is decreased reactogenicity (3, 5). Although age, compromised immune function, and even pregnancy are considered in the context of influenza vaccine efficacy and formulation, we do not adequately consider biological sex.

Sex-based differences in the immune response to influenza vaccination are documented. Among adults of reproductive ages (18–49 y), females have higher hemagglutination inhibition (HAI) and neutralizing antibody titers compared with males following receipt of the influenza trivalent inactivated vaccine (TIV) (6–8). In mice immunized with influenza TIV, females also generate higher neutralizing antibody responses to the H1N1 component of the vaccine than males (9). In mice immunized with live H1N1 or H3N2 viruses, adult females develop greater neutralizing antibody titers following vaccination (10).

Although sex differences in the humoral immune response are observed following both influenza virus infection and vaccination, there are fundamental differences in the responses that are elicited by infection versus vaccination. Inactivated influenza vaccination induces neutralizing antibodies against the highly immunogenic influenza virus membrane surface proteins hemagglutinin (HA) and neuraminidase (NA). In contrast, influenza virus infection induces robust cell-mediated immune responses in addition to the neutralizing antibody response (3). We sought to evaluate whether protection following infection or vaccination differed between the sexes and identify the immunological mechanism mediating these differences.

Results

Influenza A Virus Infection Induces Greater Activation of Germinal Center B Cells and Humoral Immune Responses in Females. Infection with influenza A viruses induces robust humoral and cellular immune responses and provides long-lasting immunity to subsequent influenza virus exposures. Understanding these protective immune responses, and specifically how biological sex may influence these responses, is essential to the development of effective influenza vaccines. To evaluate sex differences in the adaptive immune responses following influenza virus infection, male and female mice were intranasally inoculated with 2009 H1N1 influenza virus. Serum anti-2009 H1N1 IgG and neutralizing

Significance

Biological sex is typically not considered in the evaluation of vaccine responses and protection against infection. We show that female mice mount greater humoral and cell-mediated immune responses to influenza infection and vaccination than males. Females can rely solely on antibody for protection following vaccination, which is associated with greater expression of toll-like receptor 7 (*Tlr7*) caused by epigenetic mechanisms in B cells from females. Deletion of *Tlr7* reduces sex differences in vaccine-induced antibody responses and protection. The increased expression of *Tlr7* in B cells contributes to greater antibody production in females than males, which has a functional advantage for vaccine efficacy.

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The authors declare no conflict of interest.

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antibody titers were detectable as early as 14 d postinfection (dpi) and were greater in females compared with males (Fig. 1*A* and *SI Appendix*, Fig. S1*A*, $P < 0.05$). Females also generated greater anti-2009 H1N1 IgA titers and neutralizing antibody titers in the bronchoalveolar lavage (BAL) fluid than males (Fig. 1*B* and *SI Appendix*, Fig. S1*B*, $P < 0.05$).

Following H1N1 infection, higher humoral immune responses in females, particularly in the respiratory tract, may be caused by increased virus replication, damage to the endothelium, or both. Peak titers of virus as well as clearance of virus from the lungs were similar between males and females (*SI Appendix*, Fig. S1*C*). Lung endothelial damage and leakage, as measured by total protein in the BAL (*SI Appendix*, Fig. S1*D*) and the amount of Evans blue dye in the lungs during and after peak virus titers (*SI Appendix*, Fig. S1*E*), were also similar between the sexes, suggesting that the mechanism mediating the higher mucosal antibody responses in females was independent of virus replication and lung tissue damage.

To determine if greater pulmonary antibody responses in females reflected greater B cell activation and differentiation, the numbers of B cells, total $CD4^+$ T cells, and follicular helper T (Tfh) cells were quantified in lungs and mediastinal lymph nodes (mLNs). Although the total number of B cells in the lungs was similar between males and females (males = $9.05 \times 10^5 \pm 3.2 \times 10^5$; females = $9.3 \times 10^5 \pm 2.2 \times 10^5$ cells), the total number of anti-2009 H1N1 IgA secreting B cells in the lungs was significantly greater in females compared with males (Fig. 1*C*, $P < 0.05$). In the mLNs, females had more $CD4^+$ T cells (Fig. 1*D*, $P < 0.05$), but similar numbers of Tfh cells (Fig. 1*E*) as males. Females also had significantly greater numbers of germinal center B cells compared with males (Fig. 1*F*, $P < 0.05$). An analysis of mLN germinal center histology revealed that females had significantly larger germinal centers compared with males following infection (Fig. 1 *G* and *H*, $P < 0.05$). These data suggest that there are sex-specific differences in B cell activation and generation of antibody in response to an influenza A virus infection.

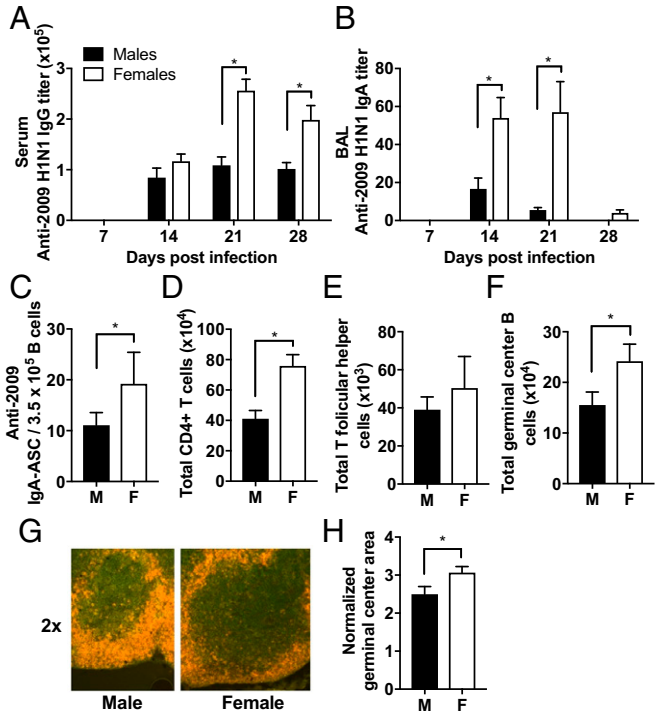

Fig. 1. Females have greater antibody responses and B cell activation in response to influenza A virus infection. Adult male and female mice were inoculated intranasally with 10 $TCID_{50}$ (i.e., the tissue culture infectious dose that causes cytopathic effects in 50% of cells) units of 2009 H1N1 virus, serum was collected to measure anti-2009 H1N1 IgG titers (*A*; $n = 10$/sex/time point), and BAL fluid was collected to analyze anti-2009 H1N1 IgA titers (*B*; $n = 9$–10/sex/time point). At 21 dpi, anti-2009 H1N1 IgA antibody-secreting (ASC) B cells ($n = 6$ pools of 3/sex) were quantified in the lungs (*C*) and $CD4^+$ T cells (*D*), T-follicular helper cells (*E*), and germinal center B cells (*F*) were analyzed in the lung-draining mLNs by flow cytometry ($n = 13$–14/sex); M = males, F = females). At 21 dpi, mLNs were isolated and frozen sections were stained for germinal centers by immunofluorescence (*G*). The ratio of each germinal center area to the area of the lymph node section was calculated using ImageJ ($n = 3$/sex) (*H*). Data represent means ± SEM from two to three independent experiments and significant differences between males and females are represented by asterisks (*).

Influenza A Virus Infection Elicits Greater Memory $CD8^+$ T Cell Activity in Females than Males. Influenza A virus infection induces $CD8^+$ T cell responses, in addition to antibody responses, that can protect against the development of symptomatic disease (3). To determine whether cell-mediated immune responses differed between the sexes during infection, male and female mice were infected with 2009 H1N1 virus and 42 d later the number of $CD8^+$ memory T cells in the lungs was evaluated. Females had significantly greater numbers of tetramer-specific $CD8^+$ T cells, $CD8^+$ T memory cells, central memory $CD8^+$ T cells, and tissue resident memory (TRM) $CD8^+$ T cells in their lungs than males (Fig. 2 *A–D* and *SI Appendix*, Table S1, $P < 0.05$ in each case).

Because females had greater B cell, $CD4^+$ T cell, and memory $CD8^+$ T cell responses following 2009 H1N1 infection, we hypothesized that females would be better protected against a secondary influenza A virus challenge. To test this hypothesis, male and female mice were infected with 2009 H1N1 virus or mock infected and challenged with a 2009 H1N1 drift variant (2009dv) virus 42 d later. Infected males and females were equally protected against the 2009dv challenge, experiencing no morbidity (Fig. 2*E*) or virus replication in the lungs (Fig. 2*F*), which contrasted significantly with the outcome of challenge in mock-infected animals. These data suggest that influenza virus infection induced adequate adaptive immunity to protect both sexes, despite these immune responses being significantly greater in females compared with males.

Inactivated 2009 H1N1 Vaccine Induces Greater Antibody Responses and Protection Against Secondary Challenge in Females. Although primary infection induces a multifaceted systemic and mucosal immune response to confer protection against subsequent influenza A virus exposures, influenza virus vaccines, formulated with inactivated virus, confer protection almost exclusively through the generation of vaccine-specific antibody responses (2). To test the hypothesis that females, but not males, could be protected from influenza A virus challenge by antibody alone, adult male and female mice were vaccinated with inactivated 2009 H1N1 virus and challenged with the 2009dv virus. Before challenge, vaccinated females developed higher total anti-2009 H1N1 IgG titers (Fig. 3*A*, $P < 0.05$) and neutralizing antibody titers (Fig. 3*B*, $P < 0.05$) compared with males. In contrast, following vaccination, virus-specific $CD8^+$ T cell responses were not different between the sexes and were significantly lower than responses following a primary infection (*SI Appendix*, Table S1). Following challenge with 2009dv, females experienced less morbidity (Fig. 3*C*, $P < 0.05$), had lower peak virus titers, and cleared virus faster from their lungs than males (Fig. 3*D*, $P < 0.05$).

To test whether vaccine-induced antibodies from females could equally protect males and females, serum was collected and pooled separately from male and female mice that were vaccinated with inactivated 2009 H1N1 and inoculated into naïve male and female mice that were then challenged with the 2009dv virus. At 3 d postchallenge, protection was assessed by measuring pulmonary virus titers. Transfer of antibodies from vaccinated females into either naïve males or females reduced pulmonary virus titers in both sexes following challenge (Fig. 3*E*, $P < 0.05$). In contrast, receipt of antibodies from vaccinated males reduced

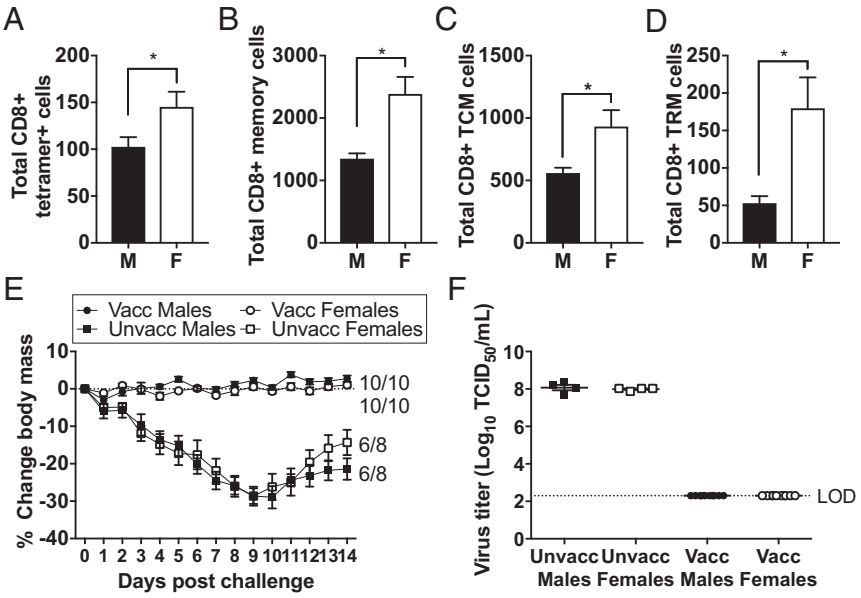

Fig. 2. Influenza A virus infection induces greater numbers $CD8^+$ T memory cells in females than males. Male and female mice were inoculated intranasally with 10 $TCID_{50}$ (i.e., the tissue culture infectious dose that causes cytopathic effects in 50% of cells) units of 2009 H1N1 at day 0 and on day 42 postinfection, half of the mice were either killed to measure virus-specific $CD8^+$ T cells or challenged with 10^5 $TCID_{50}$ units of 2009dv virus and killed 6 d postchallenge to quantify memory responses. Total numbers of live $CD8^+$ $tetramer^+$ cells (*A*), $CD8^+$ memory cells (*B*), $CD8^+$ central memory (TCM) cells (*C*), and $CD8^+$ TRM cells (*D*) were quantified; M = males, F = females. Morbidity following challenge was determined by monitoring changes in body mass (numbers represent the number of animals that survived the challenge out of the total in each group) (*E*), and virus titers were quantified in the lungs at 3 d postchallenge (*F*) in vaccinated (Live Vacc) and unvaccinated (Unvacc) mice. Data represent means ± SEM from two independent experiments (n = 8–10/sex) and significant differences between males and females and treatment groups are represented by asterisks (*).

virus titers in naïve females, but not males, following challenge, suggesting that the threshold antibody level for protection is lower for females than males. Naïve males that received serum from vaccinated males had pulmonary viral titers that were comparable to male and female mice that received serum from unvaccinated mice, further illustrating that males are not protected by vaccine-induced antibody alone. The titer of transferred 2009 H1N1 antibody was inversely correlated with lung virus titers following challenge, further demonstrating that naïve males that received serum from vaccinated males had the lowest antibody titers and the highest virus titers (Fig. 3*F*, $P < 0.05$).

Vaccinated Females Produce Higher Quality Antibodies Compared with Males. The ability of female-derived antibodies, but not male-derived antibodies, to protect both naïve males and females from influenza A virus challenge, may reflect either greater quantity or quality of the antibody generated following vaccination of female mice. To test whether class switching following vaccination differed between the sexes, we measured virus-specific IgM titers. No sex differences were observed in IgM titers following inactivated 2009 H1N1 vaccination (Fig. 4*A*), suggesting that the sex differences in virus-specific IgG antibody titers arose following antibody class switching. Following vaccination or infection in mice, the predominant antibody isotype present in the serum of animals that survive secondary influenza A virus infections is IgG2c (11, 12). IgG2c antibodies are also associated with increased vaccine efficacy (13–15). To test the hypothesis that vaccine efficacy is greater in females because of elevated IgG2c titers, we measured titers of virus-specific IgG1 and IgG2c. Vaccinated males and females had similar virus-specific IgG1 antibody titers (Fig. 4*B*), whereas vaccinated females had

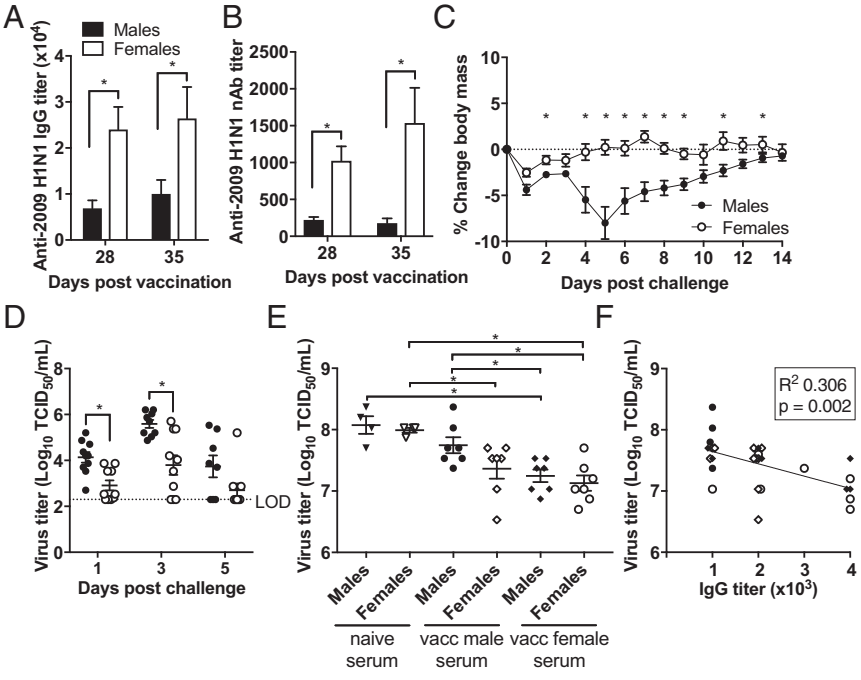

Fig. 3. Inactivated influenza virus vaccination induces greater antibody responses and better protection in females. Adult male and female mice were vaccinated with inactivated 2009 H1N1 on day 0, boosted on day 21, and challenged with 10^5 $TCID_{50}$ (i.e., the tissue culture infectious dose that causes cytopathic effects in 50% of cells) units of 2009dv virus on day 42. Anti-2009 H1N1 IgG (*A*) and neutralizing antibody (nAb; *B*) titers in serum were determined. Morbidity was determined by monitoring changes in body mass (*C*), and virus titers were quantified in the lungs after challenge (*D*). At 6 wk postvaccination, serum was pooled by sex and intraperitoneally injected into naïve mice of both sexes. Male and female control animals received serum from naïve males and females. Three hours later, mice were bled and challenged with the 2009dv virus, and 3 d later lung virus titers were quantified (*E*). Lung virus titers were correlated with IgG antibody titers (*F*). Data represent means ± SEM from two independent experiments (n = 7–10/sex) and significant differences between groups are represented by asterisks (*).

significantly greater titers of virus-specific IgG2c compared with males (Fig. 4*C*, $P < 0.05$). The ratio of IgG1 to IgG2c antibodies, however, was not significantly different between the sexes (Fig. 4*D*), although there was evidence of skewing toward Th1 immunity (i.e., IgG2c) in females compared with males. To further evaluate sex differences in antibody quality, we measured the serum binding avidity index of the vaccine-induced IgG antibodies. The IgG avidity index was significantly greater in vaccinated females compared with males (Fig. 4*E*, $P < 0.05$). Overall, these data indicate that females generate higher quality antibodies than males following influenza vaccination and these differences may explain the sex bias in vaccine outcome.

Influenza Vaccination Induces Greater Expression of *Tlr7* in B Cells, Which Is Necessary for Female-Biased Antibody Responses and Protection. Because females had greater quality vaccine-induced antibody than males, the genes associated with the generation of high-quality antibodies may be differentially expressed in B cells between the sexes. The toll-like receptor-7 (*Tlr7*) gene is encoded on the X chromosome and has been primarily studied in the context of innate immunity, but is also expressed in B cells and plays a role in isotype switching (16). To determine if influenza vaccination induced greater *Tlr7* expression in B cells from females, male and female mice were vaccinated with inactivated 2009 H1N1 and 28 d later splenic B cells were isolated, and RNA was extracted to evaluate relative gene expression. Compared with cells from vaccinated males, B cells derived from vaccinated females had significantly greater *Tlr7* expression (Fig. 5*A*, $P < 0.05$), but not toll-like receptor 8 (*Tlr8*) expression, which also is encoded on the X chromosome (Fig. 5*B*). To evaluate whether B cell induction of *Tlr7* was observed in response to subunit as well as whole inactivated influenza vaccines, we vaccinated male and female mice with the 2017–18 seasonal influenza quadrivalent inactivated vaccine (QIV). Following QIV, anti-H1N1 IgG antibody responses were greater in females than males (*SI Appendix*, Fig. S2*A*, $P < 0.05$), although responses were approximately two logs lower than responses to inactivated whole H1N1. Splenic B cells derived from females tended to have greater *Tlr7* (*SI Appendix*, Fig. S2*B*, $P = 0.08$), but not *Tlr8* expression (*SI Appendix*, Fig. S2*C*), than cells from males. The greater induction of *Tlr7* in B cells from females may not be dependent on influenza vaccine formulations.

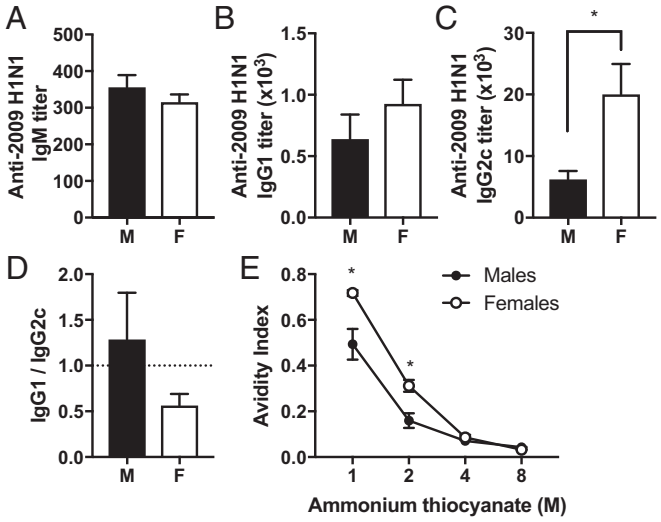

Fig. 4. Inactivated influenza virus-vaccinated females generate greater quality antibodies than vaccinated males. Adult male and female mice were vaccinated with inactivated 2009 H1N1 on day 0, boosted on day 21, and antibody responses were measured on day 28. Anti-2009 H1N1 IgM (*A*), IgG1 (*B*), IgG2c (*C*), IgG1:IgG2c ratio (*D*), and IgG avidity index (*E*) were measured in serum; M = males, F = females. Data represent means ± SEM from two independent experiments ($n = 9$–10/sex) and significant differences between males and females are represented by asterisks (*).

Higher expression of an X-linked gene, e.g., *Tlr7*, may indicate escape from X inactivation caused by epigenetic modifications, including DNA methylation. To determine whether higher expression of *Tlr7* in B cells from females reflected transcriptional regulation via DNA methylation, DNA was isolated from splenic B cells collected 28 d after prime vaccination (i.e., 7 d after boost) with either vehicle (unvaccinated) or inactivated 2009 H1N1 to quantify CpG site-specific DNA methylation in the regulatory region of *Tlr7* (*SI Appendix*, Fig. S3*A*). Eight clones from each sample were sequenced to obtain direct measures of DNA methylation at each of five CpG sites (*SI Appendix*, Fig. S3*B*), which revealed greater DNA methylation at three of five CpG sites on *Tlr7* in B cells from unvaccinated compared with vaccinated females (*SI Appendix*, Fig. S3*C*, $P < 0.05$). Vaccination did not affect DNA methylation at any of the CpG sites on *Tlr7* in B cells isolated from males (*SI Appendix*, Fig. S3*C*). As a result, vaccination significantly reduced the percentage of DNA methylation in the promoter region of *Tlr7* in B cells from females, but not males (Fig. 5*C*, $P < 0.05$). Reduced DNA methylation may be one mechanism mediating greater expression of *Tlr7* in B cells from vaccinated females compared with males.

TLR7 activity in B cells is associated with increased somatic hypermutation and induction of mutations that select for high-affinity B cells following antigen stimulation (17) as well as preferential generation of IgG2c antibodies (18). To evaluate if higher *Tlr7* expression following vaccination is necessary for greater quality antibody responses in females compared with males, we utilized *Tlr7* knockout male and female mice and compared their responses to wild-type males and females following inactivated influenza vaccination. Compared with wild-type males, wild-type females generated greater influenza-specific neutralizing, IgG, and IgG2c antibodies that were of higher avidity; sex differences in both the titers and avidity of vaccine-induced antibody were reduced among *Tlr7* knockout mice (Fig. 5 *D*, *E*, *G*, and *H*, $P < 0.05$ in each case). The impact of *Tlr7* expression in mice was greater for females than males as elimination of *Tlr7* resulted in a greater fold decrease in neutralizing, IgG, and IgG2c titers in females compared with males. Specifically, relative to wild-type males, *Tlr7* knockout males had a 2.36-fold decrease in neutralizing antibody, 6.26-fold decrease in IgG, 7.94-fold decrease in IgG2c; relative to wild-type females, *Tlr7* knockout females had a 4.98-fold decrease in neutralizing antibody, 13.47-fold decrease in IgG, 24.76-fold decrease in IgG2c). No sex differences in either wild-type or *Tlr7* knockout mice were observed in virus-specific IgG1 titers (Fig. 5*F*).

To test the hypothesis that *Tlr7* expression is required for female-biased, antibody-mediated protection following challenge, wild-type and *Tlr7* knockout male and female mice were vaccinated with inactivated 2009 H1N1 and challenged with the 2009dv virus. Overall, *Tlr7* knockout mice did not clear virus from their lungs as efficiently as wild-type mice (Fig. 5*I*). As shown previously (Fig. 3*D*), vaccinated wild-type females cleared virus from their lungs faster than wild-type males, in which the 2009dv virus was eliminated from the lungs of females, but not males, by day 5 postchallenge (Fig. 5*I*, $P < 0.05$). In contrast, sex differences in virus clearance were not present in *Tlr7* knockout mice, in which the female-biased protection was no longer observed (Fig. 5*I*). Taken together, these data illustrate that TLR7 signaling in B cells is necessary for the higher quality and quantity of antibody and subsequent protection afforded females.

Discussion

Sex differences in immune responses are documented (19), yet few vaccine studies partition and analyze data for sex-specific differences in vaccine efficacy. We evaluated sex differences in the responses to and protection induced following either influenza A virus infection or vaccination in a murine model.

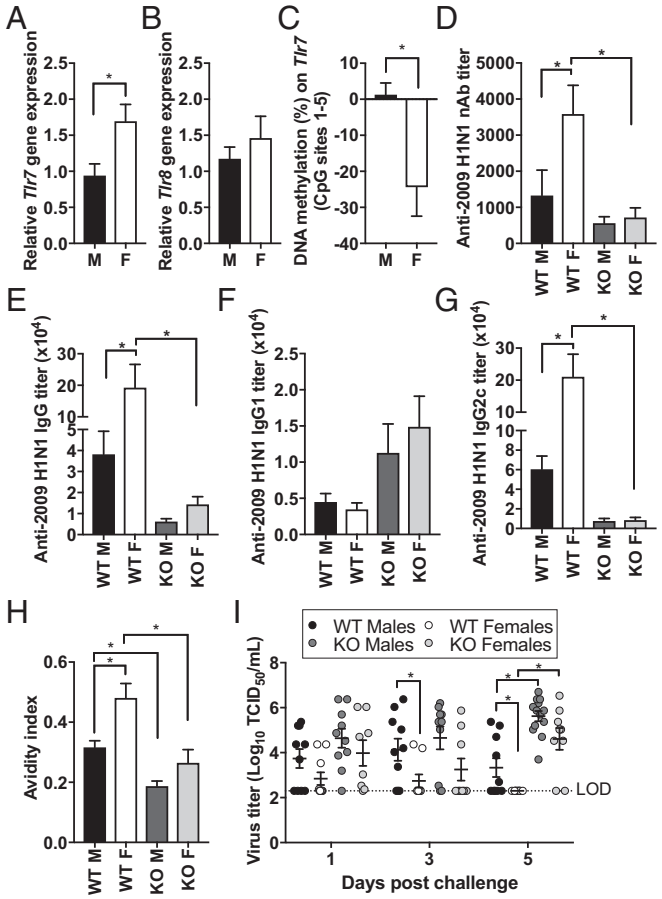

Fig. 5. *Tlr7* expression is elevated in female-derived B cells and is necessary for greater antibody titers and protection in vaccinated females than males. Adult male and female mice were vaccinated with inactivated 2009 H1N1 or vehicle on day 0, boosted on day 21, and splenic B cells were isolated on day 28. RNA was extracted from B cells to evaluate the relative gene expression of *Tlr7* (*A*) and *Tlr8* (*B*). Using DNA isolated from B cells, CpG site-specific DNA methylation of the 5′ regulatory region for *Tlr7* was assayed. Results are represented as the change in the percentage of DNA methylation on *Tlr7* in vaccinated relative to unvaccinated males or females (*C*; n = 5–9/sex). To evaluate the contribution of *Tlr7* to antibody somatic hypermutation and class switch recombination, adult male and female wild-type and *Tlr7* knockout mice were vaccinated with inactivated 2009 H1N1 on day 0, boosted on day 21, and antibody responses were measured on day 28. Anti-2009 H1N1 neutralizing antibody (*D*), IgG (*E*), IgG1 (*F*), IgG2c (*G*), and IgG avidity (*H*) responses were measured in serum. To evaluate protection following challenge, male and female wild-type (WT) and *Tlr7* knockout (KO) mice were vaccinated and boosted with inactivated 2009 H1N1 and challenged with 10^5 $TCID_{50}$ (i.e., the tissue culture infectious dose that causes cytopathic effects in 50% of cells) units of 2009dv virus on day 42. Virus titers were measured in the lungs after challenge (*I*). Data represent means ± SEM from two to three independent experiments (n = 8–14/sex/genotype) and significant differences between groups are represented by asterisks (*).

Females produce virus-specific antibodies that are of greater quantity and quality than their male counterparts, which appears to be mediated by epigenetic regulation of *Tlr7* expression in B cells derived from females. In the absence of *Tlr7*, sex differences in vaccine-induced antibody responses and protection following challenge are reduced. Because *Tlr7* is encoded on the X chromosome and can escape X inactivation in B cells, this could represent a fundamental factor mediating higher antibody responses in females than males, which has implications in the development of autoimmunity (20, 21) as well as vaccine-induced immunity (this study).

In response to influenza A virus infection, females not only had higher antibody titers but also had greater numbers of antibody-secreting B cells in their lungs and germinal center B cells in their lymph nodes compared with males. In contrast, males and females had similar numbers of total B cells and Tfh cells in the lungs and draining lymph nodes, suggesting that sex differences are most pronounced in B cells that are activated to secrete antigen-specific antibody. Females also had larger germinal centers compared with males. Germinal center size peaks at around 30 d postinfection (22) and the germinal center B cells that compose these structures are critical for the development of high-affinity IgG and IgA antibodies, suggesting that in our model, germinal center size is associated with the generation of higher amounts of antibody in females compared with males.

Despite females having greater numbers of antibody-secreting B cells, higher antibody titers, and $CD8^+$ T cell memory responses, H1N1 infected males and females were equally protected against secondary challenge with an H1N1 drift variant virus. The equivalent protection of males and females following secondary challenge was likely due to the induction of $CD8^+$ T cells. The induction of $CD8^+$ memory T cells, including TRM cells, was greater in both males and females following influenza virus infection than vaccination. TRM cells, in particular, promote local and immediate protection in the lungs with the ability to expand rapidly, kill virus-infected cells, recruit circulating memory T cells, and release cytokines, resulting in TRMs being indispensable for protection against secondary influenza virus infection (23–25).

Previous studies have reported that following influenza infection, female mice are better protected from a heterosubtypic virus challenge compared with males (10). To expand on these data, we evaluated sex differences in protection following inactivated influenza vaccination and illustrated that females generated greater virus-specific antibody responses and were better protected against an H1N1 drift variant virus than males, which involves expression of the X-linked gene, *Tlr7*. We report a mechanism mediating sex differences in inactivated influenza vaccine efficacy and suggest that when protection depends primarily on antibody responses, then females are better protected than males. When antibody from vaccinated males or females was passively transferred into either naïve males or females, the antibody from vaccinated females provided better protection than the antibody from vaccinated males. Naïve males were better protected when they received antibody from vaccinated females than vaccinated males. The differences in antibody-mediated protection were likely due to the increased antibody titers in females as well as the induction IgG antibodies of greater avidity and isotype specificity in females compared with males. Additionally, there may be other factors specific to the female milieu (e.g., sex steroids) that could contribute to antibody function and neutralization.

The induction of class switch recombination and the generation of antigen-specific antibodies rely on TLR engagement in B cells (26). Following influenza vaccination, relative *Tlr7*, but not *Tlr8*, expression was greater in B cells derived from females compared with males. Female-biased expression of *Tlr7* in B cells was greater following receipt of inactivated whole than the inactivated subunit influenza vaccine, which may reflect that there is less viral RNA in the QIV than inactivated whole influenza vaccine or that the QIV is less immunogenic than the inactivated whole influenza vaccine. The role of TLR7 in B cells has been studied in the context of sex differences in the development of autoimmune diseases, including systemic lupus erythematosus in humans and mice (20, 21), but its role in mediating sex differences in vaccine-induced immunity has not been reported. TLR7 mediated the sex differences in antibody titers observed following influenza vaccination and protection following challenge. Deletion of *Tlr7* reduced sex differences in virus-specific neutralizing, IgG and IgG2c antibody titers and avidity as well as clearance of virus from the lungs following secondary challenge. Although TLR7 and TLR8 function similarly in

humans, the roles of these TLRs in mice are controversial and require further investigation. As with autoimmunity, the extent to which sex differences in vaccine-induced immunity are caused by direct or indirect effects of sex chromosomes, sex steroid hormones, the gut microbiome composition, or a combination of factors requires consideration (1).

One prevailing hypothesis for immunological differences between the sexes is that sex steroids influence the functioning of immune cells, although few studies have evaluated the impact of sex steroids on the immune response to vaccines. Estradiol, at physiological concentrations, can induce B cells and stimulate antibody production to an inactivated influenza vaccine in female mice (27, 28). In humans, higher serum testosterone levels are correlated with reduced neutralizing antibody responses to influenza TIV (7). Our data suggest that epigenetic factors may also contribute to sex-specific responses to vaccines. Many genes, including *Tlr7*, on the X chromosome, code for immunological proteins and can have sex-specific effects on immunity. Here, we demonstrate that following influenza vaccination, epigenetic regulation of *Tlr7* expression is greater in female- than male-derived B cells, which is associated with increased influenza virus-specific antibody titers, including isotype-switched IgG2c antibodies and protection (13, 15).

Sex differences in vaccine-induced immunity are not limited to influenza vaccines in mice, as these differences are reported in humans and nonhuman primates in response to diverse vaccines (1). There is something fundamentally different about the activity of B cells in females compared with males. Even global analyses of B cell transcriptional activity in humans reveal both broader and greater up-regulated expression of genes in B cells from females compared with males (29). A cost of higher antibody production in females may be susceptibility to antibody-mediated autoimmune diseases, including Grave's disease, systemic lupus erythematosus, and rheumatoid arthritis, all of which are more prevalent in females than males (29, 30). A notable evolutionary benefit of higher antibody production in females may be the vertical transfer of antibody to offspring (31, 32). We hypothesize that females of diverse vertebrate species have evolved to have greater antibody responses to microbes than males because transfer of antibody in utero or in milk is a fundamental means of protection of offspring early in life (33). If elevated production of antibody against influenza or other microbes in females results in greater vertical transfer of antibody, improved protection, and, hence, greater survival of offspring, then evolution will favor greater activity and specificity of B cells and antibody production in females compared with males. The data from the present study provide a foundation for considering these conserved sex differences in antibody responses in the design and implementation of diverse vaccines, including universal influenza vaccines.

Experimental Procedures

All animal procedures were approved by the Johns Hopkins University Animal Care and Use Committee under animal protocol M015H236. Please refer to *SI Appendix, Supplemental Experimental Procedures* for detailed descriptions of animals, viruses, infection, vaccination and challenge, sample collection, antibody assays, flow cytometry, immunofluorescence, B cell isolation, PCR, and bisulfite genomic sequencing.

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