

Pre-existing Immunity to H1N1 Influenza Virus Improves Antibody Response to Vaccination through Germinal Center Regulation

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Article

Keywords:

Posted Date: July 15th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-10083604/v1>

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Additional Declarations: Competing interest reported. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. AAK has received personal fees from Vaccine for serving as an associate editor and from Seqirus outside the submitted work. AK and GSM are shareholders of the company BioForge Canada Limited. BioForge Canada Limited is a company that uses bioinformatics in immunological approaches in the monitoring, prevention, and treatment of infectious diseases. The authors disclose that the interests of BioForge Canada Limited had no impact on this study. AK is a Moderna Global Fellow (2024-MGF-000316 [91353]).. All other authors have no relationships to declare.

Version of Record: A version of this preprint was published at npj Vaccines on September 26th, 2026. See the published version at <https://doi.org/10.1038/s41541-026-01586-7>.

Abstract

Influenza viruses circulate annually in humans, accumulating mutations in the viral genome. In real world conditions, people develop an immune history from multiple influenza virus infections and vaccinations, yet most experimental studies utilize naïve hosts to investigate viral pathogenesis or vaccine efficacy; a scenario that does not reflect actual human exposure and immune responses. Here, we report that mice previously infected with the mouse-adapted H1N1 virus mounted an increased antibody response after vaccination with the split virion quadrivalent seasonal influenza vaccine. Antibodies targeting the H1 antigen component of the vaccine in H1N1 preimmune mice had titers peaking at 8 HAI units versus 2 HAI units in naïve-vaccinated mice at 14 days post-vaccination, whereas H3N2 pre-immune mice showed no improved vaccine responses. Increased IgG class-switched isotype antibodies in the H1N1 pre-immune-vaccinated mice correlated with protection from weight loss and mortality when these mice were challenged with an H1N1 pandemic 2009 strain. Temporal sampling and analysis of RNA from mediastinal lymph nodes from preimmune mice by RNA-seq at time points following vaccination revealed distinct regulation of lymphocyte and germinal center-specific genes, including BCL6, an essential gene for germinal-center-dependent B-cells. Taken together, we demonstrate that H1N1 A/FM/1/1947 pre-immunity led to a more efficient vaccine response, possibly regulated by germinal center mechanisms in the mediastinal lymph node; a finding with implications for future vaccine design.

Introduction

Influenza virus infection remains an unsolved public health hazard, despite ongoing vaccination efforts ¹. Influenza A and B viruses (family of *Orthomyxoviridae*), each with its own subtypes and lineages, consist of eight single-stranded RNA segments that can express up to 12 proteins ². Of these proteins, the Haemagglutinin (HA) and Neuraminidase (NA) proteins are the immunologically dominant surface proteins on the envelope of the virion that designate subtypes such as H1N1 and H3N2 ². Influenza viruses are prone to genetic variability through two mutational mechanisms: antigenic shift and antigenic drift ³. Antigenic “drift” describes discrete substitutions in viral surface antigens enabled by an error-prone viral polymerase that cause evasion of neutralizing antibodies, whereas antigenic “shift” describes more profound changes in viral surface antigens that result from the exchange of viral genome segments between different viruses as they infect the same cell at the same time ^{2,3}. In effect, repeated influenza exposures build a complex immune “imprint” (original antigenic sin) so that prior strain exposures bias which B cell clones are recalled in later responses ^{4,5}. How immune backgrounds shaped by previous exposures influence immune outcomes in response to past and future epidemic influenza strains is not understood ^{6,7}.

Due to strain diversity, immunity to one strain does not necessarily confer immunity to other strains, thereby allowing the continual circulation of seasonal influenza viruses, reinfection of susceptible hosts, and the potential development of severe respiratory disease in those infected. To control morbidity and mortality, advisory bodies such as the National Advisory Committee on Immunization (NACI) of Canada

and the World Health Organization (WHO) suggest vaccination as the most effective strategy ^{1,8}. Despite epidemiology supporting vaccination as a means of decreasing influenza morbidity ⁹, seasonal vaccines are not 100% preventative, due in part to vaccine mismatches with circulating strains and low induction of mucosal immunity, resulting in annual hospitalizations and deaths even in the vaccinated population ¹⁰. Previous influenza virus infections can also affect vaccine performance; however, the mechanisms driving this preimmune regulation of vaccine outcome have not been elucidated ¹¹.

The early stages of influenza virus infection or vaccination are initially defined by innate immune responses ¹². Subsequently, there is a shift to adaptive cellular (cytotoxic CD8⁺ T cells) and humoral responses (antibody-producing B cells) that move through phases of expansion, contraction, and memory to establish pathogen-specific responses and long-term protection ¹³. Following infection or vaccination, antibodies, some of which have neutralizing properties, are produced against viral surface proteins. Upon re-exposure to an antigenically identical virus, neutralizing antibodies can achieve sterilizing immunity ^{14,15}. However, repeated vaccination to the same influenza strains three or more times consecutively may actually “blunt” antibody responses to subsequent infection with the same strain, suggesting frequent updating of vaccines and cross-immunity between strains may be preferred ¹⁶. Alternately, it has also been shown that exposure to antigenically distinct strains sometimes elicits non-sterilizing memory B cell and CD8⁺ T-cell responses, leading to partial or no protection ¹⁷. Influenza strain-specific memory B cells are lifelong and are generated through a process called *imprinting* or the host’s first infection, rendering the host preimmune ^{2,18–20}. At secondary exposure, memory B cells can either undergo affinity maturation for antigen refinement (germinal center-dependent memory cell development) or transition directly to long-lived plasmablasts (germinal center-independent memory cell development) ²¹. Affinity maturation is regulated by key cells and immune mediators such as T follicular helper (T_{FH}) cells, a specialized subset of CD4⁺ T helper cells that reside in the germinal center ^{21–24}. T_{FH} cells, as well as developing germinal center B cells, are both dependent on B cell lymphoma 6 (BCL6), a master transcriptional regulator that drives responses of these cells ^{25,26}.

Human studies have revealed that preimmunity impacts antibody production in response to influenza virus vaccination ^{27–29}. Moreover, we previously modelled the preimmune impact on influenza virus vaccination in ferrets, showing that previous exposure to a historic H1N1 influenza virus leads to increased vaccine-elicited protection by influencing antibody isotypes ³⁰. In the present study, we seek to determine the mechanisms regulating preimmune B cells and, potentially, the germinal center reaction during influenza vaccination in C57BL/6j mice. To this end, C57BL/6J mice were imprinted with a historical mouse-adapted H1N1 virus and then vaccinated with a contemporary human influenza virus vaccine, FLUZONE Quadrivalent (FLUZONE® QIV) influenza virus vaccine. H3N2 imprinted mice were also used as a control comparison at vaccination. Responses to vaccination were then characterized in the H1N1 preimmune model, and mice were subsequently challenged with the 2009 pandemic H1N1 virus to assess vaccine effectiveness.

Results

H1N1 FM/47 preimmune mice have early, robust antibody response to vaccination.

Most humans have been exposed to influenza viruses or their antigens through infection or vaccination; however, how prior exposure to influenza antigens impacts vaccine outcomes is poorly understood. We established a preimmune mouse model of A/Fort Monmouth/1/1947 H1N1 (hereafter H1N1 FM/47) based on our previously established ferret model³⁰ (Fig. 1A). Adult, female C57Bl/6J mice were imprinted on day 0 with H1N1 FM/47 at an infectious dose of $10^{3.5}$ EID₅₀ to induce preimmune development. Mice recovered for 60 days, allowing innate immune responses to return to baseline and for adaptive memory to develop. On day 60 post-imprinting (pi), mice were vaccinated with FLUZONE® QIV and subsequently boosted on day 74 pi. On day 105 pi, mice were challenged with a lethal dose (10^6 EID₅₀) of 2009 pandemic H1N1, A/Mexico/4108/2009 (Mex/09), to assess the vaccine protection³¹. Naive-mock vaccinated, naive-vaccinated, and H1N1 FM/47 preimmune-mock vaccinated mice were also assessed as controls (Fig. 1B). Initially, H1N1 FM/47 virus imprinting at the sublethal dose was characterized in comparison to the lethal infectious dose. Mice imprinted with the sublethal dose of H1N1 FM/47 lost 10% of their original weight and had 100% survival, shown in comparison to mice inoculated with a higher lethal dose (10^6 TCID₅₀) (Supplementary Fig. 1A and B). Lung viral titer following inoculation with the sublethal dose peaked on day 3 pi (10^6 TCID₅₀), and high hemagglutination inhibition (HAI) antibody titers were evident by day 14, supporting the immune imprint (Supplementary Fig. 1C and D). On day 7 pi, there was infiltration of inflammatory cells and sloughing of epithelial cells into the bronchiolar lumen, which resolved by day 14 pi, as determined by histopathology and by upregulation of the inflammatory genes IL-6, IFN- γ , and TNF in the lung (Supplementary Fig. 1E and F).

After determining that H1N1 FM/47-induced immunity was imprinted, we assessed vaccine responses to the Sanofi quadrivalent split-virion seasonal influenza vaccine in H1N1 FM/47 preimmune (referred to as pre-vaccinated in the figures) mice vaccinated 60 days after H1N1 FM/47 infection and compared these responses with those in naïve-vaccinated mice. As expected, no significant weight changes were observed after vaccination (Supplementary Fig. 2A and B). Serum samples were collected at days 2 (62 pi), 5 (65 pi), 7 (67 pi), and 14 (74 pi) post-vaccination (pv), and HAI assays were performed against the H1 vaccine component. H1N1 FM/47 preimmune mice exhibited rapid antibody induction, with antibodies specific for the vaccine antigen (2009 H1N1) detectable in all mice by day 5 (65 pi) pv, increasing to 8 HAI units by day 14 (74 pi) (Fig. 2A, triangles, left panel). By contrast, naïve-vaccinated mice did not develop detectable 2009 H1-specific antibodies until day 14 pv (circles) with significantly lower titers (2 HAI units). Moreover, antibody titers against the imprinting virus H1N1 FM/47 remained stable since the imprinting event over the 60-day rest and were not boosted following vaccination, suggesting no detectable antibody cross-reactivity or back boosting (Fig. 2A, right panel).

Given the enhancement of vaccine-induced antibody responses observed following H1N1 FM/47 imprinting, our subsequent analyses focused on defining the immunological features associated with the

H1N1 response by examining the systemic immune environment associated with this accelerated response. Systemic analysis of serum inflammatory cytokine production post-vaccination through cytometric bead array (CBA) showed differing profiles in H1N1 FM/47 preimmune-vaccinated and naïve-vaccinated mice (Fig. 2B). H1N1 FM/47 preimmune-vaccinated mice had high proinflammatory cytokines: IL-6 (~ 25 pg/ml), MCP-1 (~ 40 pg/ml), and anti-inflammatory IL-10 (~ 500 pg/ml), peaking on day 2 pv (62 pi), whereas cytokine levels in naïve-vaccinated mice did not reach apex until day 14 pv (74 pi). Interestingly, while both groups of mice had detectable IL-12 concentrations on day 5 pv (65 pi), H1N1 FM/47 preimmune-vaccinated mice produced approximately fivefold higher levels than naïve mice (> 100 pg/mL). Antibody isotypes and subclasses were next evaluated in the serum as IgM is typically produced after a novel antigen exposure and IgG subclasses later as a result of class-switch recombination^{21,32}. H1N1 FM/47 preimmune mice had significantly higher concentrations of IgG1 (1300 ng/μL) and IgG2b (700 ng/μL) compared to naïve mice (~ 600 ng/μL for both isotypes) by day 14 pv (Fig. 2C). Changes in concentration were also observed for other isotypes, although only IgG2ak showed a significant change (Supplementary Fig. 2C). IgG1 and IgG2b concentrations peaked at day 14 pv at over 750 ng/μL, while IgG3 reached its apex on day 65 in H1N1 FM/47 preimmune-vaccinated mice, at 30 ng/μL. H1N1 FM/47 preimmune mice minimally increased IgM after vaccination at approximately 20 ng/μL. Conversely, naïve-vaccinated mice had higher IgM concentrations post-vaccination (Fig. 2C). Taken together, analyses of cytokine production and antibody isotypes and subclasses indicated that H1N1 FM/47 preimmune mice have a faster and more mature immune response, suggesting possible germinal center activation after vaccination.

To further understand if the influence of imprinting on vaccine responses was specific to H1N1 FM/47 or if it could also be recapitulated with other influenza virus subtypes, we, in parallel, imprinted mice with an H3N2 virus (H3N2 A/HongKong/1/1968 (HK/68)) (Fig. 3A) and vaccinated with FLUZONE® QIV on day 60 pi. Infectious H3N2 HK/68 virus and viral RNA were detected in the lungs of H3N2 HK/68-inoculated mice on day 3 pi, providing evidence that the mice were infected with the virus (Fig. 3B and C). Additionally, antibodies specific for H3N2 HK/68 were detected at ~ 5 HAI Units by day 14 pi, confirming that the virus imprinted the immune cells of the mice (Fig. 3D). Surprisingly, when evaluating antibodies elicited following vaccination with FLUZONE® QIV, mice with prior H3N2 HK/68 infection did not have detectable HAI titers against H1 or H3 vaccine components by day 7 pv (day 67 post H3N2 inoculation) (Fig. 3E). These results contrasted with vaccine responses in H1N1 FM/47 imprinted mice, which had augmented antibody responses to the H1 component of the seasonal vaccine. Therefore, from this data, we continued to focus on vaccine responses and outcomes in H1N1 FM/47 imprinted mice only.

H1N1 FM/47 preimmune-vaccinated mice have less morbidity and mortality compared to naïve-vaccinated mice during pandemic H1N1 Mex/09 influenza virus challenge.

To assess the effect of H1N1 FM/47 preimmunity on vaccine-mediated protection, all groups of mice, including both H1N1 FM/47-imprinted and naïve animals, were challenged on day 105 post initial infection with a lethal dose of H1N1 Mex/09 (10^5 TCID₅₀). Body weight and survival were monitored for 14 days post-challenge (pc). All control groups had significant weight loss after the challenge, with all

naïve-vaccinated mice reaching the humane endpoint of 80 percent baseline weight by day 8 pc (113 pi). By contrast, the H1N1 FM/47 preimmune-vaccinated mice did not lose weight or succumb to infection (Fig. 4A). Percentage survival analysis revealed that naïve-vaccinated mice had a survival rate of only 50% post-challenge, whereas H1N1 FM/47 preimmune-vaccinated mice were fully protected (Fig. 4B). H1N1 FM/47 preimmune-mock vaccinated mice also experienced significant weight loss; however, none of the mice in this group reached humane endpoint. Investigation of active viral infection as a determinant of susceptibility and protection revealed that all groups had detectable infectious H1N1 Mex/09 virus and viral RNA in their lungs following challenge with H1N1 Mex/09 (Fig. 4C and D). However, the H1N1 FM/47 preimmune-vaccinated mice had a lower infectious titer of 4 TCID₅₀/mL on day 3 pc (108 pi), which resolved faster than the other groups. Histopathological assessment by H&E staining of lung tissue revealed that alveolar consolidation (blue arrows) and hemorrhage (red arrows) were common responses to challenge across all groups. Both H1N1 FM/47 preimmune-mock vaccinated and naïve mice showed consolidation in the alveolar space from day 106 pi (one day pc) onward, which did not resolve by day 14 pc (119 pi). Naïve-mock vaccinated mice had observable consolidation on day 7 pc (112 pi). Alveolar consolidation was observed only in the H1N1 FM/47 preimmune-vaccinated mice on day 7 pc (112 pi) resolving by day 14 pc (119 pi). Unlike other control groups which all had hemorrhaging, H1N1 FM/47 preimmune-vaccinated mice did not have signs of hemorrhage (red arrows) or any fibrin accumulation (black arrows) in the lungs, and while epithelial cell sloughing (black arrowhead) was observed on day 1 pc (106 pi) with peribronchial infiltration (blue arrowhead) observed on days 1 pc (106 pi) and 3 pc (108 pi) with these features resolved by day 14 pc (119 pi). Taken together, the clinical outcomes and viral titer results suggest that H1N1 FM/47 preimmune-vaccinated mice had improved clinical outcomes compared to all control groups.

Cytokine responses post-challenge were examined in the lung and serum to determine potential host mechanisms of protection, pathogenesis, and disease. In the lung, proinflammatory cytokine gene expression for IL-6, TNF, and IFN- γ was assessed, given their roles in influenza pathogenesis, as previously reported^{33–35}. IL-6 expression was increased in the lungs of H1N1 FM/47 preimmune-vaccinated mice on day 7 pc (day 112 pi) (10 x), while all other groups had an earlier and greater increase in IL-6 by day 3 pc (day 108 pi) with a fold change over 100 relative to non-infected baseline (Fig. 5A). Similarly, maximum IFN- γ and TNF expression was observed in all groups on day 3 pc (day 108 pi); however, the H1N1 FM/47 preimmune-vaccinated mice had significantly lower fold changes than the other groups. Systemic presence of both protein concentrations pro- and anti-inflammatory cytokines in sera, determined by CBA assay, revealed that while H1N1 FM/47 preimmune-vaccinated mice had lower concentrations of proinflammatory cytokines IL-6 and MCP-1 compared to other groups, IL-10 (day 3 pc) and IL-12 (all time points) were increased compared to H1N1 FM/47 preimmune-mock vaccinated and naïve-vaccinated mice (Fig. 5B).

Antibody dynamics were assessed in all groups to confirm preimmune status on day 105 pi for H1N1 FM/47 infection and vaccination, and to investigate the dynamics of antibody regulation post-challenge. HAI analysis of serum antibodies found that all preimmune groups had high levels of H1N1 FM/47

antibodies at the time of challenge (Fig. 5C). Furthermore, all vaccinated mice had significant antibody levels reactive against the 2009 H1N1 pandemic virus Mex/09 on challenge day, as the challenge virus matched the H1 component of the vaccine. Interestingly, H1N1 FM/47 preimmune-mock vaccinated mice did not have cross-reactive antibodies as determined by HAI at the time of challenge. Mex/09 titers did not increase until day 14 pc in this group of mice, suggesting *de novo* antibodies were elicited following challenge (Fig. 5D). Antibody isotype and subclass analysis post-challenge revealed that IgM was significantly higher in both H1N1 FM/47 preimmune and naïve-mock vaccinated mice on day 7 pc (day 112 pi). In contrast, preimmune and naïve vaccinated mice had higher levels of all class-switched IgG isotypes (Fig. 5E). H1N1 FM/47 preimmune-vaccinated mice had maximum IgG2a and IgG3 on day 7 pc (day 112 pi), whereas IgG1 was highest on day 14 pc (day 119 pi) (106 ng/hl). Significant differences between groups were detected for all other antibody isotypes/subclasses analyzed (Supplementary Fig. 3). In summary, these data demonstrate a predominance of IgG responses in H1N1 FM/47 preimmune-vaccinated mice, whereas naïve-mock vaccinated mice exhibited primarily IgM responses, consistent with a primary immune response.

Pre-existing H1N1 FM/47 immunity enhances coordinated lymph node transcriptional programs and germinal center-associated gene activation following vaccination.

From our antibody data shown above, where H1N1 FM/47 preimmune mice had earlier, more mature antibody responses following vaccination, we hypothesized that germinal center reactions were being induced in H1N1 FM/47 preimmune-vaccinated mice immediately following vaccination. To investigate our hypothesis, we collected lymph nodes near the site of vaccination (inguinal) and near the site of infection (mediastinal) for transcriptomic analysis of gene expression regulation. In addition, lungs were also collected to assess whether tertiary lymph node responses or tissue-resident memory cells generated during imprinting were stimulated following vaccination (Fig. 6A). RNA isolated from these tissues collected on days 0, 60 (vaccination day), 62, 67, and 74 pi (or days - 60, 0, 2, 7, and 14 pv) were sequenced at a depth of 45 million reads per sample on the Illumina platform. Global gene analysis of the lung and the lymph nodes (mediastinal and inguinal) revealed that H1N1 FM/47 imprinting fundamentally reshapes the transcriptional response to vaccination producing a profile distinct from naïve-vaccinated mice (Supplementary Fig. 4A-C). In the lymph nodes, naïve-vaccinated mice exhibited a more gradual shift in gene regulation that became most apparent at later time points, whereas H1N1 FM/47 preimmune-vaccinated mice had an earlier immune-associated gene set that was evident soon after vaccination and remained through day 14 pv (74 pi) (Supplementary Fig. 4A and B). This divergence was more evident for genes related to disease, inflammation, and adaptive immunity, including pathways associated with B cells, T cells, and isotype switching (Fig. 6B; Supplementary Fig.S4A and B). Comparison of differentially expressed genes between naïve- and H1N1 FM/47 preimmune-vaccinated mice across time revealed distinct transcriptional responses in both the inguinal and mediastinal lymph nodes following vaccination, as illustrated in Venn diagrams (Fig. 6C-F). At day 0 post-vaccination, a few overlapping differentially expressed genes were observed between the two groups in each lymph node. By days 2, 7, and 14 post-vaccination, substantial numbers of shared differentially expressed genes were identified, with 14,364, 12,382, and 11,621 shared genes, respectively, in both lymph nodes (Fig. 6C and

D). However, at most post-vaccination time points, H1N1 FM/47 preimmune-vaccinated mice exhibited more uniquely regulated genes than naïve-vaccinated mice. Analysis of differential gene-expression overlap among lymph nodes within each vaccination group further supported these differences. In naïve-vaccinated mice, overlap between the inguinal and mediastinal lymph nodes increased over time following vaccination, whereas H1N1 FM/47 preimmune-vaccinated mice exhibited a high degree of overlap between lymph nodes even at day 0 post-vaccination and remained relatively stable across later time points (Fig. 6E and F).

Having established differences in gene expressions and coordination between lymph nodes in the mouse groups, we next examined which adaptive immune genes were differentially regulated following vaccination by comparing transcriptional profiles between naïve and H1N1 FM/47 preimmune-vaccinated mice (Supplementary Fig. 5A-D). Some adaptive immune genes were upregulated in both lymph nodes of naïve mice following vaccination relative to naïve baseline but downregulated in H1N1 FM/47 preimmune mice. These genes were found to be predominantly B and T-cell receptor genes (CD3, CD4, CD8, CD19, CD22, and CD79A) that are essential for lymphocyte production and development (Supplementary Fig. 5A-D). Regulation of these genes did not occur in the lungs, the site of infection (Supplementary Fig. 6A and B).

We further examined the H1N1 FM/47 preimmune state by analyzing T- and B cell gene regulation in preimmune mice on day 60, prior to vaccination, compared with naïve control mice on day 0. While analysis of the lungs found distinct regulation in both B and T cell pathways (Supplementary Fig. 6C and D), gene regulation in the mediastinal lymph node, and to a lesser degree, in the inguinal lymph node, was mostly T and B cell receptor genes (Supplementary Fig. 7A to D). Naïve mice had very few differences between day 0 and day 60, as expected, whereas the observed changes in H1N1 FM/47 preimmune mice were suggested to depend on infection with the historical H1N1 virus and host recovery from infection (Supplementary Fig. 7A to D). H1N1 FM/47 preimmune mice had multiple T cell-associated genes that were increased on day 60 compared to day 0 (Fig. 6A). Genes coding for CD3 chain molecules, CD3d, CD3e, CD3g and CD3z, as well as markers of T cell subsets CD4 and CD8 α and CD8 β 1³⁶, were all increased in the mediastinal lymph nodes. The mediastinal lymph node of H1N1 FM/47 preimmune mice had increased CD19 and CD22 gene expression which code for proteins that increase on the surface of B cells as they mature. In addition, CD79a, a key signalling molecule for B cells, was also increased in H1N1 FM/47 preimmune mice mediastinal lymph nodes (Fig. 6B). The regulation of B cell-related genes was even greater in the inguinal lymph node of H1N1 FM/47 preimmune mice as the signalling molecules BCL10, BLNK, CARD11, and CD79B were also significantly increased. Together, these data suggest that both the mediastinal and inguinal lymph nodes contribute to T and B cell development and responses in the preimmune host at vaccination.

B cells undergo affinity maturation in the germinal centers of lymph nodes through somatic hypermutation, class switching, and clonal selection, resulting in a mature antibody response^{21,37}. Affinity maturation is regulated by key cells and molecules such as T follicular helper cells (T_{FH})^{22,24,38}. Next, we examined germinal center-associated genes in naïve and H1N1 FM/47 preimmune mouse

mediastinal lymph nodes in response to vaccination to identify the specific molecules that contribute to the mature antibody response identified in H1N1 FM/47 preimmune mice. Preimmune mice had regulation of key molecules, such as IL-2, IL-4, and IL-21, that contribute to the germinal center reaction, and are produced by T_{FH} cells^{39,40} (Fig. 7A and C). CXCR5, a chemokine essential for initial germinal center formation^{41,42}, was elevated in both naïve and H1N1 FM/47 preimmune mice after vaccination; however, it elevated earlier and to a greater extent in preimmune-vaccinated mice (Fig. 7A and B). While FAS, a gene attributed to the activation of apoptosis in B cells⁴³, was upregulated by day 14 pv (74 pi) in the mediastinal lymph nodes of naïve-vaccinated mice, it was downregulated as early as day 2 pv (62 pi) in H1N1 FM/47 preimmune-vaccinated (Fig. 7B), suggesting altered regulation of B cell selection and survival during germinal center responses following vaccination. H1N1 FM/47 preimmune mice also showed increased expression of PRDM1, which encodes the plasma cell transcription factor BLIMP1, and decreased expression of IRF4, two genes encoding proteins involved in plasma cell differentiation and in the regulation of germinal center exit⁴⁴ (Fig. 7A and B). Moreover, BCL6, a master regulator of the germinal center reaction^{26,45,46} was among the most differentially regulated between naïve and H1N1 FM/47 preimmune mice upon vaccination (Fig. 7A). While naïve mice downregulated BCL6 consistently in the mediastinal lymph nodes in response to vaccination, H1N1 FM/47 preimmune mice had significant increases with its higher fold change in FPKM value on day 2 at Log2 of 1.16 (Fig. 7B). Taken together, these data show that several key molecules that drive the germinal center reaction forward are increased in H1N1 FM/47 preimmune mice in response to vaccination.

Discussion

Influenza virus infection is an ongoing threat to public health¹. While vaccination remains the best defense against infection and severe disease, the mechanisms of protection are not fully understood with the seasonal influenza vaccine being less than optimal. Evidence has accumulated over the last decade suggesting previous exposures shape one's immune response to current influenza virus exposures and vaccinations^{19,30,47–53}. Most humans are infected with an influenza virus by the age of three, meaning virtually all individuals above this age have been imprinted and have a preimmune history to influenza viruses^{54,55}. We previously established a preimmune ferret model, which demonstrated that a previous H1N1 virus infection can lead to improved antibody response to vaccination³⁰. The present study extends this work by exploring the mechanisms by which preimmunity affects vaccination outcomes. Here, we demonstrated that H1N1 FM/47 preimmune mice have an early and robust antibody response following vaccination, predominated by class-switched isotypes. Importantly, H3N2 preimmune mice did not exhibit similar antibody responses post-vaccination, suggesting unique properties of H1N1 viruses. Further, H1N1 FM/47 preimmunity increased protection against pandemic influenza virus infection in vaccinated mice. Finally, we provided transcriptome data suggesting that H1N1 FM/47 preimmunity-induced vaccine protection is associated with T_{FH} and B cells regulated by BCL6, as well as germinal center reactions, in secondary lymphoid organs. This work advances our

understanding of vaccination-induced protection in a model that more closely reflects the human population.

In the human population, it has been shown that the influenza virus one has been imprinted with substantially impacts subsequent immune responses to influenza viruses and vaccine outcomes, with stronger evidence observed for H1N1 preimmunity^{19,30,47–53}. B-cell clonotypes from early exposures can be elicited and enhanced by subsequent exposures, leading to improved antibody responses^{48,56}. However, it remains unclear how imprinting strain or subtype specificity confers protection or predisposes to poor outcomes during a secondary exposure. Consistent with prior studies demonstrating imprinting following natural infection with H1N1 and H3N2 viruses (45), antibodies were generated after both H1N1 FM/47 and H3N2 HK/68 imprinting, and serum antibody levels remained high for 60 days in our study. However, only H1N1 FM/47 preimmunity effectively primed the immune system for enhanced responses to inactivated vaccine exposure, whereas H3N2 HK/68 preimmunity did not improve vaccine-induced immune responses. These findings suggest that imprinting history shapes subsequent vaccine-induced immunity and has important implications for determining how to achieve optimal vaccine responses, particularly in current human cohorts imprinted with H3N2 virus strains. Nevertheless, while we have shown the impact of H1N1 FM/47 preimmunity on elicitation of HA head antibodies by HAI assays, further work is needed to characterize antibodies directed at the HA stalk.

The preimmune state primes the host immune system such that, when interacting with a familiar antigen, a germinal center reaction can induce a response against the existing pool of memory B cells⁵⁶. This germinal center reaction is dependent on several key molecules, such as BCL6^{25,45,46}, leading to the generation of mature class-switched antibodies^{21,37}. It has been shown that CD4 T cells play an essential role in protection against influenza virus infection in a preimmune host, with significant regulation of molecules such as Th1 Tbet and TFH cells, and FOXP3⁵⁷. Our transcriptome data revealed that H1N1 FM/47 preimmunity-induced vaccine protection is strongly associated with robust T_{FH} and B cell responses governed by BCL6 and other germinal center-promoting genes in the secondary lymphoid organs. These findings indicate that H1N1 FM/47 preimmunity establishes a transcriptional program that favors efficient T_{FH}-B cell interactions and sustained germinal center activity, providing a mechanistic basis for the enhanced vaccine-induced protection observed in this group. We chose to examine the lymph nodes proximal to the vaccination and infection sites to gain insight into germinal center dynamics and to identify mechanisms underlying differences in antibody responses. This tissue-focused approach is supported by studies from Ellebedy's group demonstrating that critical features of vaccine-induced humoral immunity are more accurately captured within draining lymph nodes than in peripheral blood⁵⁸. Ultrasound-guided fine needle aspiration was used to longitudinally sample cells from draining lymph nodes in humans after an influenza vaccination event, where these adults would have an influenza preimmune history. In this study, Turner, *et al.* found that, compared to blood, the lymph nodes proximal to the vaccination site exhibited increased levels of B cells and CD4 + T cells, with increased vaccine-induced germinal center B cells that expanded over time and exhibited ongoing somatic hypermutation consistent with affinity maturation. This study reinforces our observation that

prior H1N1 exposure shapes germinal center dynamics, promotes affinity maturation and the selection of high-affinity B cell clones following vaccination.

The exact mechanisms underlying vaccine-induced protection against influenza viruses are not well understood, especially in hosts with complex preimmune histories. Towards a better understanding of the impacts of preimmunity on vaccine responses, our study provides important new insights into possible mechanisms that may improve vaccination outcomes. While our study does not exactly capture the events in the complex preimmune host, it does identify a mechanism of improved vaccination outcomes in a host that has had a single prior H1N1 infection before vaccination. Therefore, future studies should investigate if germinal center reactions are continually induced over multiple H1N1 infections and vaccinations, which are antigenically distinct. Moreover, with our data in mind showing that H1N1 previous infection was able to prime the immune response for vaccination, novel vaccine regimens should be considered, potentially leveraging attenuated H1N1 live virus vaccines to improved antibody outcomes in combination with inactivated vaccines.

Materials and Methods

Ethics Statement

All animal research was performed in strict accordance with the Canadian Council of Animal Care Guidelines. The protocol license number 18-091 was assigned by the Animal Care Committee of Dalhousie University. The animal use protocol was approved by the Dalhousie University Animal Care Committee (ACC). All efforts were made to minimize animal suffering. Inoculations and sample collections were performed under 5% isoflurane anesthesia.

Influenza Viruses and Mice

The mouse-adapted H1N1 influenza A virus A/Fort Monmouth/1/1947 (FM/47) and H3N2 A/HongKong/01/1968 were obtained from the American Type Culture Collection (ATCC VR-97 and VR-822, respectively) through Cedarlane (Burlington, ON, Canada). The 2009 pandemic H1N1 strain A/Mexico/4108/2009 (Mex/09) was obtained from the Centers for Disease Control and Prevention (Atlanta, GA, USA) (ATCC VR-1894). Viruses were titrated in embryonated chicken eggs or Madin-Darby Canine Kidney (MDCK) cells prior to use to determine TCID₅₀ and EID₅₀. All RG2 virus work was performed in the CL2 facility. Sanofi FLUZONE® quadrivalent (QIV) influenza vaccine from the 2018–2019 influenza season was acquired from Sanofi Canada (North York, ON, Canada). The vaccine contained concentrated HA proteins from A/Michigan/45/2015 X-275 (H1N1), A/Brisbane/1/2018 X-311 (H3N2), B/Phuket/3073/2013 (B Yamagata lineage), and B/Maryland/15/2016 BX-69A (a B/Colorado/6/2017-like virus, B Victoria lineage). Adult female C57Bl/6J mice were purchased from Jackson Laboratories (Bar Harbour, ME, USA) and were housed in HEPA-filtered cage racks during infection studies. All animal procedures were performed in a certified class II biosafety cabinet.

Infection and Vaccination

Mice were anesthetized with isoflurane and intranasally inoculated with $10^{3.5}$ EID₅₀ of H1N1 FM/47, or with 10^6 EID₅₀ of 2009 pandemic H1N1 Mex/09. The volume of inoculum was 50 µL (25 µL in each nare). Mice were vaccinated and boosted with 50 µL of FLUZONE® Sanofi QIV vaccine in the hind caudal muscle. Mice were monitored for post-inoculation adverse effects from the anesthesia and inoculation procedures for 15 minutes. Additionally, mice were monitored for 14 post-inoculation days for clinical disease caused by influenza virus infection (see Clinical monitoring).

Clinical Monitoring

Weight, clinical signs such as breathing, and survival were monitored for 14 days following infection and vaccination. Weight for each day was calculated as a percentage of the original weight taken on day 0 prior to inoculation or vaccination. Mice were removed from the study if the humane endpoint of 80% of their original weight was reached. Standard error and standard deviation were calculated for weight within each group. Mice were examined at the same time each day for consistency.

Viral Titers

Viral titers were calculated from collected lung samples post-inoculation with the H1N1 FM/47 virus and the 2009 pandemic H1N1 Mex/09 virus. Lungs were snap frozen upon collection, homogenized, and supernatants were subjected to TCID₅₀ assays followed by a hemagglutinin (HA) assay to determine viral titer as previously described^{30,59}. Viral load was calculated using the Reed and Muench method⁶⁰.

HAI (Hemagglutinin Inhibition) Titers

Hemagglutinin Inhibition (HAI) titers were determined in serum samples by HAI assay as previously done⁵⁹. Assays were performed using FM/47 or Mex/09 live virus or the World Health Organization (WHO) circulating influenza virus detection kit (2018–2019). The WHO kit contains antigen for the 2009 pandemic H1N1, circulating H3N2, circulating B-Yamagata, and circulating B-Victoria strains. The collected blood was centrifuged to obtain antisera. Sera were treated for at least 4 hours with Receptor Destroying Enzyme (RDE; Denka, Seiken, Japan) at 37°C. Fresh turkey red blood cells (TRBC) were washed and diluted to 0.05% (vol/vol) in PBS. Sera were serially diluted in PBS in 96-well V-bottom plates with 8 HAU/50 µL of antigen. Following a 15-minute incubation at room temperature, 50 µL of 0.05% TRBC was added to each well. The plate was incubated for 30 minutes at room temperature in the dark. The HAI titer was determined as the reciprocal of the highest serum dilution to completely prevent agglutination. HAI Units were calculated as the Log₂ of the denominator of the HAI titer.

Antibody Isotype

Antibody isotypes were determined in serum samples by Cytometric Bead Array (CBA) analysis using the BD™ Cytometric Bead Array (CBA) Mouse Immunoglobulin Isotyping Kit (BD Biosciences) according to the manufacturer's protocol. Serum was diluted 1:100,000 with master buffer, and 50 µL was incubated with 50 µL of Mouse Ig capture beads for 15 minutes at room temperature. After a wash with master buffer, 50 µL of PE/FITC detector antibody was added, and the samples were incubated for 15 minutes at room temperature in the dark. After a final wash, samples were diluted in 500µL of master buffer and analyzed by Flow Cytometry on a BD FACSCanto™ II with BD FACSDiva™ Software (BD Biosciences). Data was analyzed using FlowJo (BD) software.

Gene Expression Analysis by qRT-PCR

Gene expression analysis in the lung was determined via quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). Lungs were collected at necropsy in RNAlater, homogenized, and RNA extracted using TRIzol (Thermo Fisher Scientific). Total lung RNA was reverse transcribed using the iScript Reverse Transcription System (Bio-Rad). qRT-PCR was performed using the CFX384 Touch Real-Time PCR Detection System (Bio-Rad). Raw data was collected with CFX Maestro™ Software. Each reaction well contained 4µL of 500 ng/µL cDNA, 0.04 µL each of forward and reverse primers (final concentration of 400 nM; Thermo Fisher Scientific) (Table 1), and 5 µL SYBR Green Master Mix (Bio-Rad), for a total reaction volume of 10 µL. Each sample was run in quadruplicate. Gene expression was normalized to β actin (BACT) housekeeping gene, represented as a fold change relative to control samples.

Table 1
qRT-PCR Primer List

Primer	Host	Sequences	
BACT	Mouse	F	5'-AGGGAAATTGTTTCGTGACATAAA-3'
		R	5'-TCATAACTCTTCTCCAAGGAGG-3'
IL-6	Mouse	F	5'-GAGGATACCACTCCCAACAGACC-3'
		R	5'-AAGTGCATCATCGTTGTTTCATACA-3'
TNF- α	Mouse	F	5'-CATCTTCTCAAAATTCGAGTGACAA-3'
		R	5'-TGGGAGTAGACAAGGTACAACCC-3'
IFN- γ	Mouse	F	5'-GAGGAACTGGCAAAAGGATG-3';
		R	5'-GCTGATGGCCTGATTGTCTT-3'
M1/M2	A/Mexico/4108/2009	F	5'-AAGACAAGACCAATCTTGTCACCTCT-3'
		R	5'-TCTACGCTGCAGTCCTCGCT-3'
NP	A/Hong Kong/8/68	F	5'-CAG(A/G)TACTGGGC(A/T/C)ATAAG(A/G)AC-3'
		R	5'-GCATTGTCTCCGAAGAAATAAG-3'

Cytokine Production

Protein cytokine concentrations in serum samples were determined by Cytometric Bead Array (CBA) analysis using BD™ Cytometric Bead Array (CBA) Mouse Inflammation Kit and BD™ Cytometric Bead Array (CBA) Mouse TH1/TH2 Kit (BD Biosciences) as per the manufacturer's protocols. Sera were diluted 1:10 with assay diluent, and 50 μ L was incubated with 50 μ L of Mouse Ig capture beads and 50 μ L of PE detector antibody at room temperature in the dark for 2 hours. After washing, samples were diluted in 300 μ L of wash buffer and analyzed by Flow Cytometry using a BD FACSCanto™ II machine and BD FACSDiva™ Software (BD Biosciences).

Histopathology

Lung samples were collected at necropsy and perfused with 10% neutral buffered formalin. Perfused lung samples were embedded in paraffin wax, sectioned, and stained with Hematoxylin and Eosin (H&E). Samples were viewed under brightfield microscopy using a Zeiss Axio Imager Z1, and images were captured with an Axioplan Colour Camera in Zen 2012.

RNA Sequencing and Transcriptome Analysis

Lung and lymph nodes (mediastinal and inguinal) were collected at necropsy and placed in RNAlater for subsequent homogenization and RNA extraction. RNA was extracted using TRIzol (Thermo Fisher Scientific) or the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocols. Sequencing was

done on the Illumina Platform (Novogene, Sacramento, California). We targeted a read depth of 20 million read pairs for mRNA sequencing. Raw FASTQ files were mapped (STAR) to the reference genome mm10 (NCBI). Differentially expressed genes were compared within and between experimental groups. Differentially expressed genes were determined for each permutation relative to an indicated baseline (EdgeR) after the gene expression for each replicate was measured in featureCounts implemented within the Galaxy platform^{61–63}. Next, the genes with p-values greater than 0.5 were filtered out using filter, and the final lists of generated DEGs were generated for each group of interest, and Venn diagrams were produced (Bioinformatics & Evolutionary genomics). Individual gene expression was quantified as fragments per kilobase of exon model per million reads mapped (FPKM) values (HTSeq). The average baseline FPKM value was calculated between 3 mice for each gene, and fold change from all-time points was calculated for each sample individually using Log2 (Sample FPKM)-Log2 (Baseline average FPKM). Analysis of specific pathways was performed using published KEGG pathway gene lists for T and B cell analysis (DAVID) or customized pathway gene lists for germinal center analysis (Bioconductor-RStudio), available in 2019. Heatmaps were generated with all values for the germinal center or T and B cell pathways. For global gene expression analysis, the top 3000 genes with the highest FPKM values across all time points and tissues were extracted (Biocmanager - RStudio). These genes were assessed via DAVID using KEGG pathways analysis for functional clustering. Seventeen initial clusters formed, comprising 500 unique genes (KEGG-DAVID). Once all duplicates/redundancies were removed, 5 functional clusters remained.

Statistical Analysis

Unpaired, unequal-variance, two-tailed Student's t-tests (two groups) or one-way ANOVA with Tukey's multiple-comparisons test (three or more groups) were conducted in GraphPad Prism (version 10, San Diego, USA), comparing groups at the same time point unless otherwise indicated. A p-value ≤ 0.05 was considered statistically significant. Data are presented as mean $\pm$ SD. Hemagglutination-inhibition (HAI) titers are summarized as the group geometric mean. For body-weight and percent-survival monitoring (Fig. 4A, Fig. 4B, Supplementary Fig. 1A, Supplementary Fig. 1B, Supplementary Fig. 2A, and Supplementary Fig. 2B), the same animals were followed longitudinally (n = 10 per group); group differences in body weight were compared at each time point, and survival was compared by the log-rank (Mantel-Cox) test. Host gene expression measured by qRT-PCR is reported as fold change relative to control samples using BACT as the housekeeping gene, with comparisons made per time point. Differentially expressed genes were identified in edgeR and filtered at a p-value threshold of 0.5. The specific statistical test and n for each panel are stated in the corresponding figure legend.

Declarations

Data availability

The datasets and materials that were generated in this study can be obtained from the corresponding authors under standard transfer agreements. Datasets are deposited on the Sequence Read Archive

(SRA) with accession ID: PRJNA1460348.

Competing Interests

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. AAK has received personal fees from Vaccine for serving as an associate editor and from Seqirus outside the submitted work. AK and GSM are shareholders of the company BioForge Canada Limited. BioForge Canada Limited is a company that uses bioinformatics in immunological approaches in the monitoring, prevention, and treatment of infectious diseases. The authors disclose that the interests of BioForge Canada Limited had no impact on this study. AK is a Moderna Global Fellow (2024-MGF-000316 [91353]).. All other authors have no relationships to declare.

Declaration of Interest:

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. AAK has received personal fees from Vaccine for serving as an associate editor and from Seqirus outside the submitted work. AK and GSM are shareholders of the company BioForge Canada Limited. BioForge Canada Limited is a company that uses bioinformatics in immunological approaches in the monitoring, prevention, and treatment of infectious diseases. The authors disclose that the interests of BioForge Canada Limited had no impact on this study. AK is a Moderna Global Fellow (2024-MGF-000316 [91353]).. All other authors have no relationships to declare.

Author Contribution

Author Contributions: Conceived and designed the experiments: AAK. Performed the experiments: AAK, MEF, MKF, MLR. Analyzed the data: AAK, OEO, MEF, AK, GSM, MKF, RS, AR, JS, GD, SEB, GKT. Wrote the manuscript: AAK, MEF, OEO. Edited the manuscript: AAK, OEO, CM, JM, JS, GD, GKT, SEB: Contributed resources: AAK.

Acknowledgement

Thank you to Andrew Zarnke, Derek Rowter, Patricia Colp, Jacqueline Pearce and Dr. Suzanne Pearce for technical assistance in this work.

Data Availability

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Figures

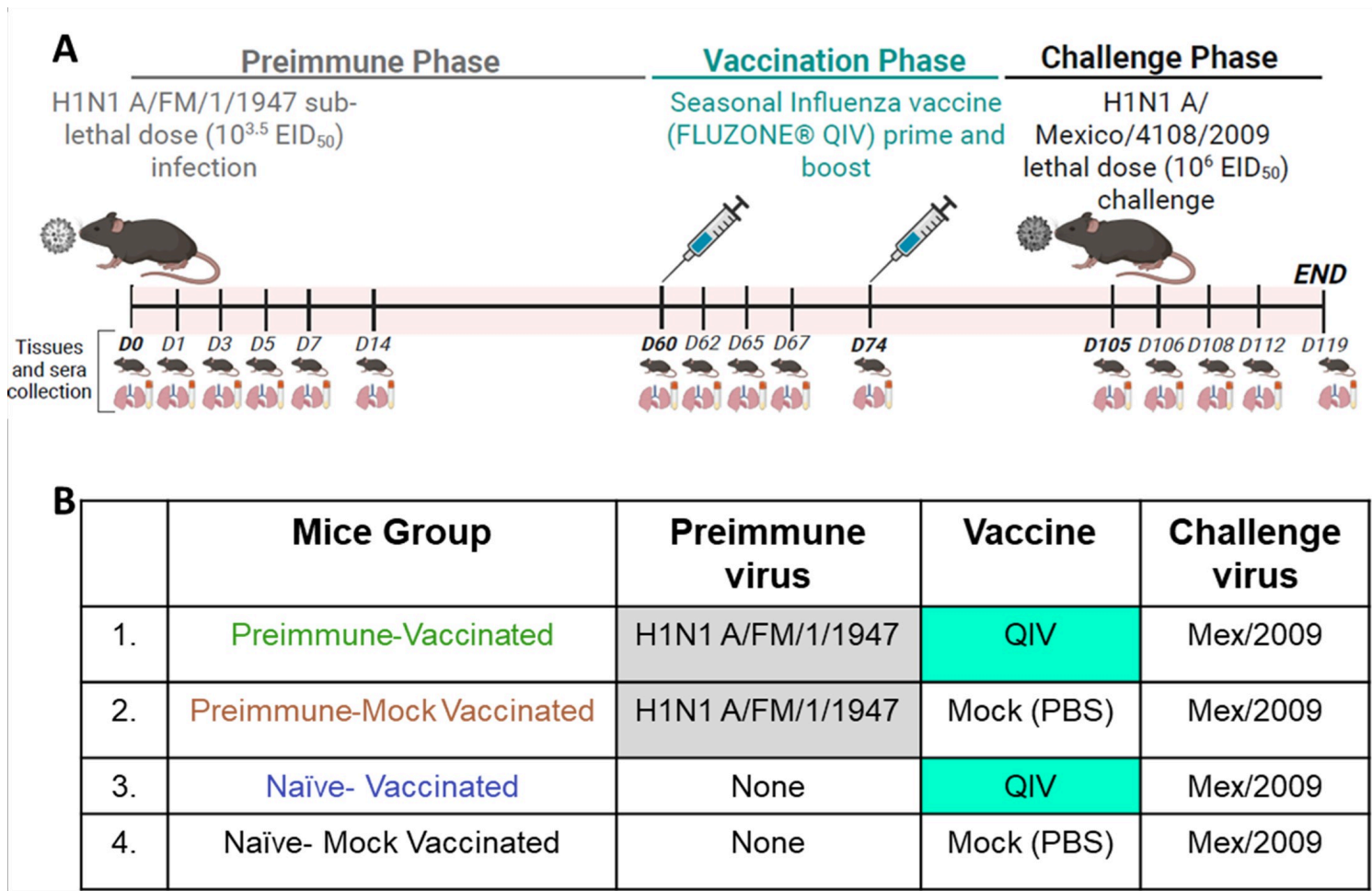


Figure 1

Study design for investigating the effects of H1N1 FM/47 pre-existing immunity on the outcomes of influenza vaccination. (A) Experimental schematic to assess the impact of H1N1 FM/47 preimmunity on influenza vaccine responses. Adult, female C57Bl/6J mice were imprinted on day 0 via intranasal inoculation at $10^{3.5}$ EID₅₀, a sublethal dose, of H1N1 A/FM/1/1947 (FM/47) to induce preimmune development. On day 60 pi, mice were vaccinated with FLUZONE® QIV and boosted on day 74. On day 105 pi, mice were challenged with 10^6 EID₅₀ of the 2009 pandemic H1N1 virus, A/Mexico/4108/2009 (Mex/09). Tissues and sera were collected at the indicated time points. Days are denoted as “D”. (B) Experimental groups for the varying conditions of preimmunity and vaccination.

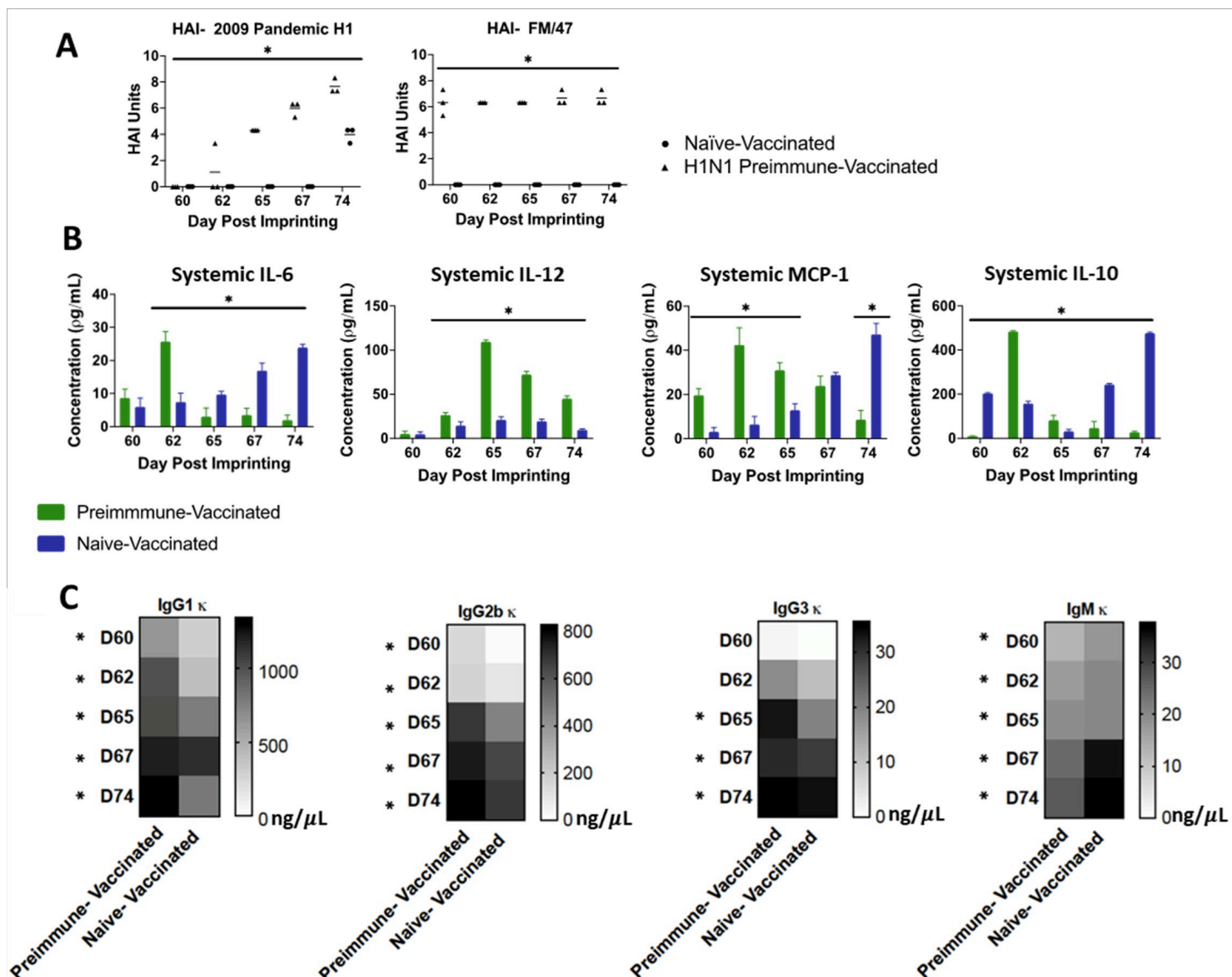


Figure 2

H1N1 FM/47 preimmune-vaccinated mice have increased antibody responses to vaccine antigens with affinity-matured antibody production and cytokine circulation following vaccination with the seasonal influenza vaccine. Mice were imprinted with H1N1 FM/47 to induce preimmunity and vaccinated on day 60 pi with 50 mL Fluzone® Quadrivalent Influenza Vaccine (QIV) (Sanofi Pasteur) as previously described. **(A)** Receptor-destroying enzyme (RDE)-treated sera collected days 60 to 74 pi were assessed by HAI assay for antibodies against the 2009 pandemic H1N1 Mex/09 vaccine strain (left) or the imprinting strain H1N1 FM/47 (right). Antibody titers are reported as HAI units. **(B)** Serum cytokine levels (IL-6, IL-12, MCP-1, and IL-10) measure in pg/ml in the sera collected post vaccination using a mouse inflammatory CBA kit and analyzed by flow cytometry. **(C)** Serum antibody isotypes (IgG1κ, IgG2bκ, IgG3κ, and IgMκ) measured in the sera collected post vaccination using a mouse antibody isotype CBA kit and analyzed by flow cytometry. Serum antibody data are presented as heatmaps showing antibody concentrations (ng/μL) over time. pv- post vaccination; pi- post imprinting. Data are mean ± SD; HAI titers (A) are shown as the group geometric mean. Because two groups (H1N1 FM/47 preimmune-

vaccinated and naïve-vaccinated) were compared, groups were compared at each time point by unpaired, two-tailed Student's t-test; $P \leq 0.05$. Different animals were sampled at each time point. $n = 3$ mice per group.

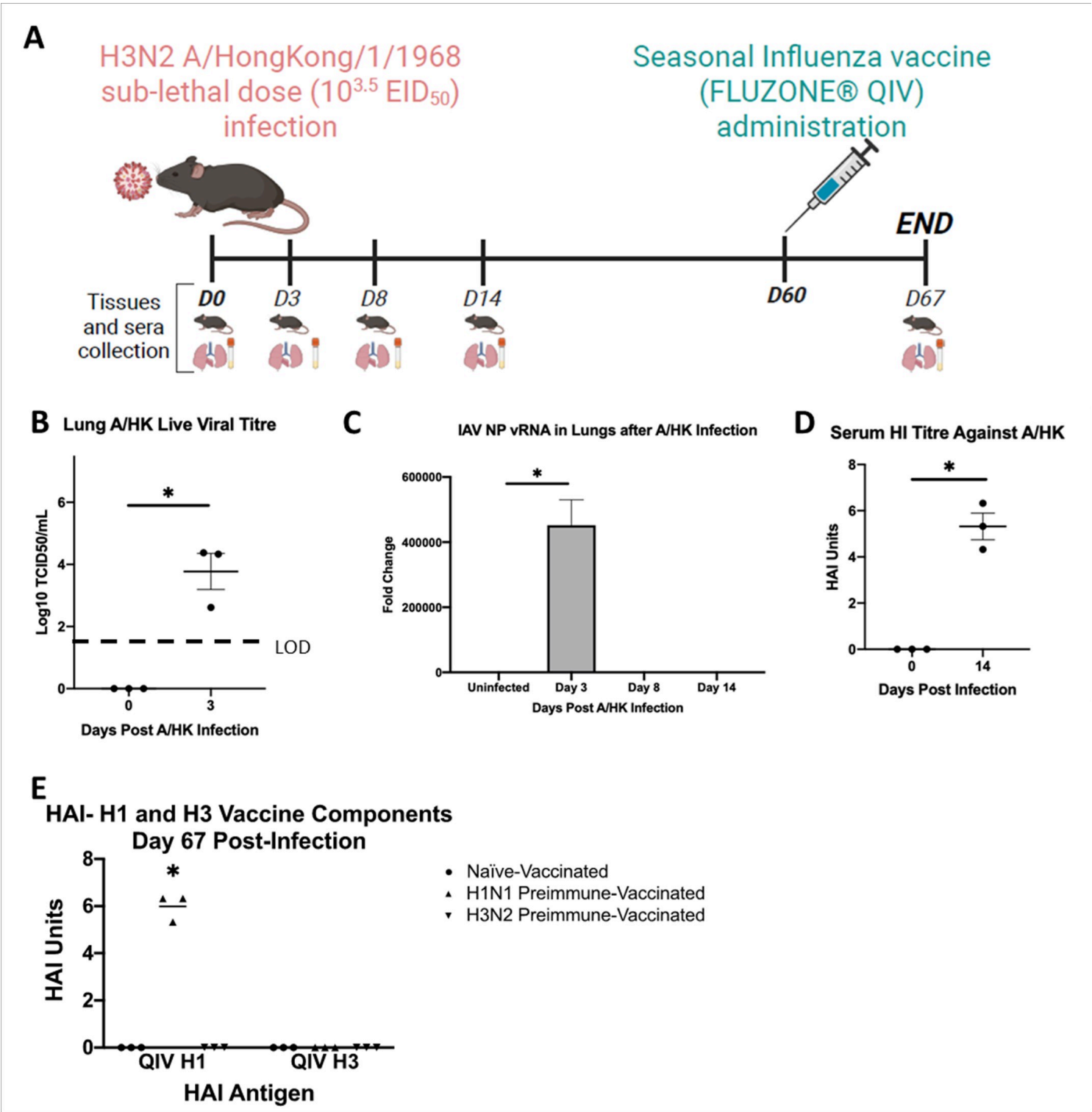


Figure 3

H3N2 preimmunity does not enhance vaccine-induced antibody responses. (A) Experimental schematic to assess the impact of H3N2 preimmunity on influenza vaccine responses. Adult female C57BL/6J

mice were infected with H3N2 A/Hong Kong/1/1968 (HK/68) on day 0 to establish preimmunity or left uninfected as controls. Following a similar study plan as with H1N1 FM/47 imprinting, mice were primary vaccinated on day 60 pi, and blood samples were collected 7 days pv. Days are denoted as “D”. **(B)** Lung infectious viral titers determined by TCID₅₀ assay day 3 following HK/68 inoculation in an independent control group of mice to optimize and confirm H3N2 HK/68 viral imprinting. The dashed line indicates the limit of detection. **(C)** Lung viral genomic RNA determined during H3N2 HK/68 infection optimization by q-PCR for influenza A nucleoprotein (np) RNA levels during the acute phase post imprinting. **(D)** HK/68-specific hemagglutination inhibition (HAI) antibody titers in serum samples collected before imprinting (day 0) and at day 14 pi to confirm that imprinting occurred and that mice elicited virus-specific antibodies during optimization studies. **(E)** Vaccine-induced HAI antibody responses to H1N1 and H3N2 components in naïve-vaccinated (circles), H1N1 FM/47 preimmune-vaccinated (triangles), and H3N2 HK/68 preimmune-vaccinated (down triangle) mice at day 67 pi. Data are mean $\pm$ SD (n = 3 mice per group). $P \leq 0.05$; comparisons were made between groups at the same time point by unpaired, two-tailed Student’s t-test (two groups; D) or one-way ANOVA with Tukey’s multiple-comparisons test (three groups; E). HAI titers are shown as the group geometric mean.

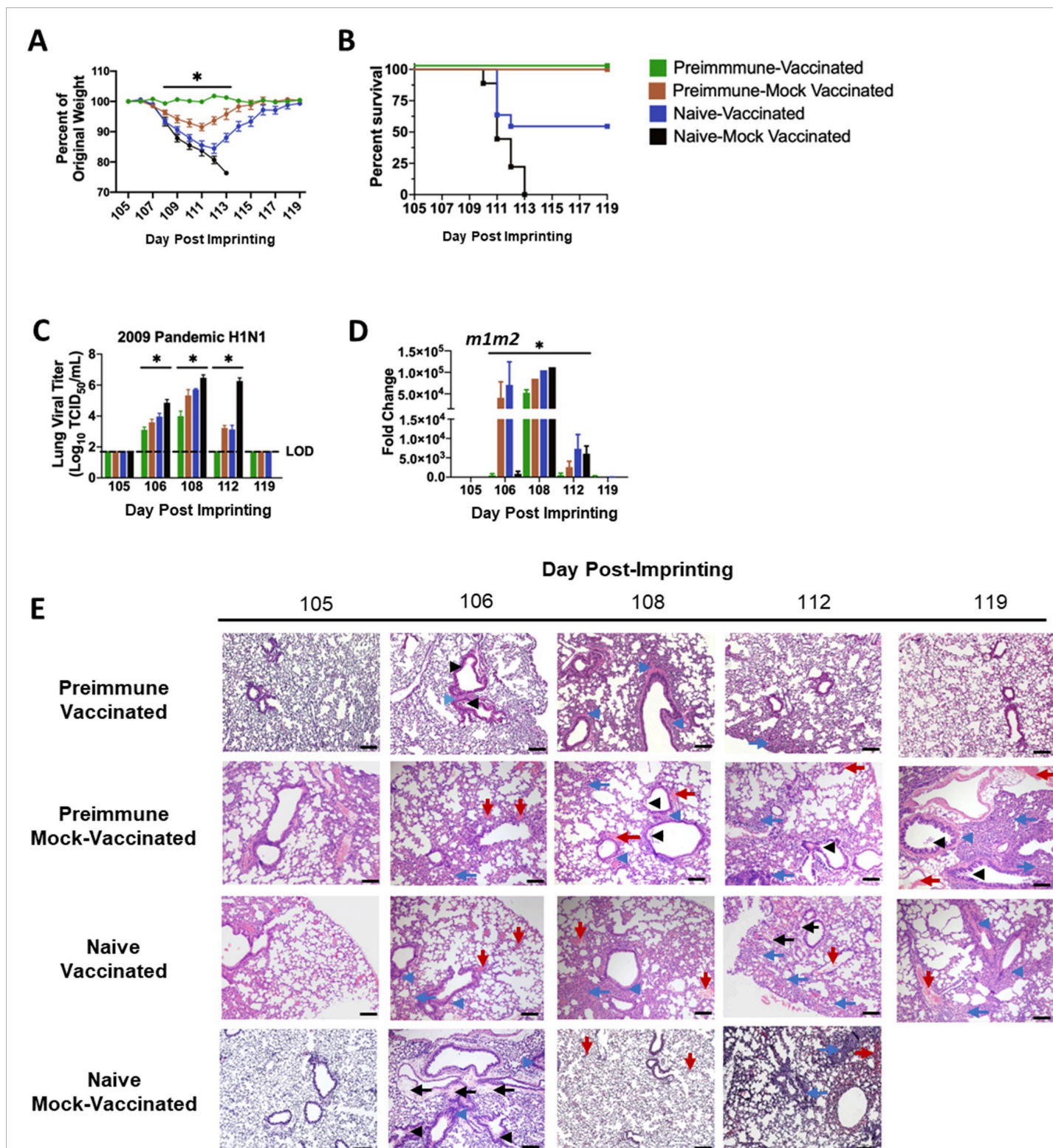


Figure 4

H1N1 FM/47 preimmune-vaccinated mice have improved clinical outcomes following lethal H1N1 challenge. (A) Body weight change following the challenge is shown as a percentage of the original weight. (B) Survival following challenge, defined by reaching the humane endpoint of 80% baseline body weight and graphed for each group as Kaplan-Meier curves. (C) Infectious viral titers following challenge as determined by TCID_{50} assay. The dashed line indicates the limit of detection (LOD). (D) The viral RNA

levels quantified by qPCR following viral challenge. **(E)** Representative lung histopathology shown for specific time points following viral challenge. Alveolar consolidation (blue arrows), hemorrhage (red arrows), peribronchiolar infiltration (blue arrowheads), epithelial cell sloughing (black arrowheads), and fibrin accumulation (black arrows) are indicated. Data are mean $\pm$ SD. $P \leq 0.05$. Body weight (A) and viral data (C, D) were compared between groups at the same time point by one-way ANOVA with Tukey's multiple-comparisons test; survival (B) was compared by the log-rank (Mantel-Cox) test. The same animals were followed longitudinally in (A, B), whereas independent animals were sampled at each time point in (C, D). Group size $n = 10$ mice per group for (A, B) and $n = 3$ mice per group for (C, D). Representative histology images in E reflect the average appearance across three mice per group at each time point.

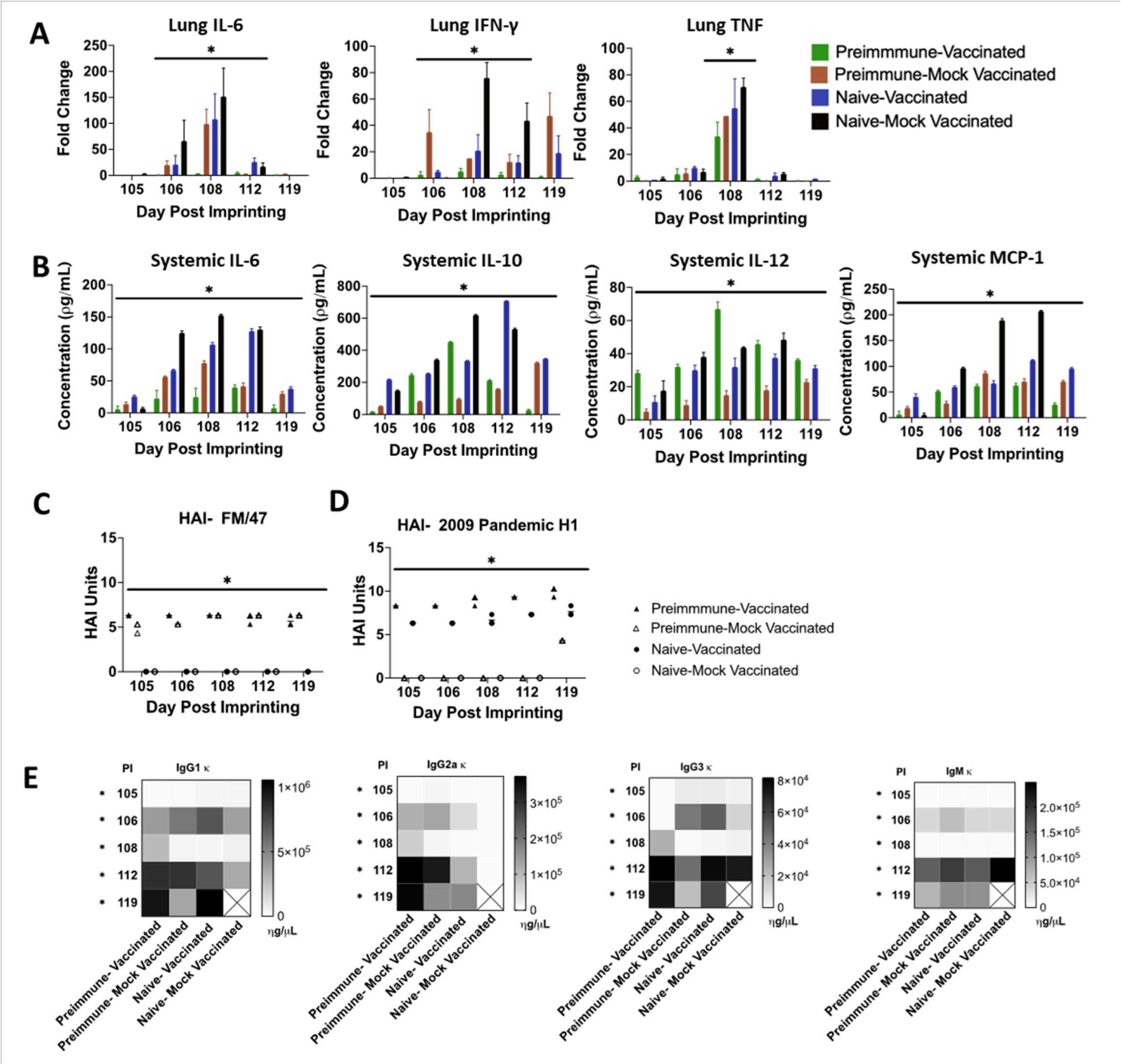


Figure 5

H1N1 FM/47 preimmune-vaccinated mice have decreased inflammatory cytokines and increased affinity-matured antibody response following lethal H1N1 challenge. **(A)** IL-6, IFN- γ and TNF mRNA levels in lung samples collected after the challenge. **(B)** Serum cytokine levels of IL-6, IL-10, IL-12, and MCP-1 measured in pg/ml following H1N1 Mex/09 challenge. **(C and D)** Influenza virus-specific antibody concentrations (in HAI units) measured against H1N1 FM/47 (C) or 2009 pandemic H1N1 (D) in serum samples collected from days 0 to 14 (105 to 119 pi) following H1N1 Mex/09 challenge. **(E)** Antibody isotype (IgG1 κ , IgG2a κ , IgG3 κ , and IgM κ) concentrations in serum samples collected following H1N1 Mex/09 challenge. Serum antibody data are presented as heatmaps showing antibody concentrations (ng/ μ L) over time. Data are mean $\pm$ SD; HAI titers (C, D) are shown as the group geometric mean. * indicates a p-value $\leq$ 0.05 as determined by one-way ANOVA with Tukey's multiple-comparisons test. n = 3 for all groups. Different animals were sampled at each time point; one-way ANOVA was applied independently at each time point, as samples were cross-sectional.

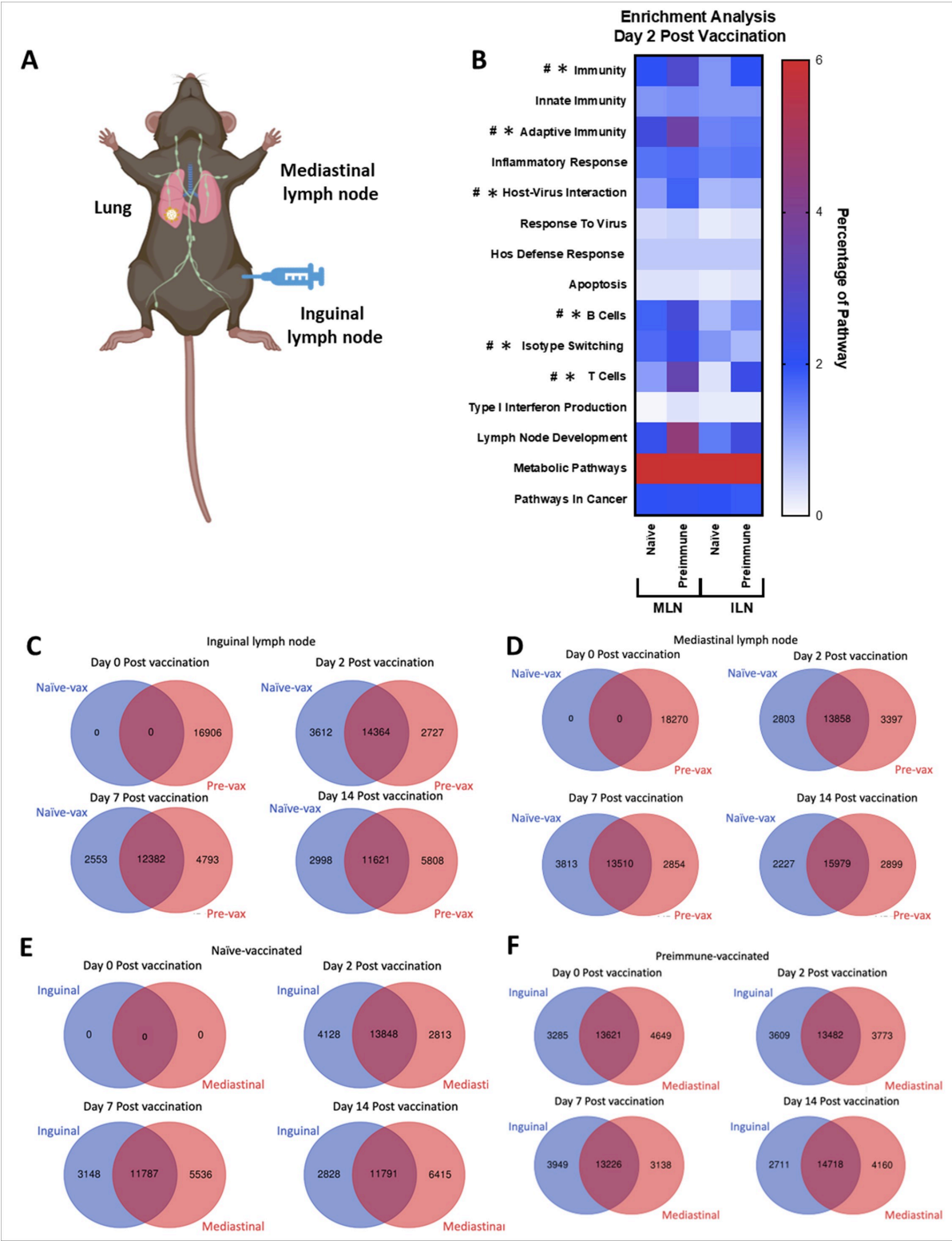


Figure 6

Distinct transcriptional responses across immunologically and virologically relevant tissues in preimmune-vaccinated mice. (A) Schematic representation of tissues collected for transcriptional analysis in preimmune and naïve-vaccinated mice before and after challenge. Lungs and draining lymph nodes were harvested at designated time points post-infection for downstream gene expression profiling. (B) Enriched KEGG pathways detected day 2 pv in mediastinal and inguinal lymph nodes from

preimmune and naïve-vaccinated mice, shown as gene counts per pathway for each tissue type and experimental group. **(C–F)** Venn diagrams depicting the overlap of differentially expressed genes at days 0, 2, 7, and 14 pv between preimmune- and naïve-vaccinated mice in the inguinal lymph nodes (C), mediastinal lymph nodes (D), inguinal and mediastinal lymph nodes within the naïve-vaccinated group (E), and inguinal and mediastinal lymph nodes within the preimmune-vaccinated group (F). * denotes significantly differentially expressed genes relative to the day 0 baseline within each group; # denotes significantly differentially expressed genes between naïve- and preimmune-vaccinated mice at the indicated time point (day 2 pv). n = 3 mice per group.

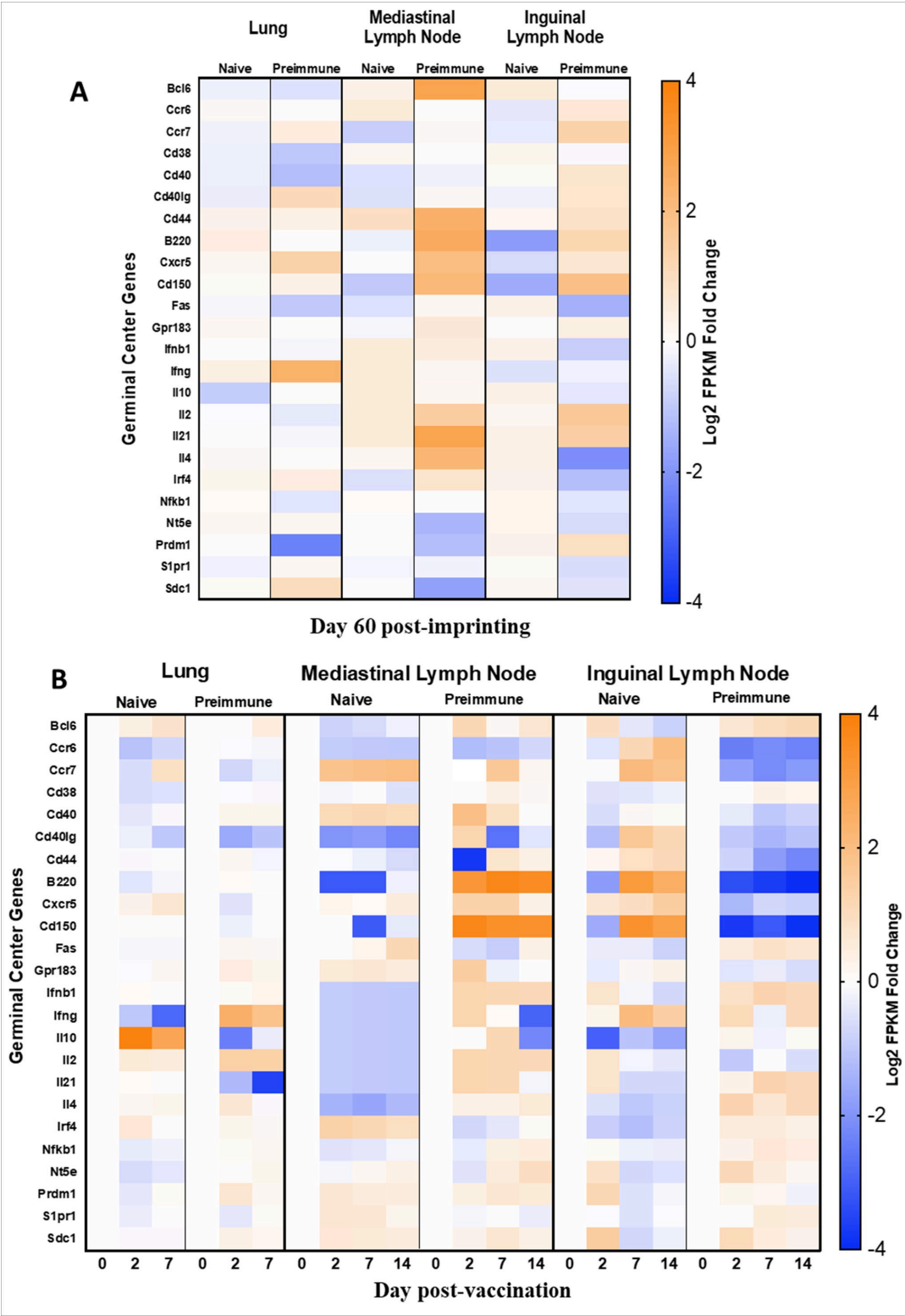


Figure 7

H1N1 preimmunity alters germinal center-associated gene expression in the draining lymph node following vaccination. (A)Baseline expression of germinal center-associated genes in lung, mediastinal lymph nodes, and inguinal lymph nodes from naïve and H1N1 preimmune mice at day 60 pi (prior to vaccination). **(B)** Temporal regulation of germinal center-associated genes in lung, mediastinal lymph nodes, and inguinal lymph nodes at days 0, 2, 7, and 14 pv in naïve-vaccinated and preimmune-

vaccinated mice. Heatmaps show gene expression quantified as $\log_2$ fold change in FPKM values relative to the corresponding baseline (day 0; leftmost column for each tissue), with values normalized to z-scores. Germinal center gene sets were curated de novo. n = 3 mice per group.

Supplementary Files

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- [OludadaFrancisetalSupplementaryInformation.docx](#)