PNAS
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Supplementary Information for

Biological sex affects vaccine efficacy and protection against influenza in mice

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This PDF file includes:

www.pnas.org/cgi/doi/10.1073/pnas.1805268115

Supplementary Information Text
Supplemental Experimental Procedures

Animals. Adult (7-9 weeks) male and female C57BL/6CR mice were purchased from Charles River Laboratories (Frederick, MD). *Tlr7* knockout mice on the C57BL6/NJ background were a gift from Patricia Gearhart, PhD (NIA) and bred at Johns Hopkins Bloomberg School of Public Health and adult (7-9 weeks) male and female C57BL6/NJ mice were purchased from Jackson Laboratory and used for comparison. All animals were housed at up to 5 mice per microisolator cage under standard biosafety level 2 housing conditions, with food and water provided *ad libitum*. All animal procedures were approved by the Johns Hopkins University Animal Care and Use Committee under animal protocol M015H236.

Viruses. Inactivated 2009 H1N1 was generated by infecting Madin-Darby Canine Kidney (MDCK) cells at a multiplicity of infection (MOI) of 0.01 and the infected cell supernatant was collected, centrifuged, and the virus containing supernatant was collected and inactivated with the addition of 0.05% β-propiolactone (1). The inactivated virions were purified by ultracentrifugation over a 20% sucrose cushion and the virus-containing pellet was resuspended in 1XPBS. Virus inactivation was confirmed by performing a 50% tissue culture infectious dose ($TCID_{50}$) assay following both virus inactivation and purification. To generate the 2009dv influenza A virus, viral RNA was extracted (QIAamp Viral RNA, Qiagen) from the 2009 H1N1 virus and transcribed into cDNA (SuperScript III, Invitrogen). HA specific cDNA was PCR amplified and purified on a 1% agarose gel. Purified HA cDNA was digested and cloned into the pHH21 plasmid vector (2). The K166Q HA mutation (3) was introduced by site directed mutagenesis (QuikChange Lightning Site-Directed Mutagenesis Kit, Agilent Technologies). Viruses encoding the K166Q HA mutation were generated entirely from cDNA using the 12-

plasmid rescue system (2, 4). The HA sequence of the rescued virus was confirmed by sequencing the coding region of the HA gene. Viral stocks were generated by infecting Madin-Darby canine kidney (MDCK) cells at a multiplicity of infection (MOI) of 0.01 and the infected cell supernatant was collected at 72 hours post-infection. The $TCID_{50}$ was calculated for both viruses on MDCK cells.

Infection, vaccination, and challenge. Mouse-adapted A/California/04/09 (2009 H1N1) generated by Dr. Andrew Pekosz from a published sequence (5) was used for the primary influenza infection. A mouse adapted A/California/04/09 drift variant virus (2009dv H1N1) containing the K166Q mutation of the HA sequence was previously generated in our lab by reverse genetics and used for the virus challenge (6). The inactivated 2009 H1N1 vaccine was generated by 0.05% β-propiolactone treatment of the 2009 H1N1 virus. For virus infection, mice were anesthetized and inoculated intranasally with 30µl of a sub-lethal dose of 2009 H1N1 as described previously (6, 7). For inactivated virus vaccination, mice were vaccinated intramuscularly with 20µg in 40µl at day 0 and day 21 (8-11) or mock vaccinated with 1XPBS. For challenge experiments, mice were anesthetized and inoculated intranasally with 30µl of a lethal dose of 2009dv (32 MLD_{50}) at day 42 following infection or the first dose of inactivated virus vaccination. For virus infection, mice were anesthetized and inoculated intranasally with 30µl of a sub-lethal dose of 2009 H1N1 as described previously (6, 7). For inactivated virus vaccination, mice were vaccinated intramuscularly with 20µg in 40µl at day 0 and day 21 (8-11) or mock vaccinated with 1XPBS. For challenge experiments, mice were anesthetized and inoculated intranasally with 30µl of a lethal dose of 2009dv (32 MLD_{50}) at day 42 following infection or the first dose of inactivated virus vaccination.

Virus quantification and purification. Virus was quantified and purified as previously

described (6, 7). To quantify virus titers, lung homogenates were serially diluted and plated onto a monolayer of MDCK cells in replicates of 6. Cells were incubated for 6 days at 32°C and 5% CO_2 after which the cells were fixed with 4% formaldehyde for 1h, stained with naphthol blue black (Sigma Aldrich) overnight, and scored for cytopathic effects. The Reed-Muench method was used to calculate the $TCID_{50}$ titer for each sample. For virus purification, viruses were used to infect MDCK cells at an MOI of 0.01 at 37°C for 3 days. Cell supernatants were collected and centrifuged at 500xg for 10 minutes. Virus containing supernatants were collected and purified by ultracentrifugation over a 20% sucrose cushion in a Beckman SW28TI rotor at 26,000rpm for 1 h at 4°C. The virus-containing pellet was resuspended in 1X PBS and the protein concentration was quantified by a bicinchoninic acid assay (Pierce).

Influenza quadravalent inactivated vaccine (QIV)

Male and female mice were vaccinated intramuscularly with 100 μL QIV containing 3.33 μg HA of each virus strain (A/Michigan/45/2015 (H1N1) pdm09-like virus, A/Hong Kong/4801/2014 (H3N2)-like virus, B/Brisbane/60/2008-like virus, B/Phuket/3073/2013-like virus).

Serum and bronchoalveolar lavage sample collection. Serum and bronchoalveolar lavage (BAL) were collected at relevant time-points to measure anti-2009 H1N1 IgG and IgA antibodies and anti-2009 H1N1 neutralizing antibodies as described previously (6, 7). Blood was collected by retro-orbital bleed and serum was isolated by centrifugation and stored at -80°C. For BAL collection, mice were euthanized by cervical dislocation and the lungs were lavaged twice with 0.5ml of a 0.9% saline solution. BAL fluid was centrifuged at 500xg for 10 minutes and the supernatant was collected and stored at -80°C.

Antibody neutralization. Serum and BAL neutralizing antibody titers were determined in tissue culture neutralizing assays with MDCK cells as described previously (6, 7). Serum and BAL samples were heat inactivated at 56 °C for 30 minutes and 2-fold serial dilutions were mixed with 100 $TCID_{50}$ of virus for 1 h at room temperature. The serum and virus mixture was then used to infect quadruplicate wells of confluent MDCK cells for 24 h at 32°C. Following the 24 h incubation, the inoculum was removed and the cells were washed with 1X PBS and new media was added. The cells were incubated for 6 days at 32°C and then fixed with 4% formaldehyde and stained with naphthol blue black overnight. The neutralizing antibody titer was calculated as the highest serum dilution that eliminated virus cytopathic effects in 50% of the wells.

Influenza ELISAs. Serum and BAL anti-2009 H1N1 IgG and IgA ELISAs were performed as described previously (6, 7). ELISA plates (Microlon 96 well high binding plates; Greiner Bio-One) were coated with 100ng of purified virus (as described above) overnight at 4°C in pH 9.6 carbonate buffer. Plates were washed with PBST (1X PBS + 0.1% Tween-20) and blocked for 1 h at 37°C with 10% nonfat milk in 1X PBS. After washing, serum samples were serially diluted and added to the wells for 1 h at 37°C. Following washing, anti-mouse horseradish peroxidase (HRP) conjugated IgG, IgG1, or IgG2c was added for 1 h at 37°C. The plates were washed with PBST and reactions were developed with 3,3',5,5' tetramethylbenzidine (TMB, BD Biosciences) and stopped using 1N hydrochloric acid (HCl). Plates were read at 450 nm absorbance and antibody titers were calculated as the highest serum dilution with an OD value above 3 times the average OD of the negative controls. For the anti-2009 H1N1 IgA ELISA, plates were washed 3 times with TBST (1X TBS + 0.1% Tween-20) and blocked with 10% nonfat milk in 1XTBS. Serially diluted BAL samples were added to the wells for 1 h at 37 °C

after which plates were washed and anti-mouse IgA (1:2,000, Southern Biotech) was added for 1 h at 37°C. Reactions were developed with p-nitrophenyl phosphate substrate (PNPP, Thermo) and stopped using 2N NaOH. Plates were read at 405 nm absorbance and antibody titers were calculated as the highest serum dilution with an OD value above 3 times the average OD of the negative controls.

Influenza ELISpot. Anti-influenza IgA secreting B cells were enumerated using the Mouse IgA ELISpot Basic kit (Mabtech). Briefly, purified 2009 H1N1 virus (5µg/mL in sterile 1XPBS) was added to polyvinylidene fluoride (PVDF) plates (Millipore) and incubated overnight at 4°C. B cells were isolated from mouse lungs using the STEMCELL Technologies EasySep Mouse B Cell Isolation kit (STEMCELL Technologies) and viable B cells were added to the wells in triplicate and allowed to incubate for 24 h at 37°C + 5% CO_2. Anti-mouse IgA biotinylated (1µg/mL, Mabtech) was added to the wells for 2 h, followed by the addition of Streptavidin-horseradish peroxidase (HRP) (1:500, Mabtech) 1 h. Plates were developed with 3,3' 5,5' tetramethylbenzidine (TMB) until distinct spots emerged and the plates were analyzed using the ImmunoSpot analyzer and software.

Flow cytometry. For 2009 H1N1-specific CD8+ T cell populations, lung cells were isolated and stained as previously described (6). For germinal center B cell (12), CD4+ T cell, and T follicular helper cell enumeration, lymph nodes were excised and single cell suspensions were made in FACS buffer (1XPBS, 2% heat inactivated FBS, 10mM Hepes). Viability and total cell counts were determined as described previously (6). Data were acquired using a FACS Fortessa (FACS DIVA Software) and analyzed using FlowJo v.10 (Tree Star, Inc.). Total cell numbers were determined by multiplying each live cell population percentage by the total live cell counts acquired by trypan blue

exclusion counts on a hemocytometer.

For influenza-specific CD8+ T cell populations, lungs were excised and single-cell suspensions were generated following red blood cell lysis. Cell viability was determined using trypan blue (Invitrogen) staining and counted using a hemocytometer. Cells were resuspended at $1x10^6$ cells/ml in RPMI 1640 (Cellgro) supplemented with 10% FBS (Fisher Scientific) and 1% penicillin/streptomycin. Cells were cultured for 5h with 2009 H1N1 peptide antigen ($NP_{366-374}$, ProImmune), PMA (Sigma) and Ionomycin (Sigma), or media alone (unstimulated) in media containing Brefeldin A (GolgiStop, BD Biosciences). Cell viability was determined by fixable Live/Dead aqua viability dye (Invitrogen) and Fc receptors were blocked using anti-CD16/32 (BD Biosciences). The CD8+ T cell populations were stained with the following antibodies: AF700 conjugated anti-CD4 (RM4-5, BD Biosciences), PerCP-Cy5.5 conjugated anti-CD8 (53-6.7, Ebiosciences), APC conjugated anti-CD44 (IM7, BD Biosciences), eVolve 605 conjugated anti-CD62L (MEL-14, Ebiosciences), eFluor 450 conjugated anti-CD69 (H1.2F3, Ebiosciences), PE conjugated anti-CD103 (M290, BD Biosciences), PE conjugated $D^bNP_{366-374}$ tetramer for 2009 H1N1 (ASNENVETM, NIH Tetramer Core Facility). Intracellular staining with PeCy7 conjugated anti-IFN-γ (XMG1.2, BD Biosciences), FITC conjugated anti-TNF-α (MP6-XT22, BD Biosciences), was performed following permeabilization and fixation with Cytofix/Cytoperm and Perm/Wash buffer (BD Biosciences).

The B cell populations were stained with Pacific Blue anti-CD4 (GKI.5, Biolegend), PerCP/Cy5.5 conjugated anti-B220 (RA3-6B2), and Alexa Fluor 488 conjugated anti-PNA (ThermoFisher). The T follicular helper cell populations were stained with Pacific Blue conjugated anti-CD4 (GKI.5, BioLegend), PerCP/Cy5.5 conjugated anti-B220 (RA3-6B2, BioLegend), biotinylated anti-CD185 (CXCR5)

(SPRCL5, Ebioscience), and PE-Cy7 conjugated anti-CD279 (PD-1)(J43, Ebioscience). Intracellular staining with PE conjugated anti-BCL6 (K112-91, BD Biosciences) was performed following permeabilization and fixation with Fixation/Permeabilization (Ebioscience) buffers.

Immunofluoresence. Mediastinal lymph nodes (mLN) were embedded and frozen in optimal cutting temperature compound before sectioning. Sections were incubated with primary antibodies (IgD, eBioscience) at 4°C overnight and the following day incubated for 3 h at room temperature in fluorochrome-conjugated secondary antibodies (Peanut agglutinin (PNA)-FITC, anti-rat Alexa Fluor 488). Sections were mounted with anti-fade medium containing 4',6-diamidino-2-phenylindole (DAPI) (ProLong Gold anti-fade CST 8961S). Researchers were blinded to the sex of the animals from which mLN sections were obtained. Sections were viewed using a Zeiss Axioplan 2 microscope (Jena, Germany) and images were taken using a Zeiss AxioCam MRM. The area of each germinal center was calculated using ImageJ (NIH) software and normalized (divided by) to the total area of the lymph node section.

Passive serum transfer. Male and female mice were vaccinated with inactivated 2009 H1N1 and serum was collected at 42 days post-vaccination. Serum was pooled by sex and an ELISA was performed to determine serum IgG titers prior to transfer. Naïve male and female mice were injected I.P. with 150µL of serum from the vaccinated male or female mice (13); thus, male mice received either pooled male or female serum, and female mice received either pooled male or female serum. Mice were rested for 3 hours and then challenged with 10^5 $TCID_{50}$ 2009dv. Mice were euthanized 3 days post challenge and virus titers in the lungs were measured.

Influenza avidity. ELISA plates were coated with 100ng of purified virus (as described above) overnight at 4°C in pH 9.6 carbonate buffer. Plates were washed 3 times with PBST and blocked for 1 h at 37°C with 10% nonfat milk in 1X PBS. Plates were washed 3 times and serum samples were added to the wells at a 1:60 dilution for 1 h at 37°C. To measure antibody avidity, 4M ammonium thiocyanate (NH_4SCN, Sigma) or 1XPBS was added to the plates for exactly 15 minutes at room temperature. The pates were washed 8 times with PBST and anti-mouse HRP conjugated secondary IgG (1:250, Thermo) was added for 1 h at 37°C. The plates were washed 3 times with PBST and reactions were developed with TMB and stopped using 1N HCl. Plates were read at 450 nm absorbance and the antibody avidity index was determined by normalizing the NH_4SCN treated absorbance values to the corresponding 1XPBS (untreated) values for each sample in duplicate.

B cell isolation and real-time RT-PCR. Vaccinated or mock-vaccinated male and female mice were euthanized 28dpv and spleens were excised and single cell suspensions were generated following red blood cell lysis. B cells were isolated using the STEMCELL Technologies EasySep Mouse B Cell Isolation kit (STEMCELL Technologies) and RNA was extracted and purified using the PureLink RNA Mini Kit (Invitrogen). cDNA was generated and used to asses TLR7 and TLR8 gene expression by qPCR. Pre-designed TLR7 and TLR8 specific primers were purchased from integrated DNA Technologies (Mm.PT.58.10526075). Relative TLR7 and TLR8 gene expression was normalized to *Gapdh* and mock-vaccinated animals using the ΔΔCT method.

Bisulfite genomic sequencing

CpG site-specific DNA methylation status of 5' regulatory region for Tlr7 was assayed by bisulfite genomic sequencing. In silico analyses were used to reveal Cytosine Guanine dinucleotides (CpG) in 5' flanking region of Tlr7 (NC_000086.7. 167331574-167330824) A total of 5 CpG sites were found in this region. PCR primers were designed to amplify region spanning from -808 to -276 bp from the transcription start site (TSS), which contains 5 CpG sites. Primer sequences were BSPCR_Tlr7-P1-Forward: 5'-TTTGGAAATTAAAGTGAGGGATTTA -3', BSPCR_Tlr7-P1-Reverse: 5'-AAAAAAATTTCAAATACACACCCTATC-3'. In brief, 200 ng of genomic DNA was used for bisulfite treatment using the EZ DNA Methylation™ Kit (Zymo Research, Irvine, CA). The bisulfite treated DNA was amplified with PCR primers using GoTaq Green Master Mix (Promega, WI) and optimized PCR conditions (95°C for 10 minutes, 35 cycles of 95°C for 1 minute, 62°C for 2 minute and 72°C for 2 minutes, followed by an extension at 72°C for 12minutes). PCR products were purified (GeneJET Gel Extraction Kit, ThermoFisher Scientific, MA) and subcloned into pCR 2.1 vector (ThermoFisher Scientific, MA). 8 clones from each sample were sequenced (Macrogen, MD) to obtain direct measures of DNA methylation at each CpG site. Sequencing data was analyzed with the QUMA (14) to examine the methylation status.

Statistical Analyses. Changes in body mass were analyzed with a MANOVA followed by multiple comparisons. Antibody titers, virus titers, or cell populations were analyzed using two-way ANOVAs or t-tests and significant interactions were further analyzed using the Bonferroni post hoc test for pairwise multiple comparisons. Mean differences were considered statistically significant if $p<0.05$.

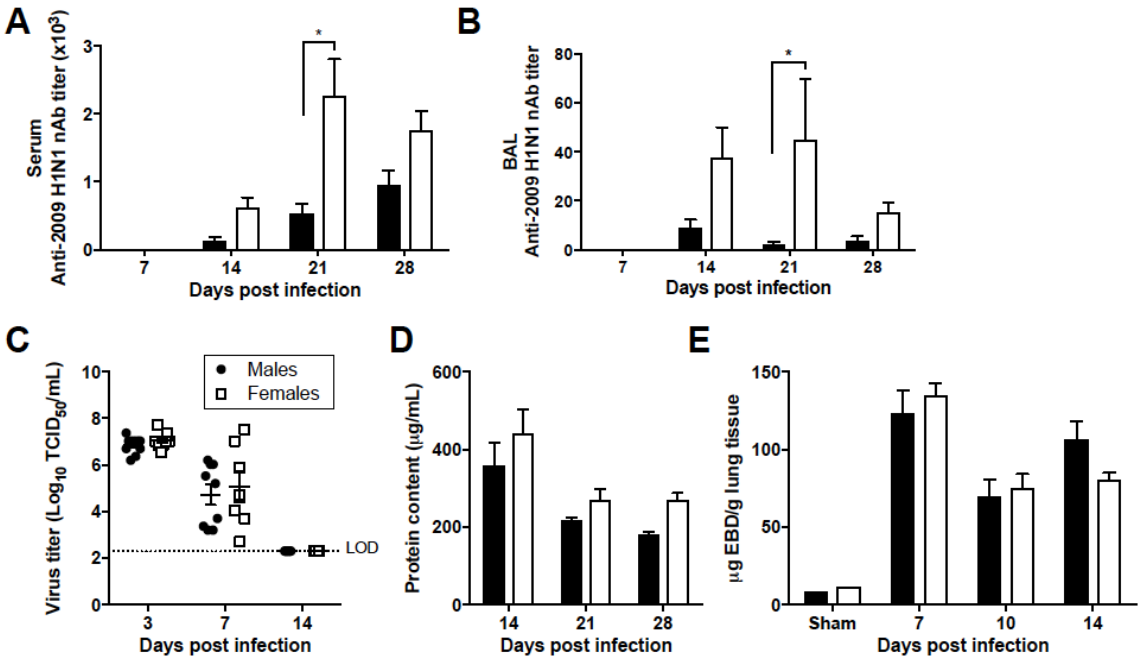


Figure S1.

Adult male (shaded) and female (open) mice were inoculated intranasally with 10 $TCID_{50}$ units of 2009 H1N1 virus. Serum was collected at 7, 14, 21, and 28 days post infection (dpi) and anti-2009 H1N1 neutralizing antibody titers were measured (A). Bronchoalveolar lavage (BAL) fluid was collected at 7, 14, 21, and 28 dpi and analyzed for anti-2009 H1N1 neutralizing antibody titers (B). Infectious virus titers in the lungs were measured at 3, 7, and 14 dpi. The stippled line represents the limit of detection (LOD) (C). Bronchoalveolar lavage fluid was collected at 14, 21, and 28 dpi and total protein content was measured by BCA assay (D). The amount of Evans Blue dye present in the lungs, and indicator of vascular leakage, was quantified at 7, 10, and 14 (E). Data represent means ±SEM from two or three independent experiments (n = 9-10/sex) and significant differences between males and females are represented by asterisks (*).

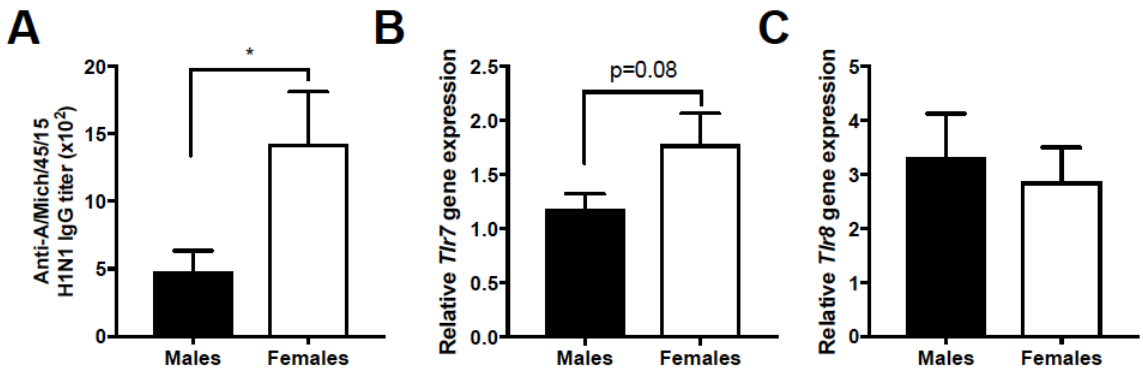
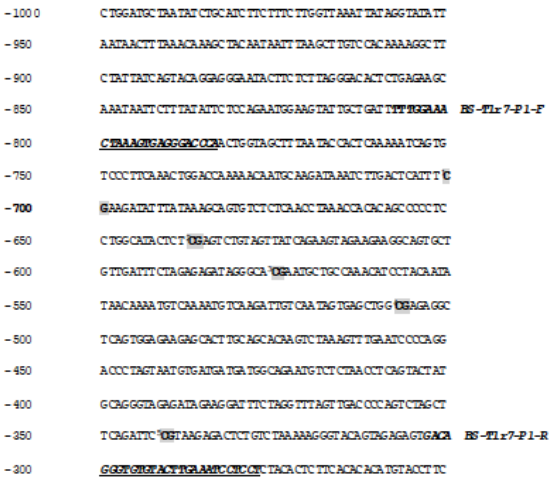


Figure S2.

Adult male and female mice were vaccinated intramuscularly with 100µL QIV containing 3.33µg HA of each virus strain (A/Michigan/45/2015 (H1N1) pdm09-like virus, A/Hong Kong/4801/2014 (H3N2)-like virus, B/Brisbane/60/2008-like virus, B/Phuket/3073/2013-like virus). At 28 days post vaccination, serum anti-A/Michigan/45/2015 H1N1 IgG titers were measured (A) and RNA was isolated from splenic B cells to measure relative *Tlr7* (B) and *Tlr8* (C) gene expression using the ΔΔCT method. Data represent means ±SEM from n=10/sex and significant differences between groups are represented by asterisks (*).

A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-850 AAAT AATT CTT TAT ATTC TCC AGAATGG AAG TAT TGCT GAT TTT TGGAAA BS-T1x7-P1-F
-800 CTAAAGTGAGGGACCCA ACTG GTA GCTT TAA TAC CACT CAA AAA TCAG TG
-750 TCCC TTCA AAC TGG ACCA AAA ACAATGC AAGATA AATC TTG ACT CATT T C
-700 GAAGATAT TTA TAA AGCA GTG TCT CTCA ACC TAA ACCA CAC AGC CCCC TC
-650 C TGG CATA CTC T CGAGT CTGT AGT TAT CAGAAGT AGAAGAA GGC AGT GCT
-600 G TTG ATTT CTA GAG AGAT AGG GCA CGA ATGC TGC CAA ACAT CCT ACA ATA
-550 T AAC AAAA TGT CAA AATG TCA AGA TTGT CAA TAG TGAG CTG G CGAGA GGC
-500 TCAG TGGA GAA GAG CACT TGC AGC ACAA GTC TAA AGTT TGA ATC CCCA GG
-450 ACCC TAGT AAT GTG ATGA TGA TGG CAGA ATG TCT CTAA CCT CAG TACT AT
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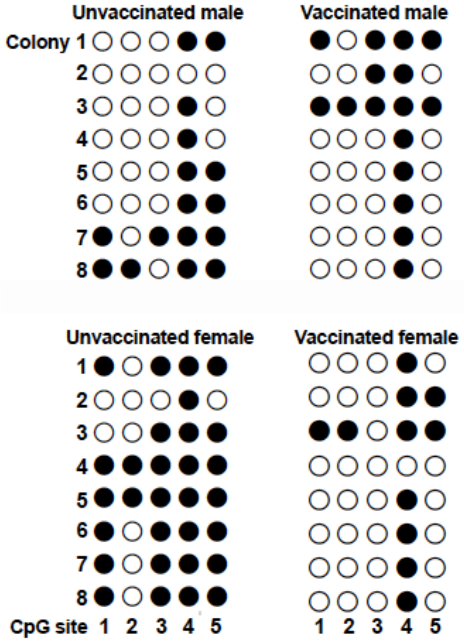
B



C

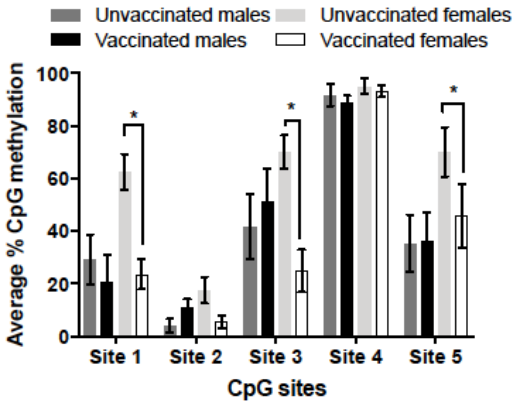


Figure S3.

Genomic DNA sequence of the 5' flanking region of Tlr7 (NC_000086.7. 167331574-167330824) (A). The sequence of the primers used for bisulfite genomic sequencing (BS-Tlr1-P1-F, BS- Tlr7-P1-R; and BS-Tlr1-P2-F, BS- Tlr7-P2-R) are underlined and in italic bold type. There are 5 CpG nucleotides (shaded in gray) located at the promoter region of *Tlr7* encompassing transcription start site (TSS). Methylation status of individual CpG site at the 5' flanking region of *Tlr7* in B cells isolated from spleen tissues was assayed by bisulfite sequencing. Representative images from clones obtained from vaccinated and unvaccinated males and females (n=5-9/sex). Unmethylated (open circles) or methylated (solid circles) CpGs are indicated. Each row of circles represents an individual clone sequenced, and 8 individual clones from each mouse were picked for sequencing. Percentage methylation of CpG sites along the 5' regulatory region was assayed by bisulfite genomic sequencing (C). Bar represents the mean of methylation level in each group, unvaccinated males (n=6), vaccinated males (n=9), unvaccinated females (n=5) and vaccinated females (n=9). Data represent means ±SEM from two independent experiments and significant differences between vaccinated and unvaccinated females are represented by asterisks (*).

Table S1. Male and female mice were infected with either 10 $TCID_{50}$ 2009 H1N1 at day 0 or vaccinated with inactivated 2009 H1N1 at days 0 and 21. On day 42 post infection or vaccination, mice were euthanized and lungs were excised for CD8+ T cell enumeration. Single cell suspensions were stimulated *ex vivo* with 2009 H1N1 specific NP antigen (ASNENVETM) in the presence of BFA. Total numbers of live CD8+ tetramer+ cells, CD8+ cells, CD8+ IFN-γ+ cells, CD8+ TNF-α+ cells, CD8+ IFN-γ+ TNF-α+ cells, CD8+ memory cells, CD8+ memory IFN-γ+ cells, CD8+ memory TNF-α+ cells, CD8+ memory TNF-α+ IFN-γ+ cells, CD8+ tissue resident memory (TRM) cells, and CD8+ central memory (TCM) cells were quantified by flow cytometry. Data represent means ±SEM from two independent experiments (n=8/sex/treatment) and significant differences ($p<0.05$) between males and females within the group of infected animals are represented by asterisks (*) and significant differences ($p<0.05$) between animals that were either infected or vaccinated are represented by pound (#).

Total Cells	Sex	Infection	Vaccination
CD8+ tetramer+ T cells	Males	102.5 ± 10 #	25.0 ± 5 #
	Females	145.2 ± 5 *#	57.2 ± 9 #
CD8+ T cells	Males	4136.3 ± 463	3416.5 ± 337
	Females	6271.2 ± 768 *	4957.0 ± 544
CD8+ IFN-γ+ T cells	Males	20.0 ± 2	6.8 ± 1 #
	Females	33.3 ± 6 #	14.4 ± 4 #
CD8+ TNF-α+ T cells	Males	92.8 ± 13 #	40.2 ± 8 #
	Females	95.8 ± 13 #	44.69 ± 7 #
CD8+ IFN-γ+ TNF-α+ T cells	Males	56.0 ± 12	28.6 ± 5
	Females	67.1 ± 6 #	28.4 ± 6 #
CD8+ memory T cells	Males	1347.8 ± 56	1000.6 ± 120
	Females	2385.9 ± 272 *#	1378.9 ± 126 #
CD8+ IFN-γ+ memory T cells	Males	28.1 ± 7	4 ± 1
	Females	55.9 ± 19 #	12.0 ± 2 #
CD8+ TNF-α+ memory T cells	Males	44.1 ± 4 #	13.63 ± 2 #
	Females	58.3 ± 2 #	16.38± 3 #
CD8+ IFN-γ+ TNF-α+ memory T cells	Males	24.9 ± 5	7.56 ± 2
	Females	39.0 ± 12 #	6.6 ± 1 #
CD8+ TRM cells	Males	53.0 ± 9 #	1.1 ± .5 #
	Females	179.7 ± 41 *#	4.1 ± 1 #
CD8+ TCM cells	Males	557.7 ± 42	490.5 ± 49
	Females	931.3 ± 132 *#	567.4 ± 36 #

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