Heliyon

Efficient protection of mice from influenza A/H1N1pdm09 virus challenge infection via high avidity serum antibodies induced by booster immunizations with inactivated whole virus vaccine

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Abstract

The immunogenicities of inactivated whole and split virus vaccines derived from influenza A/H1N1pdm09 virus were compared in a mouse model. We demonstrated the unique properties of whole virus vaccine boosters on the serum memory antibody response in mice. Consistent with previous studies, booster immunization with either whole or split virus vaccines of A/H1N1pdm09 virus produced comparable titers of serum antibodies with hemagglutination inhibition and virus-neutralizing activities. However, superior protection against the challenge infection was unexpectedly observed in mice primed and boosted with whole virus vaccines compared with those treated with split virus vaccines, despite similar levels of antibody titers in each group. Immune serum antibodies were shown to be primarily responsible for this protection via passive transfer experiments of immune serum antibodies to naive recipient mice. Moreover, this

protection correlated with elevated affinity maturation of the antibodies. Thus, booster immunization with whole virus vaccines elicited a robust serum antibody response with high avidity to the virus, which was not measurable via conventional serological assays.

1. Introduction

Vaccination is considered the most effective prophylactic measure for reducing morbidity and mortality associated with influenza virus infections [1]. Although several types of influenza vaccines are currently available, inactivated vaccines, including whole and split virus vaccines, are the most commonly used worldwide. Whole virus vaccines are produced from influenza viruses grown in embryonated chicken eggs or in cell cultures by inactivating purified viral particles with formaldehyde or β-propiolactone. Split virus vaccines are manufactured by disrupting purified viral particles with ether and/or detergents to remove the viral lipid envelope.

Serum antibodies induced by immunization with the inactivated vaccines provide the primary defense against influenza virus infections. The antibody response once primed by infection or vaccination is recalled by booster immunization with inactivated vaccines, resulting in a robust virus-neutralizing serum antibody response. Both whole and split virus vaccines are generally thought to be equally immunogenic for eliciting serum antibodies in primed but not naive hosts [2, 3, 4, 5]. Split virus vaccines have reduced adverse reactions compared with whole virus vaccines, and thus are widely used in many countries [6, 7]; however, they are less immunogenic in naive hosts and two doses are required for optimal antibody responses [8]. Furthermore, split virus vaccines are less effective in young children than in adults [8, 9]. It has been demonstrated that viral RNAs inside whole virus vaccines are the key viral components for vaccine priming, but not for boosting, as endogenous adjuvants via a TLR7-dependent mechanism, but these are lost during the manufacturing processes [10, 11, 12].

In a search to find new quality control assays to improve the immunogenicity of vaccines, we compared whole and split virus vaccines derived from A/H1N1pdm09 virus in a mouse model. Unexpectedly, mice that had been primed and boosted with whole virus vaccines had superior protection compared with those treated with split virus vaccines, although both groups showed similar serum virus-binding, hemagglutination inhibition (HI) and virus-neutralizing antibody titers. Furthermore, we found that the protection provided by whole virus vaccines correlated more closely with the avidity of serum antibodies than conventional antibody titers. It has been shown that antibody affinity constitutes an important parameter that affects the

outcome of viral infections [13, 14]. In particular, severe cases of pandemic 2009 H1N1 influenza were reported to have higher titers of non-protective serum antibodies with low avidity to the virus than mild cases, and led to severe immune complex-mediated diseases [15]. Thus, our findings imply that whole virus vaccines have a novel ability to recall the serum antibody response, and antibody avidity is a useful serological indicator of the efficacy of influenza vaccines.

2. Materials and methods

2.1. Viruses and vaccines

Influenza viruses A/California/7/2009-derived high growth reassortant virus X-179A (H1N1)pdm09, chicken egg-isolate A/Narita/01/2009 (H1N1)pdm09 [16], and A/PR/8/34 (PR8) (H1N1) were propagated in 10-day-old embryonated chicken eggs. Whole and split virus vaccines were prepared from X-179A and PR8 viruses as follows: Briefly, viruses were propagated in allantoic cavities of embryonated chicken eggs and pelleted using ultracentrifugation. Pelleted viruses were resuspended, further purified by zonal centrifugation with a sucrose density gradient, and inactivated with formalin for the preparation of the whole virus vaccine [17]. To prepare the split virus vaccine, purified virus was treated with ether and polysorbate 80, and the resulting aqueous phase was recovered and treated with formalin [18]. Inactivation of the vaccine preparations was confirmed by the absence of virus growth in eggs inoculated with the specimens demonstrating no hemagglutinating activity. The X-179A vaccine was generously provided by the Kitasato Institute (Kitamoto, Japan). The protein concentration of whole and split virus vaccines was measured with a DC Protein Assay (Bio-Rad).

2.2. Animals, vaccination, and challenge infection

Female BALB/c mice (7−10 weeks old) were purchased from SLC (Shizuoka, Japan). All animal experiments were performed in accordance with the institutional guidelines for the animal facility at the National Institute of Infectious Diseases (NIID) and were approved by the Animal Care and Use Committee of the NIID (approval No. 112033, 112117, 213025, 213026). Mice were injected subcutaneously with whole or split virus vaccines at various doses. Twenty-eight days later, the mice received booster shots with the same dose of either whole or split virus vaccine. Serum antibody titers were determined by enzyme-linked immunosorbent assay (ELISA), HI, and microneutralization (MN) assays on days 28 and 42 after the initial immunization. Protection experiments were performed as described previously [16]. Briefly, groups of mice (n = 5 per group) injected with either whole or split virus vaccine according to the prime and booster immunization protocol were intranasally challenged with 20 μL (20LD$_{50}$) of A/Narita/01/2009 on day 42. Lung

tissue samples were collected 3 days post-challenge, and 10% (w/v) lung homogenate suspensions were prepared and tested for virus infectivity by 50% tissue culture infectious dose ($TCID_{50}$) assay. Naive mice (n = 6 per group) were included as a control group. Body weights were recorded on the day of challenge infection and at 3 days after.

For passive transfer experiments, sera were recovered from 9 to 10 BALB/c mice 14 days after booster shots with 3 μg of whole or split virus vaccine. Non-immune sera were also obtained from age-matched BALB/c mice as a control group. Mice were injected intraperitoneally with either naive or immune sera (n = 5 per group, 200 μL serum per mouse), and challenged with $20LD_{50}$ of A/Narita/01/2009 one day later. Virus titers in lung homogenates were measured 3 days post-challenge. Serum samples were taken to measure antibody titers and avidity. Body weights were recorded on the day of challenge and 3 days after.

2.3. Microneutralization and hemagglutination inhibition assays

Serum samples were pretreated with receptor-destroying enzyme (Denka Seiken, Tokyo, Japan) overnight at 37 °C before being heated at 56 °C for 30 min and diluted with minimum essential medium to a final serum dilution of 1:10. Serum antibody titers were measured by MN and HI assays based on standard procedures [19]. For the MN assay, 50 μL of serial 2-fold dilutions of the sera were prepared starting at 1:10, and incubated with equal volumes of 100 $TCID_{50}$ of A/Narita/01/2009 (H1N1)pdm09 virus containing 20 μg/mL acetylated trypsin (Sigma) for 30 min at 37 °C. Confluent monolayers of MDCK cells in 96-well plates were washed with PBS and serum/virus mixtures were transferred into the wells. After incubation for 6 days at 34 °C, cytopathic effects (CPE) in individual wells were observed under an inverted microscope. MN antibody titers were expressed as the reciprocal of the highest serum dilution causing complete protection from virus-induced CPE. Naive mouse sera showed less than 10. For HI assays, serum samples were treated with packed turkey red blood cells to remove non-specific hemagglutinating activity. Using U-bottom 96-well microtiter plates, 2-fold serial dilutions of sera (25 μL) starting at 1:10 were diluted with PBS and mixed with an equal volume of 4 hemagglutinating units of A/Narita/01/2009 (H1N1)pdm09 virus. The mixture of diluted serum and virus was incubated for 30 min at room temperature. Fifty microliters of 0.5% turkey red blood cells was added to the antigen/serum mixture and incubated for 45 min at room temperature. HI antibody titer was expressed as the reciprocal of the highest serum dilution that could prevent hemagglutination. Naive mouse sera showed less than 10.

2.4. ELISA

Virus-binding antibody was measured by ELISA as follows: ELISA plates were coated with 10 μg/mL of whole virus vaccine in 0.05 M carbonate buffer (pH 9.6)

at 4 °C. After blocking nonspecific binding with Block Ace (DS Pharma Bio Medical, Japan) and subsequent washes, 4-fold serial dilutions of sera at 1:10 or 1:100 were added to the wells. Bound IgG was detected using biotin-conjugated anti-mouse IgG (Jackson Laboratories, Bar Harbor, ME), followed by alkaline phosphatase-conjugated streptavidin (Zymed Laboratories, San Francisco, CA) and *p*-nitrophenylphosphate substrate (Sigma Chemical Co., St. Louis, MO). To measure virus-binding antibody to A/Narita/01/2009, mouse serum 28 days post-infection was diluted to 1:500 and used as a standard (32 U/mL). Similarly, purified PR8 HA-specific monoclonal IgG (32 ng/mL) was used as a standard for measuring virus antibody-binding to PR8 [20]. The antibody titers of the specimens were determined from the standard regression curve constructed by serial 2-fold dilutions of the standard serum or monoclonal antibody for each assay. Naive mouse sera showed less than 0.25U/ml for X-179A and 0.25 ng/ml for PR8, respectively.

To evaluate antibody avidity, wells were incubated with 7 M urea for 15 min at room temperature to detach low avidity antibodies from the plate [14]. The avidity index was expressed as the % bound IgG after washing with urea.

2.5. TCID$_{50}$ assay

Confluent monolayers of MDCK cells in 96-well plates were inoculated with serial half log dilutions of virus samples and incubated for 6 days at 34 °C in 5% CO_2 in the presence of 10 μg/mL acetylated trypsin (Sigma). Virus titers were calculated for each sample using the Reed-Muench method [21], based on the CPE in individual wells observed under an inverted microscope.

2.6. Statistical analysis

Statistical significance was determined using unpaired two-tailed Student's *t*-tests and Spearman rank correlation tests.

3. Results

3.1. Whole virus vaccine elicits higher primary antibody response but not recall response compared with split virus vaccine

To characterize the antibody responses induced by whole and split virus vaccines, we compared antibody titers against the virus between sera of mice immunized with either whole or split virus vaccines derived from X-179A (H1N1)pdm09 virus. Our preliminary experiments indicated that both types of A/H1N1pdm09 vaccines had lower immunogenicity than corresponding well-characterized PR8 vaccines, and higher dosages were required for both whole virus and split A/H1N1pdm09 vaccines to elicit detectable serum IgG antibody responses comparable to those of PR8

vaccines (data not shown). Therefore, mice were immunized with 10 μg of either whole or split virus vaccine. Immune serum samples were collected 28 days post-immunization and subjected to ELISA, HI, and MN assays to measure antibody titers (Fig. 1). Consistent with previous reports, whole virus vaccines tended to induce higher titers of virus-binding IgG, HI, and MN antibodies, confirming their superior immunogenicity in the initial priming of humoral immune responses [22].

To compare the effects of boosters of the two types of vaccines, mice were initially primed with whole or split virus vaccines and mice in each group were boosted with either split or whole virus vaccines on 28 days post-priming. Antibody titers in the post-booster sera after 14 days were determined by ELISA, HI, and MN assays (Fig. 2). Although whole virus vaccine had a superior priming effect, it induced virus-binding IgG antibody titers comparable to or lower than those by split virus vaccine after the booster, regardless of which type of vaccine was used in the priming immunization (Fig. 2A). Similarly, booster immunization with a smaller amount (3 or 1 μg) of the whole virus vaccine induced lower HI and MN antibody titers than the split virus vaccine in primed mice (Fig. 2B and C). However, a higher amount (10μg) of whole vaccine induced higher HI and MN antibody titers, and the differences between whole and split viruses and the booster effects at all doses were not statistically significant. Collectively, boosters of both vaccines of X-179A were shown to possess a similar ability to recall secondary antibody responses. This effect was not virus strain-specific for X-179A as it was also seen in a different H1N1 virus, PR8 (Fig. 3). These findings were consistent with previous reports in which booster immunization with either whole or split virus vaccines recall similar HI and MN antibody titers in humans and mice, although whole virus vaccines have a more potent priming effect on immunogenicity than the split virus vaccine [23].
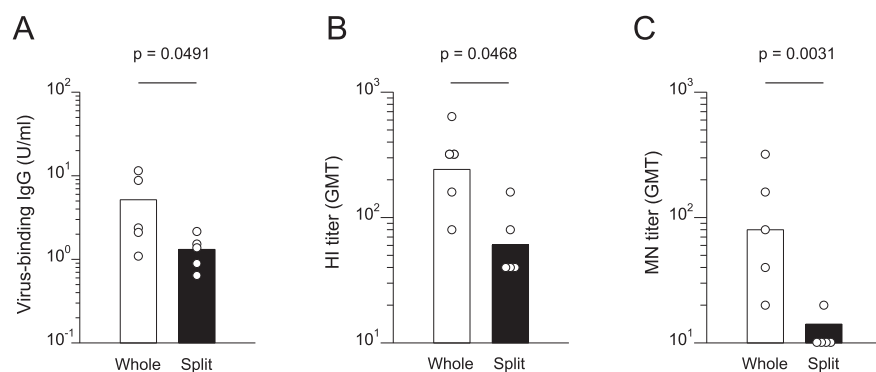

Fig. 1. Whole virus vaccine elicits primary antibody responses at higher magnitudes than split virus vaccine. Sera were obtained from BALB/c mice on day 28 post-prime with 10 μg of whole or split virus vaccine from X-179A (H1N1)pdm09 virus. Serum antibody titers were determined by ELISA (A), HI (B), and MN assays (C). Each circle represents the titers from an individual mouse (n = 5 or 6). Student t-tests were performed between two types of vaccines and revealed statistical significance for virus-binding IgG (P = 0.0491), HI (P = 0.0468), and MN titers (P = 0.0031), respectively.
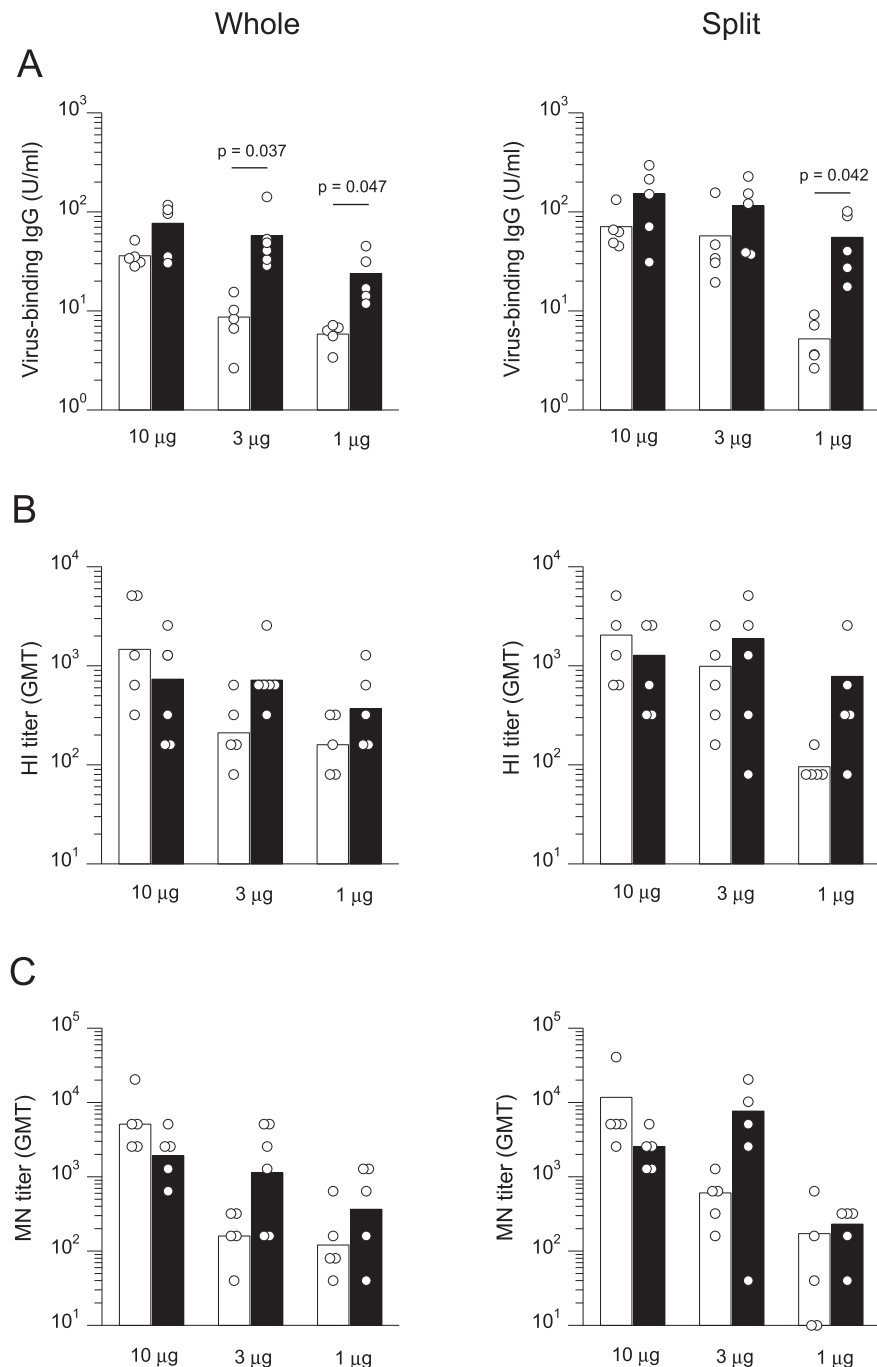
Fig. 2. Whole virus vaccines elicit recall of secondary antibody responses comparable to or lower than split virus vaccine. Mice were primed with the indicated doses of whole (left) or split (right) virus vaccine and then boosted with the same doses of whole (open) or split virus (closed) vaccine on day 28 after priming. The immune sera were obtained on day 14 post-booster. Serum antibody titers were determined by ELISA (A), HI (B), and MN assay (C). Each circle represents the titers from an individual mouse (n = 5 or 6). Student t-tests were performed between two types of booster vaccines and revealed statistical significance only for virus-binding IgG titers in the groups of mice primed with 1 (P = 0.047) or 3 μg (P = 0.037) of whole virus vaccine, and 1 μg (P = 0.042) of split virus vaccine.
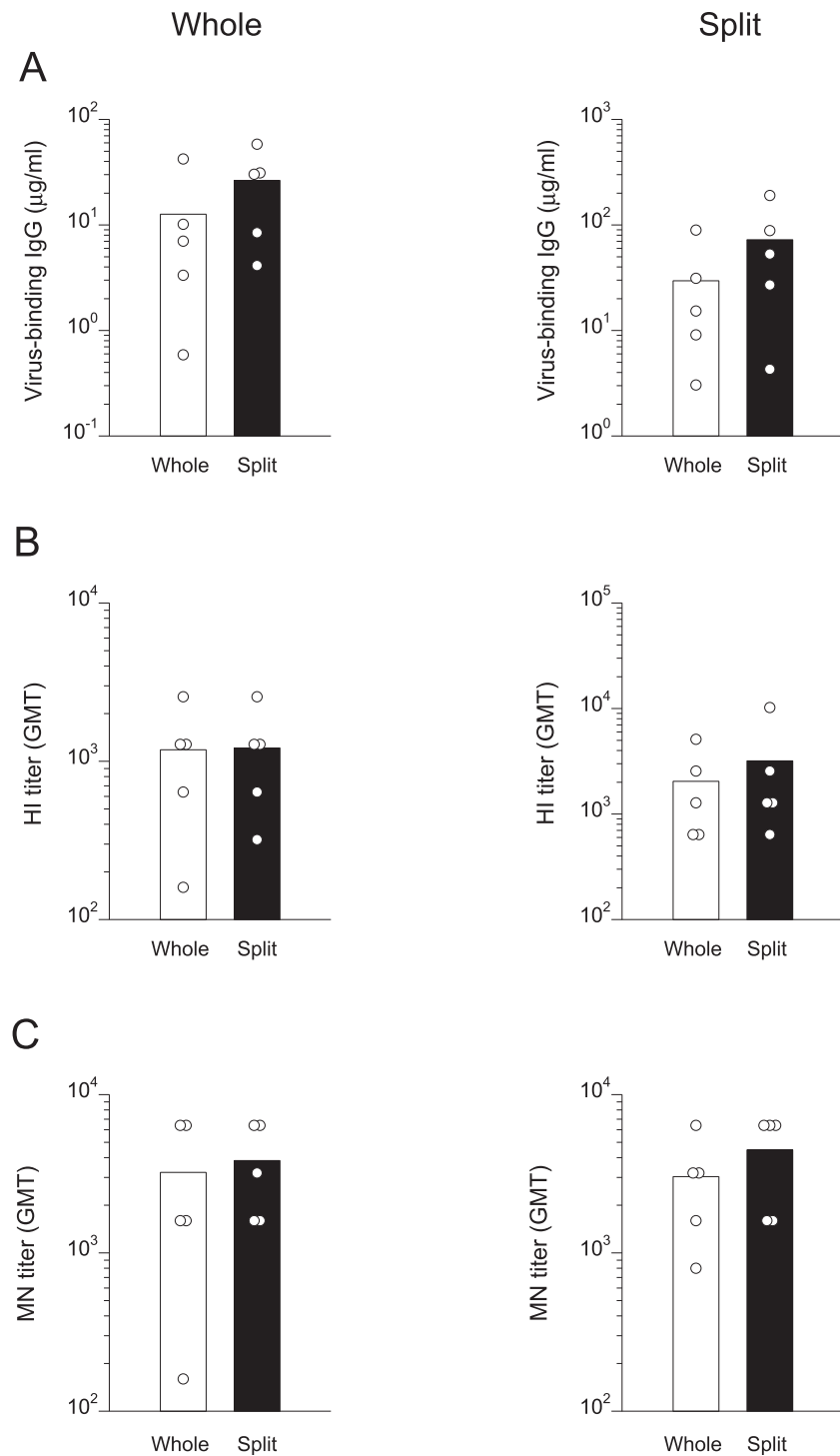
Fig. 3. Whole virus vaccines derived from PR8 virus recall secondary antibody responses similarly to or less than split virus vaccines as seen in X-179A (H1N1)pdm09 virus. Mice were primed and boosted by 1 μg of whole or split virus vaccine of PR8 virus and serum antibody titers were determined by ELISA (A), HI (B), and MN assay (C). Each circle represents the titers from an individual mouse (n = 5 or 6). Student *t*-tests were performed between the two types of vaccines and revealed no statistically significant differences among any of the antibody titers.

3.2. Booster immunization with whole virus vaccine confers better protection against challenge infection via serum antibodies than split virus vaccine

We next tried to assess the immune protection elicited in mice against a challenge infection among different booster vaccinations with whole or split virus vaccines. We initially expected similar levels of protection among the groups because there was no significant difference among serum HI and MN antibody titers (Fig. 2), and the strain-specific virus-neutralizing antibodies directed against HA have been shown to be the primary immune mediators of protection against infection [24]. Mice were immunized and boosted with 3μg of either whole or split virus vaccines, and then challenged with A/Narita/01/2009 (HlN1)pdm09 virus, which is antigenically indistinguishable from vaccine virus X-179A. Protection was evaluated as the reduction of virus lung titers 3 days post-challenge. As expected, mice primed and boosted with whole or split virus vaccines regardless of the combination showed significant reductions in virus titers in the lungs compared with naive mice (Fig. 4A). Contrary to our expectations, however, much greater protection was observed in mice primed and boosted with whole virus vaccine than in those treated with split virus vaccine. In addition, booster immunization with whole virus vaccine conferred better protection than the split virus vaccine against the challenge infection in mice primed with either whole or split virus vaccine although statistically not significant. It was also noted that mice vaccinated with whole virus vaccine exhibited very little loss of body weight, which is not a phenomenon usually observed in lethal infections of this virus in naive mice, at 3 days post-challenge. In contrast, a greater loss of body weight was observed in mice treated with the split virus vaccine than in the whole virus vaccine group, but it was less than in naive mice (data not shown). To explore whether any antibody titer can be correlated with the protection, we compared virus-binding IgG, HI and MN antibody titers among the groups (Fig. 4B, C, D). Booster immunization with whole virus vaccine induced lower virus-binding IgG, HI and MN antibody titers than those with split virus vaccine. Any immunological correlates of protection with antibody titers were not found. Taken together, booster immunization with whole virus vaccine conferred better protection than the split virus vaccine against the challenge infection despite of lower virus-binding IgG, HI and MN antibody titers.

To identify the immune components of this protection, we conducted passive transfer experiments of immune sera. Serum samples from mice after booster immunization were recovered and passively transferred into naive recipient mice. Mice were then challenged one day after the transfer in the same manner as the protection experiment. Significant protection was observed in groups of mice after sera transfer from mice immunized with both whole and split virus vaccines compared with naive mice, as indicated by the low loss of body weight (Fig. 5B). Mice that received
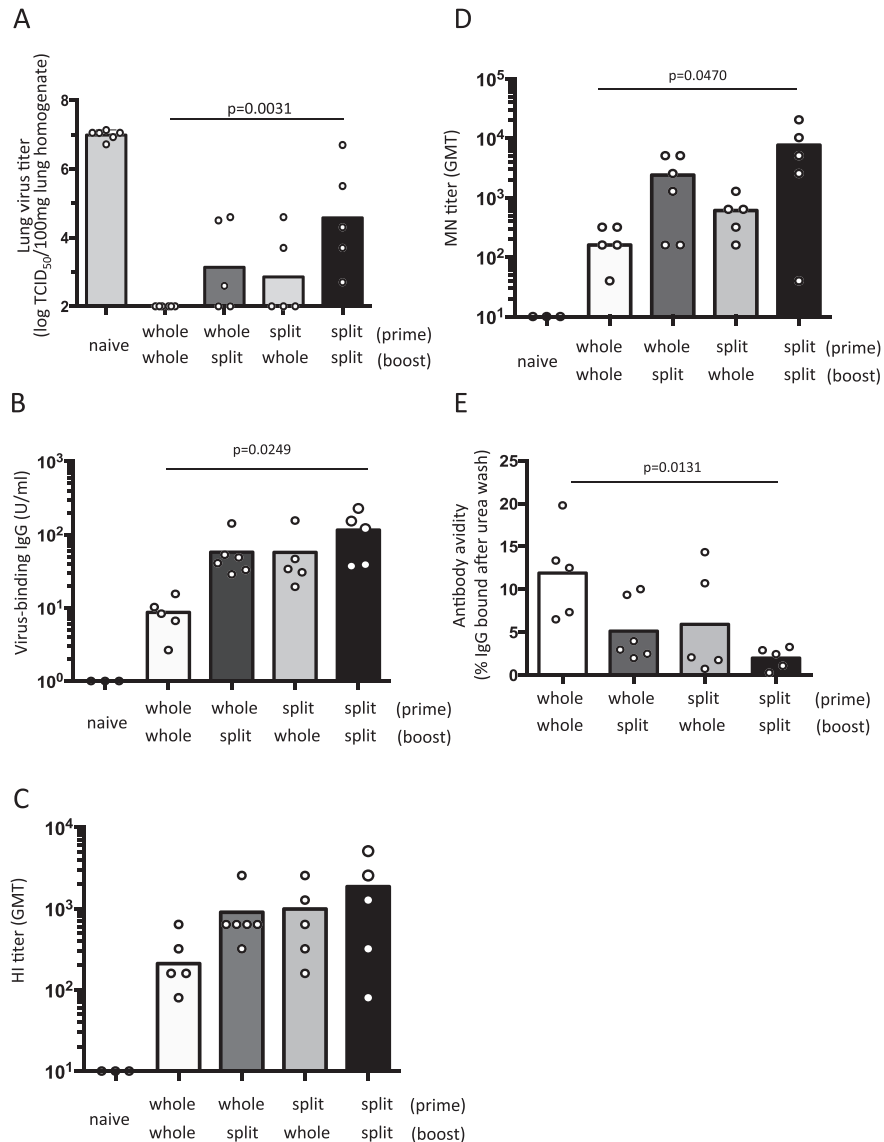
Fig. 4. Whole virus vaccine recalls secondary antibody responses with increased protective ability. (A) Five mice per group were primed and boosted with either 3 μg of whole or split virus vaccines. Unvaccinated 6 naive mice served as a control. Fourteen days after boosters, mice were challenged with A/Narita/1/2009 virus, and lungs were harvested and homogenized 3 days post-challenge. Lung virus titers were measured as protection indexes. Statistically significant differences between each group and naive group are not shown (whole-whole, whole-split, split-whole; $P < 0.0001$, split-split; $P = 0.0059$). (B to D) Sera were obtained from 5 to 6 mice per group immunized as described above on day 14 post-booster and 3 mice for unvaccinated control (naive). Serum antibody titers were determined by ELISA (B), HI (C), and MN assay (D). (E) Sera were measured for avidity to the virus. The amount of IgG bound after 7M urea wash were divided by those not washed and plotted as avidity indexes. Each circle represents the lung virus titers or antibody titer from an individual mouse. Statistically significant differences ($P < 0.05$) between groups are shown.
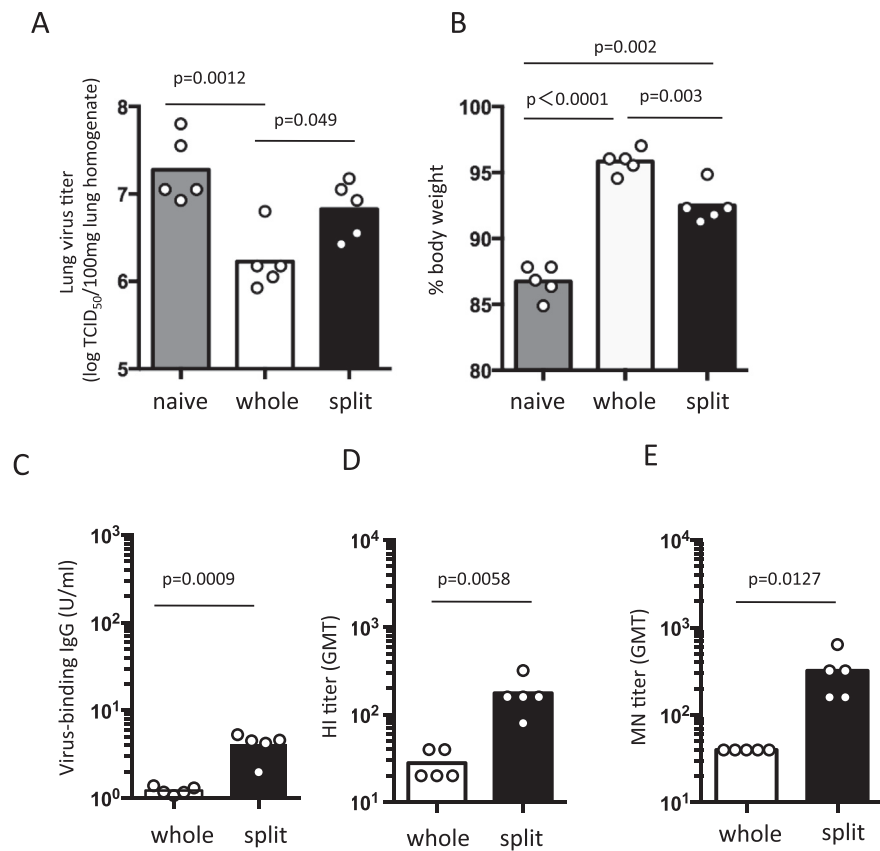
Fig. 5. Serum antibody mediates protection conferred by booster immunization with whole virus vaccine. Mice were injected intraperitoneally with the sera collected from naive (gray) or immune mice, which were primed and boosted with either 3 μg of whole (open) or split (closed) virus vaccine. One day later, mice were challenged with A/Narita/1/2009 virus. For assessing the level of protection, lung virus titers (A) and body weights (B) were measured 3 days post-challenge. Furthermore, serum antibody titers were determined by ELISA (C), HI (D), and MN assays (E) 3 days post-challenge. Each circle represents the lung virus titer, body weight, or antibody titer from an individual mouse (n = 5). Statistically significant differences (P < 0.05) between the groups are shown.

sera from whole virus vaccine-immunized mice showed significant protection against virus replication in the lungs compared with naive mice; however, the mice that received sera from the split virus vaccine-immunized group did not show any such protection (Fig. 5A). Compared with the outcome from split virus vaccines, sera from mice immunized with whole virus vaccines conferred much better protection against body weight loss (P = 0.003) and virus replication in the lungs (P = 0.049). Thus, this experiment strongly suggested that the superior protection afforded by the whole virus vaccine was mainly mediated by serum antibodies. Nevertheless, the magnitude of protection in the passively transferred recipient mice was lower than that in actively immunized mice (Figs. 4A and 5A). To explain the differences among the magnitudes of protection, we measured serum antibody titers at 3 days post-challenge in mice after passive transfer of each immune serum (Fig. 5C, D, E). Since antibody titers did not increase drastically during the 3 days

post-challenge, these antibody titers can be considered pre-challenge titers (data not shown). Antibody titers in the immune serum-transferred mice were much lower than in mice actively immunized with 3 μg of either whole or split virus vaccines, because transferred serum was diluted in the recipient mice (Figs. 4B, C, D and 5C, D, E). On the contrary, a modest level of protection (Fig. 6A) was observed in mice immunized with the lower dose 1 μg of whole virus vaccine despite of virus-binding IgG, HI and MN antibody titers comparable to the higher dose 3 μg-immunized mice (Fig. 6B, C, D). The amount of reduction in pulmonary viral load might be profoundly affected near the borderline of protective antibody titer. Taken together, the differences among the magnitudes of reduction in viral load were mainly attributable to the dilution effect of transferred serum but not a lack of other immune mediators for protection. Thus, these results indicated that serum
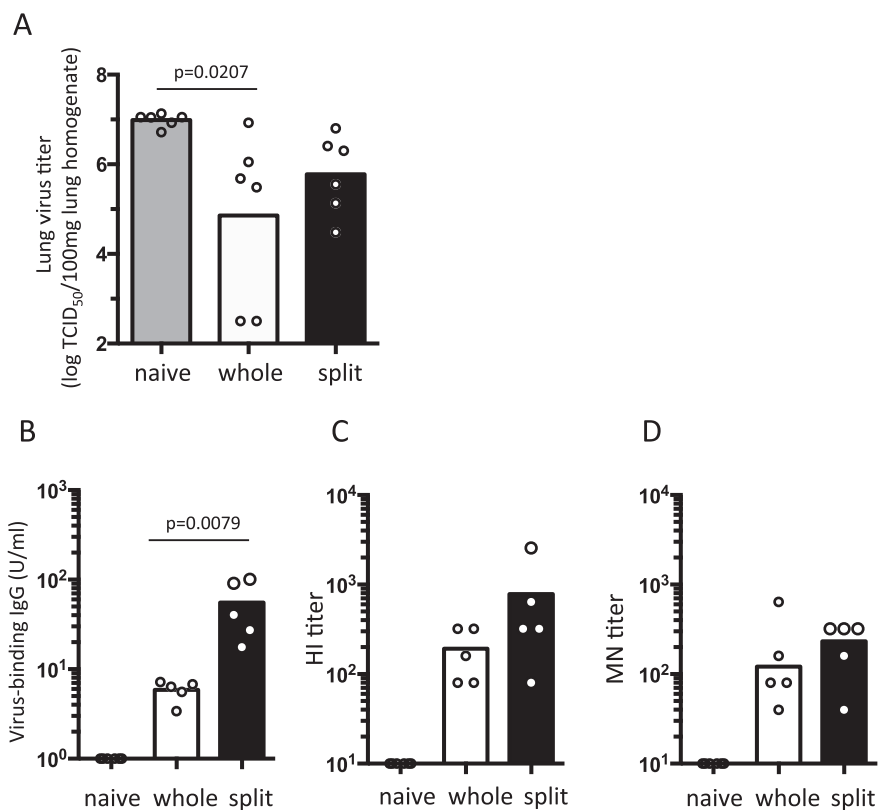

Fig. 6. Lower dose of whole virus vaccine still elicits antibody responses comparable to the higher dose, but confers a modest level of protection. (A) Mice were primed and boosted with either 1 μg of whole (open) or split (closed) virus vaccines. Unvaccinated naive mice served as a control (gray). Fourteen days after boosters, mice were challenged with A/Narita/1/2009 virus, and lungs were harvested and homogenized 3 days post-challenge. Lung virus titers were measured as protection indexes. Data from two independent experiments (n = 3) were pooled and plotted. (B to D) Sera were obtained from 5 mice per group immunized as described above on day 14 post-booster and 6 mice for unvaccinated control (naive). Serum antibody titers were determined by ELISA (B), HI (C), and MN assay (D). Each circle represents the lung virus titer or antibody titer from an individual mouse. Statistically significant differences (P < 0.05) between the groups are shown.

antibodies were primarily responsible for the protection afforded by both whole and split virus vaccines.

3.3. Whole virus vaccine recalls high avidity serum antibody responses against challenge infection

Next, we searched for possible immune correlates of protection with antibody, because the conventional virus-binding IgG, HI and MN antibody titers did not correlate with protection (Fig. 4A, B, C, D). We therefore hypothesized that the better protection afforded by booster immunizations with whole virus vaccine correlates with a higher binding affinity of the antibody to the viral antigens. To test this theory, we compared the avidity of virus-binding IgG antibodies induced by booster immunization with the whole and split virus vaccines. Treatment with a chaotropic agent (7M urea) dissociates low-avidity antibodies from ELISA plates, enabling the evaluation of antibody avidity to viral antigens. Intriguingly, virus-binding IgG antibodies boosted by whole virus vaccines were more resistant to urea treatment than those boosted by split virus vaccines (Figs. 4E and 7). This indicated that the IgG
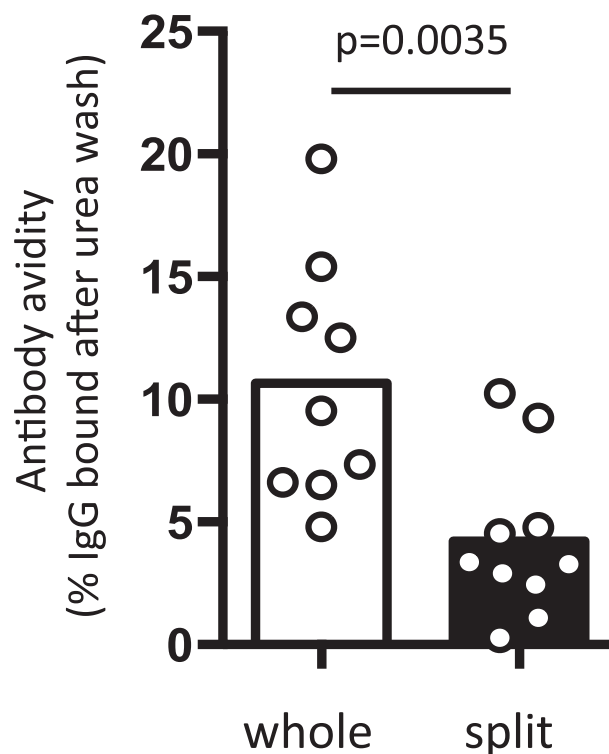

Fig. 7. Antibody avidity of serum samples from mice after booster immunization with whole and split virus vaccines, which were used for passive transfer experiments. Sera collected from mice primed and boosted with 3 μg of whole (n = 9) or split virus vaccine (n = 10) were measured for avidity to the virus. The amounts of IgG bound after 7 M urea wash were divided by those not washed and plotted as avidity indexes. Sera were pooled from either whole or split virus vaccine-immunized mice and used for passive transfer experiments of immune serum.

antibodies recalled by booster immunization with the whole virus vaccine possessed a stronger binding ability to the virus with higher affinity compared with split vaccine-boosted IgG antibodies.

To demonstrate that the increased protection afforded by the whole virus vaccine correlated with higher antibody affinity, we measured antibody avidity in the sera and lung virus titers as indexes of protection in mice that had received passive transfer of immune sera at 3 days post-challenge. Notably, a significant correlation between antibody avidity and protection was observed in plots of the combined data from sera from mice immunized with either vaccine (Fig. 8).

4. Discussion

Similar to many licensed vaccines available, influenza vaccines are administered to prime and boost virus-neutralizing antibodies that have crucial protective roles in the control of influenza infections. Inactivated whole and split virus vaccines are used across the world, although split or subunit vaccines are mainly used, and their immunogenic properties and priming/boosting effects have been extensively characterized. While previous studies have established that whole virus vaccines induce superior primary antibody responses, they are reported to be equal to or less effective
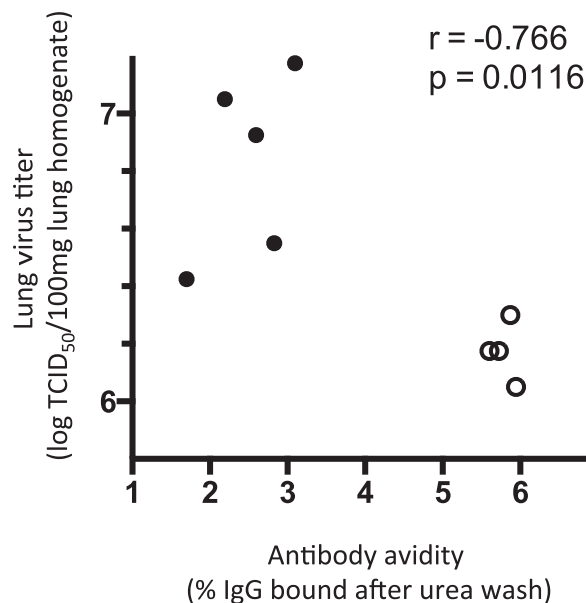

Fig. 8. Whole virus vaccine recalls a secondary serum antibody response with elevated binding avidity that correlates with better protection. Mice given sera from whole (open) and split (closed) virus vaccine-immunized mice were challenged with A/Narita/1/2009 virus as shown in Fig. 5(A). Three days post-challenge, lung virus titers and the binding avidity of serum antibodies to the virus in each mouse were measured. Lung virus titers as protection indexes were plotted against binding avidity indexes to the virus. Significant correlation was observed between protection and binding avidity indexes against the virus (Spearman rank correlation test: P = 0.0116).

for boosters in primed individuals based on serum HI and MN antibody titers, which are widely used as surrogate markers of immune correlates of influenza vaccine efficacy [25]. Here, we confirmed previous observations on antibody responses in a mouse model immunized with whole and split virus vaccines derived from A/H1N1pdm09 vaccine virus. However, we unexpectedly found that the protection conferred by booster immunization with whole virus vaccine was more effective than that by split virus vaccine, despite similar or lower levels of serum antibody titers. In addition, the levels of protection afforded by the different types of vaccine correlated better with the affinity maturation level of the serum IgG antibody rather than the antibody HI and MN titers. Both elevated IgG affinity maturation and superior protection were induced by whole virus vaccine, while these effects were weak in the split virus vaccine. Recently, Memoli *et al.* reported the importance of NA antibody titer as a correlate of protection in addition to HI antibody titer in a healthy human challenge model of influenza A/H1N1pdm09 virus, and the importance of examining other immunological correlates of protection rather than HI titers solely [26]. Thus, our results shed some light on the previously unrecognized prime/booster effect of whole virus vaccines on the recall of superior protective immunity correlating with the affinity maturation of antibodies.

Although we do not have direct evidence of why affinity-matured antibodies protect against influenza virus infections more effectively, observations that support this phenomenon have been reported. Affinity maturation and isotype-switching of antibodies require the expression of activation-induced cytidine deaminase (AID) in B cells. The production of high-affinity isotype-switched antibodies is completely lacking in AID-deficient mice [27] and, notably, AID-deficient mice showed severe morbidity, such as loss of body weight and virus replication in the lung, although they can survive challenge infections with influenza viruses, which highlights the importance of affinity-matured isotype-switched antibodies for protection [28]. This notion is further supported by a report that severe human cases of A/H1N1pdm09 virus infection are associated with high titers of non-protective serum antibodies with low avidity and C4d deposition in the lung, which is a marker of complement activation mediated by immune complexes, and results in limited prevention of virus replication and severe morbidity [15]. Collectively, these findings suggest that better protective immunity without the induction of immunopathological processes could be elicited through the recall of affinity-matured antibodies.

As observed in this study, the uncoupling of virus-neutralizing activities *in vitro* and protective functions *in vivo* are well documented for several HA-binding monoclonal antibodies and some of the underlying mechanisms relate to complement-mediated modulation of virus neutralization activity [29] and antibody-dependent cell-mediated cytotoxicity [30]. Moreover, antibody-mediated protection could be enhanced by the presence of NA-binding antibodies not detected by HI and MN assays [31]. Thus, several mechanisms potentially explain the discrepancy between

conventional serological parameters and *in vivo* protection, but the details need to be clarified in future experiments.

How does the whole virus vaccine induce high affinity memory antibody responses protective to lethal virus infections better than the split virus vaccine? The whole virus vaccine, but not the split virus vaccine, conserves several antigenic properties such as particulate structures with high-density B-cell epitopes and the possession of viral RNA as a TLR agonist. Importantly, memory B cells are able to sense these viral antigenic signatures through B-cell antigen receptors and Toll-like receptors (TLRs) in a B-cell-intrinsic manner. Indeed, Onodera *et al.* recently demonstrated that whole virus H5N1 vaccine can recall high affinity antibody responses by targeting memory B cells through TLR signaling [32]. Therefore, TLR agonists inside whole virus vaccines contribute, at least in part, to the affinity maturation of IgG responses from booster vaccinations.

Split virus vaccines have been preferentially used for booster vaccinations due to their lower reactogenicity and comparable induction of antibodies with conventional serological indicators [6, 7]. The data presented here provide important information for improving the protective efficacies of split virus vaccines, while keeping their reactogenicity low. Given the functional importance of TLR-mediated B cell stimulation, this process may be a potential new target for the induction of protective serological memory. The use of conventional TLR agonists stimulates many types of TLR-expressing cells and leads to strong inflammatory responses associated with reactogenicity [33]. Therefore, the design of novel TLR agonists that can selectively engage B-cell intrinsic TLRs could be valuable for enhancing the immunogenicity of booster vaccinations while keeping reactogenicity low.

The causative virus of the 2009 pandemic influenza, A/H1N1pdm09 virus has been circulating among humans without prominent antigenic change since 2009 to date despite high antibody prevalence to the virus [34]. In contrast, antigenic variants of human influenza A/H3N2 and B viruses have been emerging frequently due to antibody selection. This study provides a possible explanation for the stable antigenic feature of A/H1N1pdm09 viruses. Antibodies elicited by infection or vaccination with A/H1N1pdm09 virus in humans might be an inefficient pressure for the selection of antigenic variants, and is likely attributed to the low avidity of the antibodies despite their high prevalence in humans.

Declarations

Author contribution statement

Shigeyuki Itamura, Yoshimasa Takahashi: Conceived and designed the experiments; Wrote the paper.

Kayoko Sato: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Yu Adachi: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Hideki Asanuma: Performed the experiments; Contributed reagents, materials, analysis tools or data.

Masato Tashiro, Manabu Ato: Analyzed and interpreted the data.

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Competing interest statement

The authors declare no conflict of interest. The funders had no role in the design of the study, data collection/interpretation, or the decision to submit the work for publication.

Additional information

No additional information is available for this paper.

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