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Prior infection with influenza virus but not vaccination leaves a long-term immunological imprint that intensifies the protective efficacy of antigenically drifted vaccine strains[☆]

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ABSTRACT

The role of pre-existing immunity for influenza vaccine responses is of great importance for public health, and thus has been studied in various contexts, yet the impact of differential priming on vaccine responses in the midst of antigenic drift remains to be elucidated. To address this with antigenically related viruses, mice were first primed by either infection or immunization with A/Puerto Rico/8/34 (PR8) virus, then immunized with whole-inactivated A/Fort Monmouth/1/47 (FM1) virus. The ensuing vaccine responses and the protective efficacy of FM1 were superior in PR8 infection-primed mice compared to PR8 immunization-primed or unprimed mice. Increased FM1-specific Ab responses of PR8 infection-primed mice also broadened cross-reactivity against contemporary as well as antigenically more drifted strains. Further, prior infection heightened the protective efficacy of antigenically distant strains, such as A/Brisbane/59/2006 infection followed by immunization with split pandemic H1N1 vaccine (A/California/07/2009). Therefore, influenza infection is a significant priming event that intensifies future vaccine responses against drift strains.

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1. Introduction

Recommendations by the US Advisory Committee on Immunization Practices (ACIP) has helped improve overall influenza vaccine coverage and reduce disease burden and mortality [1,2], yet influenza remains a significant threat to public health [3]. Influenza vaccine effectiveness (VE), currently estimated at 50–60%

by the World Health Organization (WHO), is influenced by multiple confounding factors including vaccine recipients' age and health status, virulence of the circulating strain as well as the VE study design itself [4]. The antigenic relatedness between vaccine and circulating strain also impacts the VE, such that emergence of antigenically drifted strains have caused vaccine mismatches, resulting in increased infections and reduced VE [5–7]. The intermittent infections by drifted strains may seem discouraging for the vaccination effort, but it remains unclear how and to what extent these infections can influence subsequent vaccine responses. A better understanding of this issue can provide an important rationale for continual seasonal influenza vaccinations.

While the role of a primary infection on heterosubtypic immunity has been established in animal studies [8–12], its role on subsequent vaccine responses is not well-known. Human studies on this subject have been challenging, as it requires multi-year longitudinal studies in a defined cohort. However, recent studies provide valuable insights on what extent a single infection can induce hemagglutination inhibition (HI) titers against historic as well as contemporary strains in unvaccinated individuals, termed 'back-boost' [13]. Back-boost was also detectable following

Abbreviations: HA, hemagglutinin; NA, neuraminidase; HI, hemagglutination inhibition; HAU, hemagglutination unit; MN, microneutralization; LD₅₀, 50% lethal dose; PFU, plaque forming unit.

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vaccination, but as often is the case in clinical studies, the individuals' infection history and proper controls were not readily feasible, making it difficult to delineate infection history as a compounding factor for their Ab responses. In this study, we found that prior infection, but not immunization with PR8, intensified immunogenicity and efficacy of killed FM1 vaccine and broadened Ab cross-reactivity against antigenically further drifted strains in mice. Interestingly, prior infection enhanced vaccine efficacy of even antigenically distant strains. The impact of prior infection was also long-lasting, as immunization as late as 1 year post PR8-infection enhanced FM1-specific Ab responses. Since primary infections hardly occur in naïve hosts, we also addressed whether primary infection could be modulated in immune host using vaccine mismatch scenarios. Collectively, our findings suggest that an influenza infection strengthens the subsequent vaccine responses against variants in quantity and quality, while the impact of infection can be attenuated by the host's pre-existing immunity.

2. Methods

2.1. Cells, viruses and vaccines

Madin-Darby canine kidney (MDCK) cells were maintained in Dulbecco's modified Eagle's medium containing antibiotics, glutamine and 10% fetal bovine serum (FBS). Influenza viruses were propagated in 11-day-old embryonic chicken eggs and clarified allantoic fluid was used for virus infection of mice. For preparation of whole-inactivated viruses (WIV), clarified allantoic fluid was purified on discontinuous sucrose-gradient composed of 15%, 30% and 60% sucrose and inactivated with 4% w/v (=10%, v/v) formalin until no infectivity was detected in MDCK cells. After titration of the HA unit (HAU) by hemagglutination (HA) assay, WIVs (700–1400 HAU/100 μ l/mouse) were used as immunogens as previously described [14]. For the experiments assessing the impact of prior infection on the immunization with antigenically distant strain, commercially available pandemic H1N1 (pH1N1) monovalent split vaccine (A/California/07/2009; Cal07, 15 μ g HA/500 μ l) was used to immunize mice.

2.2. Mice, immunizations, infection and tissue collection, bronchoalveolar lavage (BAL), nasal wash

Balb/c mice, infection and immunization were previously described [14]. Spleen, lung and lymph nodes were collected after euthanizing mice with a lethal dose of Avertin (Sigma-Aldrich). BAL was collected by injecting 1 mL PBS + 0.5% bovine serum albumin (BSA) through the trachea with an 18G catheter. Nasal washes were collected by passing 0.5 mL PBS + 0.5% BSA through the nasal passage. All animal studies were performed with the approval and guidance of the Institutional Animal Care and Use Committees in an Association for Assessment and Accreditation of Laboratory Animal Care International-accredited animal facility of Emory University and the CDC.

2.3. Ab responses

Serum microneutralization (MN) and HI titers were previously described [14]. Mucosal IgG and IgA responses of BAL and nasal washes were analyzed by ELISA. Nunc 96 well plates (Maxi-sorb) were coated with 100HAU whole inactivated virus (WIV), then blocked with 4% BSA in PBS-Tween for 1 h. Ten-fold dilutions of samples are added to the plates for 2 h. Plates were then developed by biotin- α -mouse IgG/IgA followed by streptavidin (SA)-HRP (Southern Biotech). The signals were developed using 1 \times TMB (ebioscience) and measured at 450 nm using a plate reader (Biotek).

2.4. Ab-secreting cells (ASCs) by ELISpot assay

ELISpot plates (Millipore) were coated with 100 HAU WIV overnight and blocked with cRPMI-1640 media. Dilutions of cells were added to plates and incubated overnight at 37 °C. Plate-bound Abs were probed by biotin- α -mouse IgG, SA-alkaline phosphatase (Southern Biotech), then Vector Blue Substrate Kit (Vector Lab). Spots were counted using an ImmunoSpot® ELISPOT reader (Cellular Technology Ltd.).

2.5. Lung lysates and plaque assay

Lung lysates were prepared by homogenizing lung tissues through a 40 μ m cell strainer with syringe plungers. Homogenates were spun at 450 \times g for 20 min at 4 °C and the supernatants were assessed for virus titers via plaque assay as previously described [14].

2.6. Monoclonal Ab staining and flow cytometry

Cells were stained with CD11b, CD95, CXCR5, CD3, CD8a (BD Bioscience); CD103, I-Ad, GL7, CD138, CD69, CD86, CD19 (Biolegend); CD49b, F4/80, CD11b, CD11c, CD45, PD-1 (eBioscience). Virus-specific CD8⁺ T cells were identified using H-2K^d/IYSTVASSL (HA) and H-2K^d/TYQRTRALV (NP) pentamers (Proimmune). For in vitro stimulation, cells were infected with PR8 or FM1 virus at a multiplicity of infection (MOI) of 1 for 1 h. After incubation overnight and addition of Golgi-Plug (BD) for the last 6 h, cells were stained with CD4 and CD8, permeabilized and stained with IFN γ , IL-2, TNF α . Cells were analyzed with a FORTRESSA flow cytometer (BD) and FlowJo software (Tree Star, Inc.).

2.7. Statistics

Student's *t* test was used to compare the Ab titers between PR8 vs. FM1-specific responses following log₂ transformation. For multiple groups, one way analysis of variances with Bonferroni post-test was used. For statistical designations, * denotes $p < 0.05$; ** denotes $p < 0.02$; *** denotes $p < 0.001$.

3. Results

3.1. Prior infection, but not immunization with PR8 enhanced the local and systemic Ab responses and virus-specific T cell response following FM1-WIV immunization

To compare the vaccine responses in differential priming contexts, mice were either infected or immunized with PR8. Infection dose (0.01 \times LD₅₀) was chosen to achieve subclinical infection ($\leq 5\%$ body weight (BW) loss; data not shown). At memory phase (>d28), mice were immunized with FM1-WIV and the acute local Ab responses in inguinal lymph nodes were assessed on d5 post-immunization. The %plasma cells were significantly higher in PR8_{inf}/FM1_{imm} than in PBS/FM1_{imm} or PR8_{imm}/FM1_{imm} mice (Fig. 1A). The differential local Ab response was also reflected systemically in spleen by significant Ag-specific ASC responses (Fig. 1B). While FM1-WIV in naïve mice (PBS/FM1_{imm}) induced minimal ASC responses, it intensified PR8- and FM1-specific ASC responses in PR8_{inf}/FM1_{imm}, but not in PR8_{imm}/FM1_{imm} mice. The virus-specific (NP⁺) CD8 T cell response in local lymph nodes was also significantly higher in PR8_{inf}/FM1_{imm} compared to control groups (Fig. 1C). However, virus-specific CD4 and CD8 T cells in spleen were readily recalled upon in vitro stimulation as long as the mice were previously infected with PR8 (Fig. 1D, Supplemental Fig. 1A and B). Both PR8 and FM1 stimulated T cells at comparable levels, indicating significant cross-reactivity of T

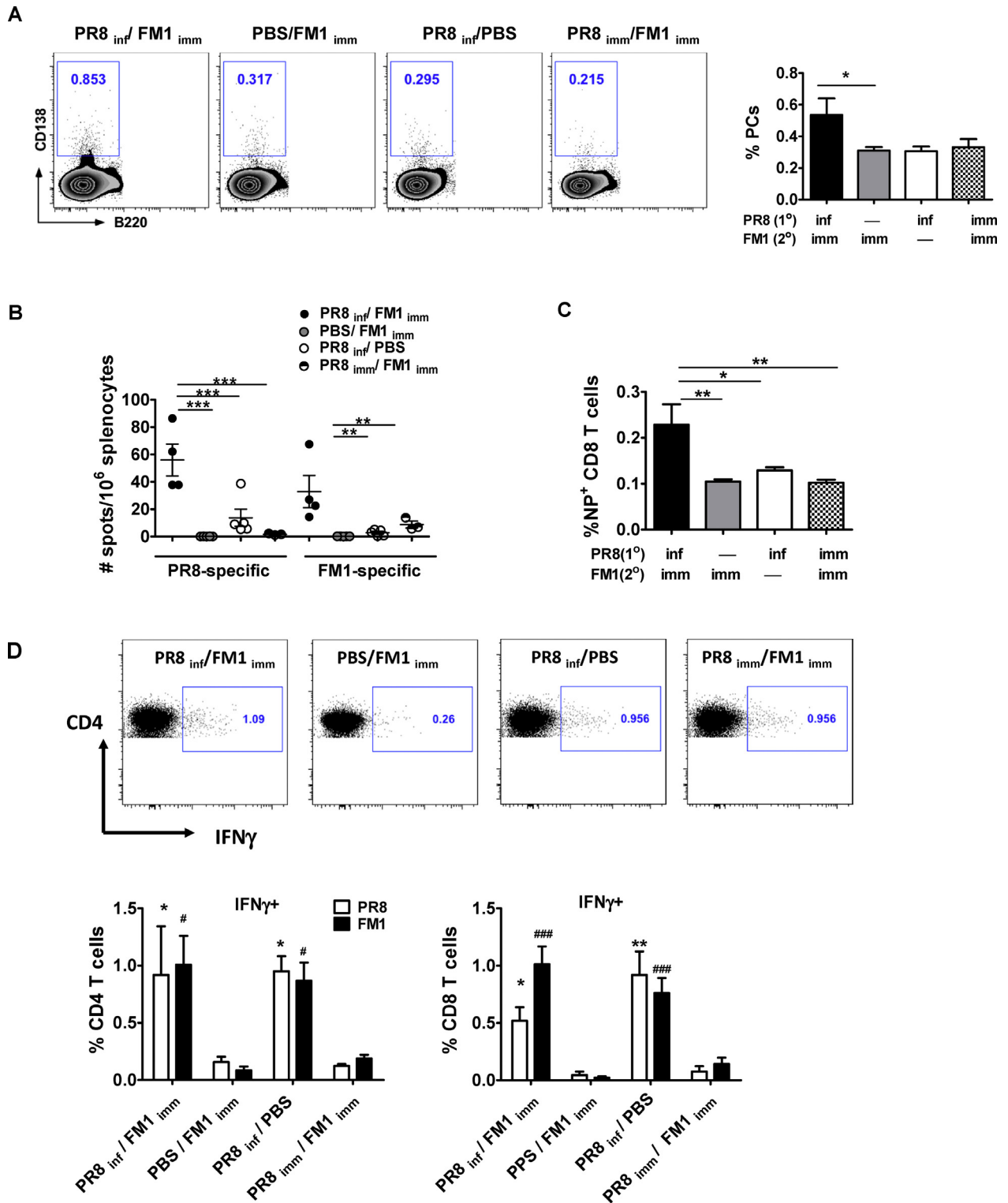


Fig. 1. Prior infection, but not immunization with PR8 enhanced the local and systemic Ab responses and virus-specific T cell responses following FM1-WIV immunization. Balb/c mice (5 mice/group) were infected i.n. with $0.01 \times LD_{50}$ mouse-adapted PR8 or immunized i.m. with 1400 HAU PR8-WIV or mock-infected. A month later, all mice except controls (PR8_{inf}/PBS) were immunized i.m. with 1400 HAU FM1-WIV. (A) Inguinal lymph nodes were collected at d5 post immunization and the frequency of plasma cells (B220⁺ CD138⁺) was analyzed by flow cytometry. (B) Spleens were also collected at the same time and PR8 vs. FM1-specific ASCs were analyzed by ELISPOT assay. (C) Influenza virus NP-specific CD8 T cells in lymph nodes were stained with NP+ pentamers and analyzed by flow cytometry. (D) Splenocytes were stimulated in vitro with egg-grown PR8 or FM1 at MOI 1 overnight then IFN γ -secreting CD4 or CD8 T cells were analyzed by intracellular cytokine staining and flow cytometry.

cell epitopes between the two viruses. On the other hand, prior immunization with PR8 failed to recall T cell responses upon FM1 immunization (PR8_{imm}/FM1_{imm}). Local follicular helper T cells (T_{FH}) were also marginally induced in PR8_{inf}/FM1_{imm} compared

to controls (Supplemental Fig. 1C). These data demonstrate that prior infection, but not immunization elicits superior Ab responses upon immunization with a drift strain. Concomitantly, virus-specific CD8 T cells, normally poorly induced by killed vaccine,

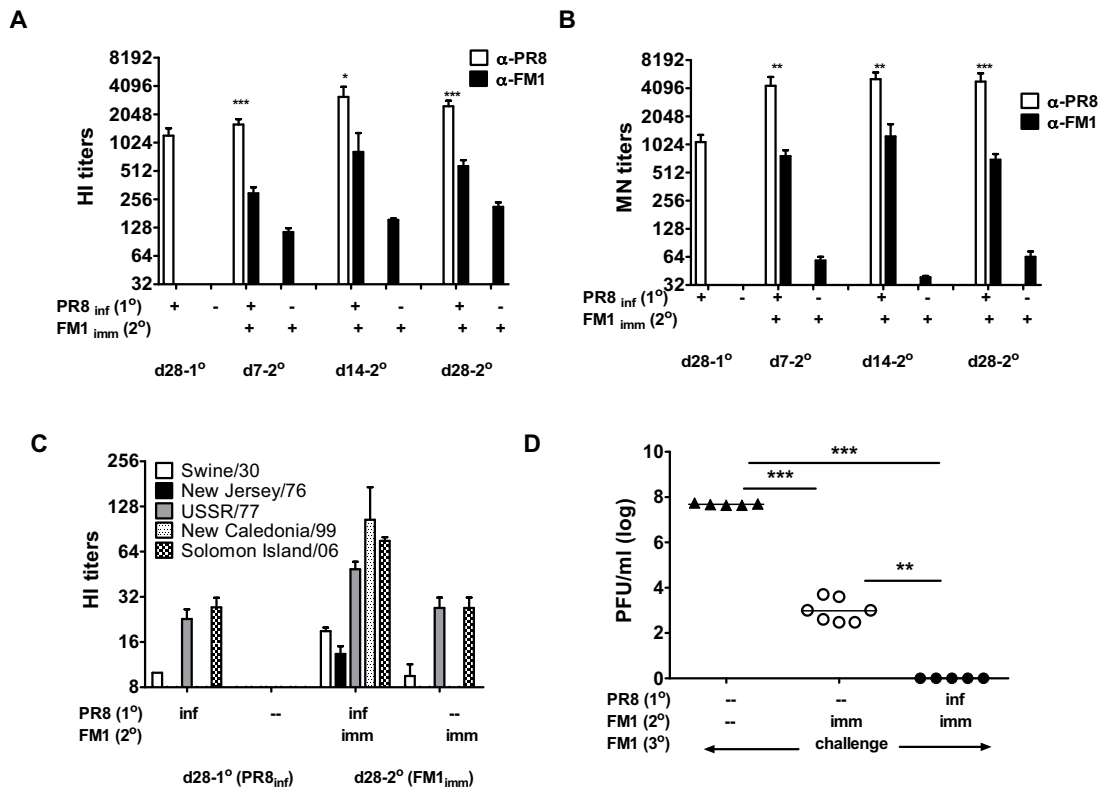


Fig. 2. Prior infection with PR8 heightened and broadened the Ab responses upon FM1 immunization and enhanced protective efficacy of vaccine Ag. Balb/c mice (5 mice/group) were infected i.n. with $0.1 \times LD_{50}$ PR8 virus and then a month later immunized with 1400HAU FM1-WIV. (A and B) Mice were bled on d28 post PR8 infection (d28-1°) and days 7, 14 and 28 post FM1 immunization (d7-2°, d14-2°, d28-2°) and tested for HI (A) and MN titers (B) against MDCK-cell grown PR8 and FM1 viruses. (C) The sera collected before (d28-1°) and after (d28-2°) FM1 immunization were further tested for HI titers against other historic and antigenically drifted seasonal human H1N1 viruses as well as swine virus (Swine/30, New Jersey/76, USSR/77, New Caledonia/99 and Solomon Island/06). (D) All immune mice and naïve control mice were challenged with a lethal dose ($100 \times LD_{50}$) of FM1 virus at d28 post immunization. Lung lysates were collected on d4 following challenge and tested for virus titers via plaque assay.

are recruited to the local immunization site at significantly higher levels.

3.2. Prior infection with PR8 heightened and broadened the Ab response upon FM1 immunization and enhanced protective efficacy of vaccine Ag

The enhanced acute responses in PR8_{inf}/FM1_{imm} mice (Fig. 1) led to development of robust FM1-neutralizing Abs (Fig. 2A and B). Upon FM1 immunization, a mixture of secondary and primary Ab responses was detected in PR8_{inf}/FM1_{imm} mice; PR8-HI and MN titers were immediately boosted, while FM1-titers followed primary response kinetics. However, their FM1-titers were significantly higher than those of the PBS/FM1_{imm} mice (Fig. 2A and B) which presented a genuine primary FM1-Ab response, and the rest of the control groups (data not shown). The quantitative increase in Ab titers was accompanied with expansion of cross-reactivity (Fig. 2C). Sera collected before and after FM1-WIV were tested against A/Swine/Iowa/15/30, a historic swine H1N1 strain during the 1930–1940s [15], as well as more antigenically drifted strains that had circulated in later years in swine and/or humans (A/New Jersey/8/1976, A/USSR/90/1977, A/New Caledonia/20/1999 and A/Solomon Island/03/2006). The genetic relationship and distances of these viruses were recently reported [16]. While PR8 infection elicited USSR/77- and Solomon Island/06-HI titers (d28-1°), FM1 immunization induced additional cross-reactivity against Swine/30, New Jersey/76, and New Caledonia/99, and further increased USSR/77- and Solomon Island/06-HI titers (d28-2°). In contrast, PBS/FM1_{imm} mice developed cross-reactivity against only USSR/77 and Solomon Island/06. The protective efficacy of

FM1-WIV was, not surprisingly, superior in PR8_{inf}/FM1_{imm} than in PBS/FM1_{imm} mice, in a subsequent lethal challenge (Fig. 2D). Lung virus titers at d4 post-challenge were undetectable in PR8_{inf}/FM1_{imm} mice whereas 9.5×10^2 PFU/ml and 5×10^7 PFU/ml were present in PBS/FM1_{imm} and naïve mice, respectively. Thus, while FM1-WIV reduced a lethal FM1 infection, prior PR8-infection intensified the protective efficacy of FM1-WIV. Since an infection leaves life-long immune memory, the long-term impact of infection was tested by immunizing PR8-infected mice with FM1-WIV either 3 months or 1 year later (the length of this study). Regardless of the length of time, prior PR8-infection elicited significant FM1-Ab titers upon FM1-WIV immunization (Supplemental Fig. 2A), and all PR8_{inf}/FM1_{imm} mice survived lethal challenge (data not shown). Furthermore, any change in the infection dose (0.01 – $0.1 \times LD_{50}$) or immunization dose (700–1400 HAU) did not affect protective efficacy (Supplemental Fig. 2B and C).

3.3. Prior infection with Bris59 enhanced Ab responses and protective efficacy to the antigenically distant strain, Cal07, upon immunization

Since antigenic distance is a major determinant for vaccine efficacy [17], the impact of prior infection on vaccine responses may be confined to antigenically related strains such as PR8/FM1 [14]. We tested whether this effect could be extended to antigenically and serologically distant viruses such as Bris59 and Cal07 (pH1N1) [16,18]. Infection with Bris59 followed by immunization with pH1N1 vaccine immediately intensified Cal07-HI titers, whereas titers of PBS/Cal07_{imm} mice were fully developed at d28 post vaccination (Fig. 3A). Consistent with the serological distance

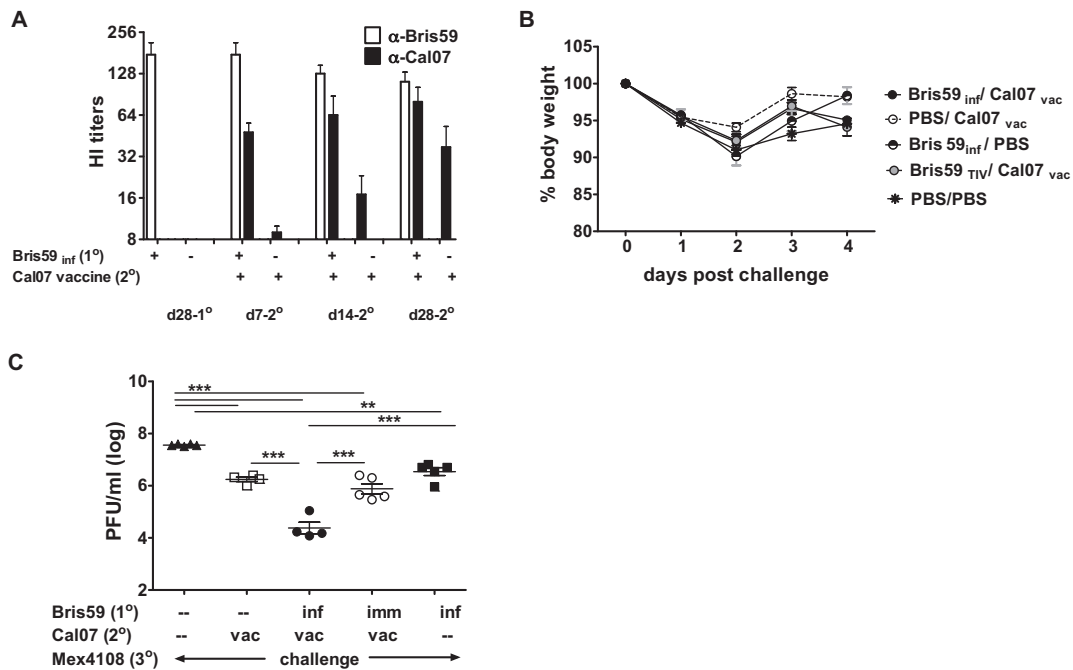


Fig. 3. Prior infection with Bris59 enhanced the Ab response and protective efficacy to the antigenically distant pandemic H1N1 strain, Cal07 upon immunization. Balb/c mice (4–5 mice/group) were infected i.n. with Bris59 at 5×10^4 PFU or mock-infected with PBS or immunized i.m. with 09–10 seasonal trivalent inactivated vaccine containing 3 μ g Bris59. A month later, all mice except control Bris59_{inf}/PBS were immunized i.m. with monovalent pH1N1 (A/California/07/09) vaccine containing 3 μ g HA. (A) Sera samples before and after immunization with Cal07 were collected and HI titers were tested against egg-grown Bris59 and Cal07 viruses. (B) A month after immunization, all mice including naïve mice were challenged with A/Mexico/4108/09 at 8×10^6 PFU and their BWs were monitored. (C) Lung lysates were collected on day 4 following challenge and lung virus titers were analyzed via plaque assay.

between Bris59 and Cal07, Bris59-HI titers were not boosted by pH1N1 immunization. When protective efficacy of pH1N1 vaccines was tested in subsequent lethal challenge with A/Mexico/4108/09 (pH1N1), Bris59_{inf}/Cal07_{imm} mice showed modest BW loss (Fig. 3B), yet had the lowest lung virus titers compared to various control groups (Fig. 3C). These data emphasize the benefit of subclinical infection in aiding subsequent vaccine responses even when the vaccine strain is antigenically distant from the infecting strain.

3.4. Attenuation of infection by prior immunization moderated development of protective immunity in an infection-dose dependent manner

Several serosurveillance studies show that the general population is immune to influenza virus likely due to prior infection or immunization [19–23]. Therefore, while prior infection intensifies subsequent vaccine responses (Figs. 1–3), seasonal infections hardly occur in naïve hosts, but rather as a result of vaccine mismatch. We, therefore, assessed whether and to what extent an infection can be modulated following mismatched vaccination, by immunizing mice first with PR8-WIV, then infecting with 0.01 or 0.1 \times LD₅₀ FM1. Following PR8-WIV-immunization, sera PR8-HI and MN titers were readily detected, yet FM1 cross-reactivity was detected only by MN assay (Fig. 4A). However, mucosal (BAL and nasal washes) cross-reactive IgGs were comparable against both viruses (Fig. 4A) with little IgA induction at either site (data not shown). Upon FM1 infection, all mice showed minimal weight loss, except PBS/FM1_{inf} (0.1) mice, which lost 5–6% BW (Supplemental Fig. 3A and B). However, lung virus titers of PR8_{imm}/FM1_{inf} mice at d5 post-infection were significantly lower than PBS/FM1_{inf} mice at each dose (Fig. 4B). Approximately 44% of PR8_{imm}/FM1_{inf} (0.01) as well as 80% of PR8_{imm}/PR8_{inf} (0.1) mice (positive control) efficiently cleared infection. The lung immune parameters reflected the differential level of ongoing infections. Although %lung resident

APC subsets (CD103⁺ vs. CD11b⁺) [24] was comparable among groups (Supplemental Fig. 4A), their activation (CD86) was significantly lower in PR8_{imm}/FM1_{inf} compared to PBS/FM1_{inf} mice at each dose (Fig. 4C, Supplemental Fig. 4B). Likewise, lung inducible T_{FH} cells [25] were induced at comparable levels, yet their activation (CD69) was reduced in PR8_{imm}/FM1_{inf} compared to PBS/FM1_{inf} mice (Fig. 4C, Supplemental Fig. 4C). Germinal center (GC)-B cells were not immediately affected by differentially activated T_{FH} cells, yet a differential level of activated B cells started to appear (Supplemental Fig. 4D). While the majority of immune parameters followed this fashion, CD16/32 (FcγR II/III) expression (Fig. 4D, Supplemental Fig. 4E) on CD11b⁺ cells was significantly higher in PR8_{imm}/FM1_{inf} compared to PBS/FM1_{inf} mice. Coincidentally, significantly more NP⁺ CD8 T cells were found in PR8_{imm}/FM1_{inf} than in PBS/FM1_{inf} mice (Fig. 5D). Since CD16/32 binds to immune complexes (ICs), this implies that IC-mediated cross-presentation [26,27] is enhanced in PR8_{imm}/FM1_{inf} mice. Virus-specific CD8 T cells recruited in this manner may contribute to further viral clearance. The mice with undetectable virus titers in Fig. 4B induced little to no activation of APCs, NP⁺ CD8 T cells, GC-B cells or T_{FH} cells (Supplemental Fig. 5A–D). Altogether, these data demonstrate that the degree of infection is mitigated even by mismatched vaccinations, yet in turn, the development of humoral immunity against the infecting strain is attenuated in an infection dose-dependent manner.

3.5. The infection dose-dependent acute responses led to the differential profile of FM1-specific protective immunity

Since attenuation of local immunity was evident, the development of FM1-Ab responses was monitored at days 7, 14 and 28 post-infection. In PR8_{imm}/FM1_{inf} (0.01) mice, FM1-HI and MN titers were significantly less than those of PBS/FM1_{inf} (0.01) mice, while PR8-titers were boosted (Fig. 5A, Supplemental Fig. 6A). In contrast, FM1-titers of PR8_{imm}/FM1_{inf} (0.1) mice were comparable to

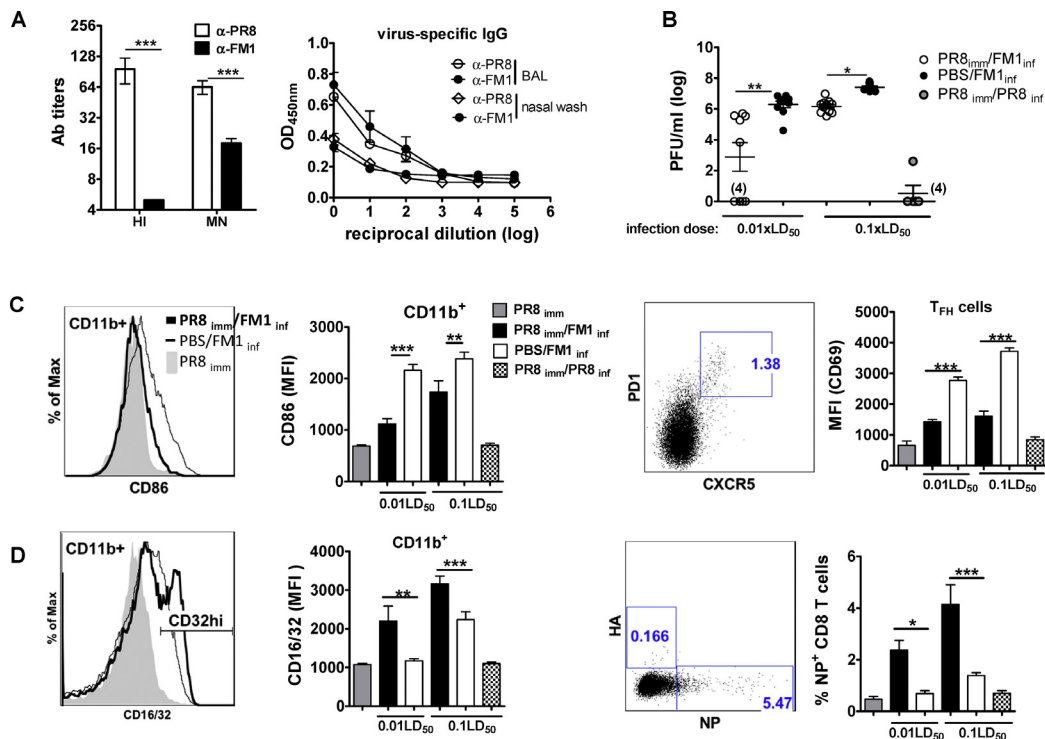


Fig. 4. Attenuation of infection by prior immunization moderated the development of protective immunity in an infection-dose dependent manner. Balb/c mice (9–10 mice/group) were immunized i.m. with 1400 HAU PR8-WIV or mock-immunized. (A) Sera, BAL and nasal wash samples were collected for assessment of Ab responses at d28 post immunization. Sera HI and MN titers were tested against PR8 and FM1 viruses (left panel). BAL and nasal washes were tested for virus-specific IgG by ELISA (right panel). (B–D) All mice were infected i.n. with 0.01 or 0.1 × LD₅₀ mouse-adapted FM1 virus. Mock infected mice (PR8_{imm}/PBS) and PR8_{imm}/PR8_{inf} (0.1) were set up as additional controls. (B) Lung lysates were collected at d5 post infection and lung virus titers were analyzed by plaque assay. The numbers indicate mice with undetectable virus titers. (C and D) Lung single cell suspensions were stained for APC subsets (CD11c⁺CD11b⁺, CD11c⁺CD103⁺), T_{FH} cells (CD4⁺CXCR5⁺PD1⁺) and NP-specific CD8 T cells. (C) A representative histogram of CD86 expression and its mean fluorescence intensity (MFI) on CD11b⁺ subsets of all groups, a representative flow chart of lung T_{FH} (CD4⁺CXCR5⁺PD1⁺) cells and their activation (CD69 MFI) of all groups are summarized. (D) A representative histogram of CD16/32 expression and its MFI on CD11b⁺ subsets of all groups, a representative flow chart of lung CD8⁺ T cells stained by NP or HA-pentamers and their summary of all groups are shown.

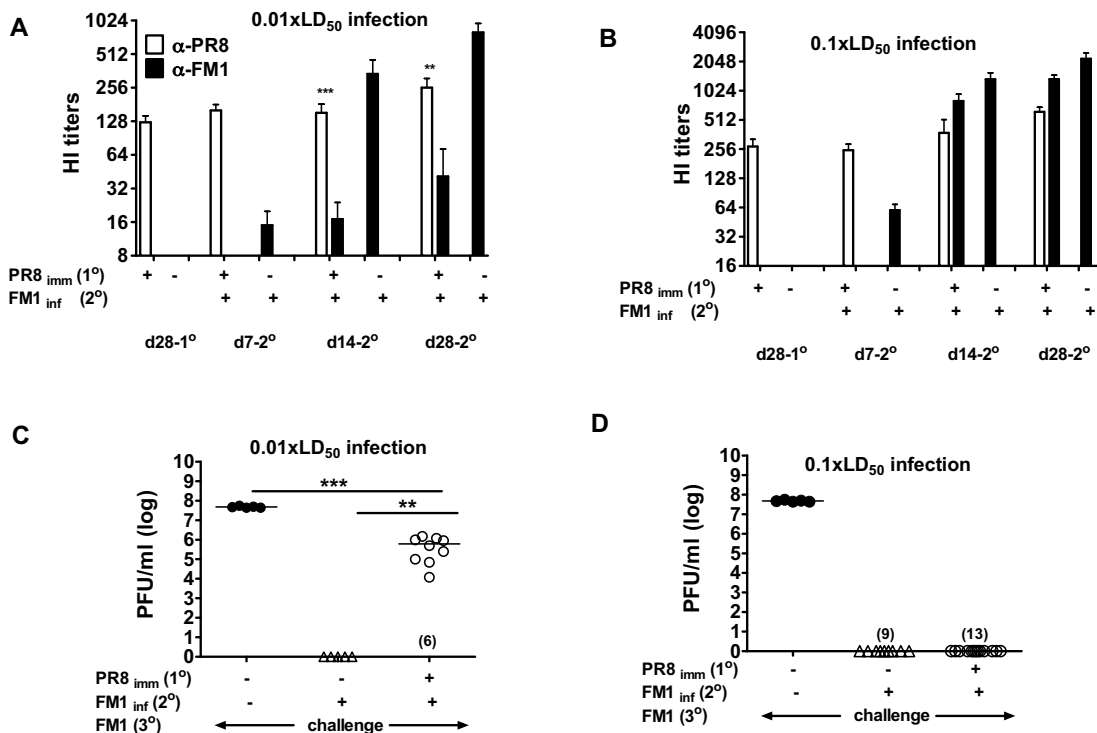


Fig. 5. The infection dose-dependent acute responses led to a differential profile of FM1-specific protective immunity. Balb/c mice (6–10 mice/group) were first immunized with 1400 HAU PR8-WIV and then infected with 0.01 or 0.1 × LD₅₀ FM1 virus at d28 post immunization. (A and B) Serum samples were collected at d28 post immunization (d28-1°), and days 7, 14 and 28 post FM1 infection (d7-2°, d14-2°, d28-2°) for assessment of Ab responses. HI titers were tested against PR8 and FM1 viruses and shown separately depending on infection dose for clarity. (C and D) A month after FM1 infection, mice were lethally challenged with 100 × LD₅₀ FM1 virus and lung lysates were collected at d4 post challenge. Lung viral titers were assessed via plaque assay and plotted separately for clarity. The numbers indicate the mice with no detectable titers.

those of PBS/FM1_{inf} (0.1) mice at 2 weeks and onward (Fig. 5B, Supplemental Fig. 6B). This dichotomy of the FM1-Ab responses was sharply contrasted in protective immunity against FM1. Upon lethal challenge, lung viral titers of PR8_{imm}/FM1_{inf} (0.01) mice were significantly higher (6×10^5 PFU/ml) than PBS/FM1_{inf} (0.01) mice (Fig. 5C). In contrast, lung titers of PR8_{imm}/FM1_{inf} (0.1) mice were undetectable as in PBS/FM1_{inf} (0.1) mice (Fig. 5D). Therefore, infection can be immediately attenuated even by mismatched vaccination, yet protective immunity against the infecting strain is consequently compromised in an infection dose-dependent manner.

4. Discussion

Our findings demonstrate that infection leaves a long-lasting immunological imprint that boosts subsequent vaccine responses against variants. Priming by infection, but not immunization conferred superior cell-mediated immune responses that were readily recruited to the local immunization site and were associated with the augmented vaccine response. Clinical significance of cellular immunity has been recently demonstrated in a human challenge study and during the 2009 pH1N1 infection wave, by direct correlation of pre-existing CD4 or CD8 T cell responses against conserved T cell epitopes with reduced virus shedding, disease symptoms and severity in neutralizing Ab-naïve individuals [28,29]. Therefore, broadly cross-reactive cellular immunity conferred by prior infection may be directly responsible for the enhanced efficacy of antigenically related as well as distant vaccine strains (Figs. 2–3).

On the other hand, inefficient development of cellular immunity by prior vaccination was associated with the immunogenicity and protective efficacy of killed virus that was comparable to that of naïve hosts (Figs. 2–3). Consistent to our findings, recent reports showed that virus-specific CD8 T cell immunity was absent in children who were annually vaccinated with inactivated influenza vaccines (IIV), but observed in unvaccinated healthy children, who had presumably experienced infections during childhood [30]. Further, a recent study in children found that repeated prior IIV vaccination during the previous 5 years was associated with lower VE in the current season compared to individuals with no prior vaccination history [31]. It remains unknown whether an infection episode of the latter might have further impacted the current VE. Considering that live-attenuated influenza vaccine (LAIV) formulation is available for healthy children age ≥ 2 and that LAIV has been shown to induce significantly better T cell immunity than IIV in children [32–34], it will be of great clinical significance to longitudinally investigate vaccine responses against antigenically drifted strains in the LAIV-administered population.

Recent findings from limited vaccine trials showed that back-boost was achieved by immunization, with the highest effect within antigenic clusters [13]. Since the degree of back boost was quite variable among individuals, it would be interesting to examine the correlation of individuals' infection history and the degree of back boost. Our findings highlight the impact of infection in expanding the breadth of Ab responses to antigenically far-evolved strains from the infecting strain, as well as increasing the magnitude of Ab responses to the vaccine strain itself (Fig. 2A–C). In addition, prior infection potentiated the protective efficacy of a vaccine strain of a far distant antigenic cluster (Fig. 3). The increased breadth of Ab responses were not maintained beyond a year in human serology studies [13]. However, our experiments extending the time between infection and immunization up to 1 year showed that the protective immunity was easily recalled long after the initial infection (Supplemental Fig. 2A). Similar observations were made by a recent trial showing that the primary Ab response was undetectable in pandemic LAIV (pLAIV)-recipients, yet upon vaccination

with inactivated subvirion influenza A (H5N1) vaccine 4 years later, HI titers as well as Ab affinity were significantly better in pLAIV-primed recipients than unprimed recipients who received 2 doses of H5N1 vaccine [35].

While infection leaves a profound imprint in naïve hosts, natural infections in the general public are likely secondary responses in nature due to pre-existing immunity to influenza. Therefore, infection events also need to be considered in the face of pre-existing, cross-reactive immunity. Under vaccine mismatch scenarios, FM1 infection was attenuated by cross-reactive Abs in an infection-dose dependent manner. While this presents the benefit of mismatched vaccination, reduced infections, in turn, dampened the subsequent FM1-Ab responses and protective immunity, especially in mice infected with $0.01 \times LD_{50}$ FM1. While FM1-Ab responses were reduced according to available viral Ag, %virus-specific CD8 T cells were much higher as infection was attenuated in an infection-dose dependent manner (Fig. 4D, panel 4). Although killed vaccine (PR8_{imm}) poorly induced CD8 T cells, subsequent FM1-infection accelerated the recruitment of cross-reactive CD8 T cells (PR8_{imm}/FM1_{inf}), demonstrating a previously unappreciated role of mismatched vaccination. Considering that lung CD103⁺ DC subsets are non-permissive to influenza infection, but prone to cross-presentation to CD8 T cells [36] and our current findings on upregulation of FcγRs in CD11b⁺ (Fig. 4D, panels 1–2) as well as CD103⁺ DC subsets (data not shown) of PR8_{imm}/FM1_{inf} mice, the IC-mediated cross presentation by these DC subsets is a likely mechanism for the recruitment/expansion of pre-existing CD8 T cells that are otherwise present at low levels. Of note, sera from PR8-immunized mice exhibited comparable binding activity to PR8 and FM1 viruses measured by ELISA (data not shown). Therefore, the benefit of mismatched vaccination can be at two levels; cross-neutralizing Abs immediately reduce the viral load, while non-neutralizing, cross-reactive Abs recruit CD8 T cells. Since vaccine mismatches are not uncommon as exemplified during the 2014–2015 influenza season [37], it would be of great interest to examine CD8 T cell responses of infection cases (vaccine failures) and their long-term memory responses compared to unvaccinated, infection cases.

Overall, our findings advocate the ACIP recommendation of influenza vaccination despite intermittent vaccine mismatches and seasonal infections. For hosts with established pre-existing immunity, even mismatched vaccinations provide partial protection against infection by pre-existing Abs and elicitation of cross-reactive T cells, yet improving vaccine formulation is worthwhile to consider to maximize the benefit of seasonal vaccinations.

Disclaimer: The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention or the Agency for Toxic Substances and Disease Registry.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.vaccine.2015.11.077>.

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