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Cross-protection Mechanisms of Influenza Virus Vaccines

Yu-Jin Kim

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CROSS-PROTECTION MECHANISMS OF INFLUENZA VIRUS VACCINES

by

YU-JIN KIM

Under the Direction of Sang-Moo Kang, PhD

ABSTRACT

Since current influenza vaccine strategy is effective in conferring protection against vaccine strain-matched influenza viruses but not against antigenically different viruses, the development of broad cross-protective vaccines is of a high priority to improve vaccination efficacy and to prevent future pandemic outbreaks. In this study, I investigated the cross-protective efficacy and the immune mechanism of three different target antigens including hemagglutinin (HA) based inactivated virus vaccines, neuraminidase (NA) protein vaccines, and tandem repeat extracellular domains of the ion channel protein M2 (M2e5x) on virus-like particle (M2e5x VLP) vaccines. Anti-NA antibodies could confer better cross-protection against multiple heterologous influenza viruses correlating with NA inhibition activity compared to anti-HA antibodies. Whereas anti-HA antibodies were superior to NA in conferring homologous protection. Anti-NA and M2e antibodies showed comparable survival protection. To better understand cross-protective vaccine efficacy, M2e and HA vaccines were tested in different genetic backgrounds. BALB/c mice

showed higher IgG responses and cross-protection than C57BL/6 mice after M2e vaccine immunization. M2e vaccine immune mice after primary challenges developed strong immunity to a secondary heterosubtypic virus as a future pandemic.

The classical complement pathway is activated to eliminate antigen-antibody immune complexes, subsequently followed by complement-dependent cytotoxicity in addition to Fc receptor-mediated antibody-dependent cell-mediated cytotoxicity. However, the role of complement system remains largely unknown in influenza virus M2e-mediated cross protective immunity. This study demonstrated that complement protein C3 is essential in inducing immune responses to influenza M2e5x VLP vaccination and influenza virus infection, which include M2e-specific isotype-switched antibody production and M2e-specific effector CD4 and CD8 T cell responses. C3 deficient (KO) mice showed lower levels of M2e-specific IgG isotype antibodies after M2e5x VLP vaccination, no control of lung viral replication, and severe weight loss upon challenge infection compared to those in wild type (WT) mice. Whereas, C3 KO mice were protected against homologous virus after immunization with hemagglutinin-based virus vaccine despite lower levels of neutralizing antibodies than those in WT mice. In addition, C3 KO mice showed impaired recruitment of macrophages and different subsets of dendritic cells. The findings in this study suggest that C3 is a key regulator in developing protective immunity by non-neutralizing antibody-based vaccination.

INDEX WORDS: Influenza vaccine, Hemagglutinin, Neuraminidase, M2e, Complement system

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by

Yu-Jin Kim

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DEDICATION

I dedicate this to my family and all my dear friends.

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1 INTRODUCTION

1.1 Influenza virus

Influenza virus contains eight segmented negative-sense RNA genomes and belongs to the family *Orthomyxoviridae*. Influenza A viruses are divided into different subtypes based on hemagglutinin (HA) and neuraminidase (NA) proteins on the surface of the virus.[1] At present, antigenically different influenza viruses with one combination out of 18 HA (H1-H18) and 11 NA (N1-N11) subtype molecules are known to exist and continue to mutate in diverse hosts including humans, birds, and pigs.[1,2] In addition to the high mutation rates of HA and NA, segmented structures of their genome facilitate genetic reassortment when the host is infected with more than one strain of influenza A viruses.[3] This unique feature of influenza viruses has been considered as one of the main reasons for the emergence of novel virus strains which are more likely to cause pandemics in humans.[4]

Current influenza vaccination is based on immunity to highly changeable HA and confers protection limitedly against homologous virus infection so that annual updates of influenza vaccines are required to match the antigenicity of the virus strains which are predicted to circulate.[5] Moreover, this strategy is not effective in preventing the pandemic outbreaks, raising the need for developing broadly cross-protective influenza vaccines.[6]

1.2 Target antigens for cross-protective influenza vaccines

Among the eleven influenza viral proteins, three proteins HA, NA, and M2 are expressed on the surface of virions. Therefore, these 3 surface proteins were evaluated and compared as the target antigens for cross-protective influenza vaccines in this study. The features and immune responses induced by each antigen are discussed below.

1.2.1 HA

HA is the most dominant homotrimeric viral surface hemagglutinin glycoprotein which mediates the attachment of the influenza virions to host cells. It binds to sialic acids on glycan structures of hosts' cellular receptors. After binding to the receptors, HA leads to the fusion between viral and cellular endosomal membranes, causing the release of the viral genome into the cytoplasm.[7] Immunization with HA vaccines (whole inactivated virus, inactivated split virus vaccine, live attenuated influenza virus vaccine) primarily induces immunity to the globular head domain of HA which is surrounded by highly variable antigenic regions so that the immune responses are strain-specific to the influenza viruses.[5] It has been known that broadly neutralizing monoclonal antibodies which are specific for the highly conserved HA stem region can be induced in mice and humans[8-10] in a lower frequency than the globular head domain strain-specific antibodies.[11] The HA stem region is less immunogenic than the globular head domain. One possible reason is that the globular head physically masks the stem region on the influenza virion. Another reason can be the close proximity of the HA stem region to the viral membrane so that hosts' immune cells hardly recognize it.[12]

1.2.2 NA

NA is a homotetrameric viral surface glycoprotein which has the sialidase activity.[13] It is required to transport the influenza virions through mucosal surfaces and to release of budding virus particles from the host cell surface.[1] Epidemiologic studies indicated that anti-NA immunity and NA inhibitors such as zanamivir, oseltamivir, or peramivir prevent severe disease or death by influenza viral infection.[14] NA targeting antibodies do not have virus neutralizing activity, providing infection-permissive protection.[15] Studies have demonstrated that NA immunity induces

a broad spectrum of cross protection within the same subtype.[16,17] Nonetheless, contribution of NA antibodies to cross protection is not well understood yet in comparison with other viral surface antigens.

1.2.3   M2e

M2 is an integral membrane protein, which has the pH-dependent proton channel activity, and required for HA maturation and viral genome release into the cytoplasm.[12] After influenza virus enters into the host cells, M2 decreases the pH of the virus interior by opening the proton channel across the viral membrane. Membrane fusion between the virus and the host cell is facilitated in the low pH condition.[5] The extracellular domain of M2 (M2e) has been considered as one of the promising antigens for cross-protective influenza vaccines. One main reason is that passive administration of anti-M2e antibodies can reduce viral replication and pathological symptoms in mice without viral neutralizing activity.[18] Another reason is that the sequence of M2e is highly conserved ($\sim$ 99%) among human influenza A viruses.[19,20] However, M2 itself is a poor immunogen. To enhance immune responses, M2e vaccines have been reported using a variety of carrier vehicles such as hepatitis B virus core particles[21,22], human papillomavirus L proteins[23], phage Qβ-derived protein cores[24], keyhole limpet hemocyanin[25], bacterial outer membrane complexes[26], liposomes[27], cholera toxin subunit[28], and flagellin[29]. Also, different adjuvants were used in the M2e vaccines, which include Freund's adjuvant[30], monophosphoryl lipid A[27,28], cholera toxin subunits[31,32], and heat-labile endotoxin[22,33]. In addition, virus-like particle (VLP) vaccines presenting heterologous tandem repeat M2e (M2e5x VLP) were effective in inducing cross protection against different subtypes of influenza viruses in the absence of adjuvants.[34]

1.3   Possible mechanisms of cross-protective influenza vaccines

Since cross-protective antibodies do not have virus neutralizing activity, those antibody-mediated immune protection has been considered to be connected with antibody-dependent cell cytotoxicity (ADCC) or complement-mediated lysis.[35-38] Innate immune cells such as macrophages, dendritic cells, neutrophils, and NK cells lead to lyse antibody-bound infected cells via interaction between Fc receptors (FcR) on their surface and antigens on the infected cells.[13,39,40] Based on these findings, FcR-mediated ADCC can be a possible mechanism of the cross-protective immunity. Complement-mediated cell lysis is also a key mechanism for virus elimination.[41,42] In the case of M2e-mediated immunity, it is still controversial whether the complement system is required for protection.[35,38] In addition, several studies have shown that M2e-specific T cells play a role in enhancing cross protection against influenza infection.[28,43,44]

Cross protective immune mechanisms induced by NA are rarely known. However, NA contains two highly conserved sequences and even one of them shows 100% conservation across all influenza A and B viruses.[45] This fact suggests that NA can be a promising target for universal influenza vaccines. Indeed, there have been a few reports that NA immunity can confer cross protection to a certain extent.[16,17,46] However, further studies are still needed to understand detailed working mechanisms of NA-mediated immunity.

1.4   The role of the complement system in the regulation of adaptive immune responses.

Complement is well known as a primitive surveillance system which is a key for the clearance of infected pathogens during inflammation responses.[47,48] Moreover, the complement system regulates both humoral and T cell immunity.[49] The complement system is involved in the B cell responses via complement receptors CD21 and CD35[50], and by localizing antigens to follicular

dendritic cells (FDCs), which are specialized cells secreting chemoattractant chemokines for B lymphocytes[51]. Although the mechanism is still unclear, complement C3 protein was reported to be important for inducing CD4 and CD8 T cell responses to influenza virus infection.[52]

2   EXPERIMENTAL METHODS

2.1   Animals and reagents

A/California/04/2009 (A/Cal) H1N1, A/Puerto Rico/8/1934 (A/PR8) H1N1, A/Philippines/2/1982 (A/Phil) H3N2, A/Wisconsin/67/2005 (A/Wis) H3N2, and reassortant A/Vietnam H5N1 (rgH5N1) containing H5 HA with removed polybasic residues and NA from H5N1 A/Vietnam/1203/2004 and six internal genes from A/PR8 H1N1 were propagated in embryonated hen's eggs as previously described.[53,54] Inactivated virus vaccines (A/PR8, A/Phil) were prepared by treating the virus with formalin at a final concentration of 1:4000 (v/v) as previously described.[55] M2e5x VLP containing tandem repeat of heterologous M2e derived from human (2xM2e), swine (1x), and avian (2xM2e) influenza virus was prepared as detailed in previous study.[34] H5 VLP presenting H5 type of HA protein from A/Indonesia/05/2005 was previously described.[54] Briefly, Sf9 insect cells were co-infected with recombinant baculoviruses expressing influenza M1 matrix core protein and M2e5x or H5 HA. M2e5x VLP and H5 VLP vaccines were purified from cell culture supernatants containing released VLP by sucrose gradient ultracentrifugation and characterized as reported.[34,54] N1 NA protein derived from A/Cal (NR-19234) and N2 NA protein derived from A/Wis (NR-19237) were provided from BEI resources. Commercial human influenza split A/Cal vaccine was obtained from a vaccine manufacturing company (Green Flu-S; Green Cross, South Korea).

2.2 Immunization and challenge

Adult wild type and mutant mice (6-10 weeks old) used in this study include BALB/c, C57BL/6, CD4KO (B6.129S2-$Cd4^{tm1Mak}$/J), CD8KO mice (B6.129S2-$Cd8a^{tm1Mak}$/J), and C3KO (B6.129S4-$C3^{tm1Crr}$/J) and were obtained from the Jackson Laboratory (Sacramento, CA). FcRγ-deficient mice (FcRγ$^{-/-}$ encoded by *Fcer1g* on the BALB/c genetic background) were purchased from Taconic Farms (Hudson, NY). Groups of each strain of mice (n=5 or 10, males and females) were intramuscularly (i.m.) immunized with 10 μg (total proteins) of M2e5x VLP, H5 VLP (H5 HA from A/Vietnam/1203/2004), H3N2i (whole inactivated A/Philippines/2/1982 virus), 5 μg of N1 or N2 NA protein with adjuvant MF59 (1:1 vol), or 1 μg of split vaccines derived from A/Cal H1N1 or A/PR8 H1N1 by prime – boost regimen at a 3-week interval. At 4 weeks after boost immunization, immunized mice were then challenged intranasally with a sublethal dose of A/Philippines/2/1982 H3N2 (0.8 X LD_{50}) or rgH5N1 (0.8 X LD_{50}), or a lethal dose of A/Cal H1N1 (17 X LD_{50}) or A/Phil H3N2 (10 X LD_{50}). For secondary challenge, mice survived from primary infection were challenged with a lethal dose of A/California/04/2009 H1N1 (10 X LD_{50}) at 7 weeks after the primary infection. Survival rate and body weight loss were daily monitored for 14 days upon infection. All animal experimental procedures in this study were approved by the Georgia State University Institutional Animal Care and Use Committee review boards.

2.3 Determination of antibody responses

Influenza virus-specific or M2e-specific antibody levels were determined by enzyme-linked immunosorbent assay (ELISA). Immune sera were serially diluted and then applied to the 96 well plate (Corning Incorporated, Tewksbury, MA) that were coated with M2e peptide, inactivated A/California H1N1, A/PR8 H1N1, A/Philippines H3N2, A/Wisconsin H3N2, A/Indonesia

rgH5N1, or A/Vietnam rgH5N1 virus as previously described.[56,57] IgG and IgG isotype levels were determined by HRP conjugated anti-mouse IgG, IgG1, IgG2a, IgG2b, or IgG2c (SouthernBiotech, Birmingham, AL) and tetramethylbenzidine (eBioscience, San Diego, CA) as a substrate.[58]

2.4 Lung virus titers

Lung samples were collected from the groups of mice at 7 days after challenge. Viral titers were determined as described previously.[34] Briefly, lung extracts were serially diluted in 10-fold and injected into 10 days old embryonated chicken eggs. The 50% of egg infectious dose (EID_{50}) was calculated by the Reed-Muench method.

2.5 Hemagglutination inhibition assay

Hemagglutination inhibition (HI) assay was performed as previously described.[59] Immune sera were mixed with receptor destroying enzyme (Sigma Aldrich, St. Louis, MO) and then incubated at 37 °C. At 16 hours after incubation, samples were heat inactivated at 56 °C for 30 min. Serially 2-fold diluted sera were incubated with 8 HA units of A/California H1N1, A/PR8 H1N1, A/Philippines H3N2, or rgH5N1 for 30 min, followed by adding 0.5% chicken red blood cells (Lampire Biological Laboratories, Pipersville, PA) to determine HI titers.

2.6 Neuraminidase inhibition assay

The optimal concentrations of viruses for the subsequent Neuraminidase inhibition (NI) assays were determined based on the NA activity of each virus. NI activity of immune sera was measured using fetuin-based assay procedure as described.[16] Briefly, 96-well plates were coated with fetuin (50 µg/ml) (Sigma Aldrich, St. Louis, MO) and incubated overnight at 4 °C. After washing, plates

were blocked with PBS containing 1% BSA for 1 hour. 2-fold serially diluted immune sera were incubated with an equal volume of virus for 1.5 hours and then added to the fetuin-coated plates and incubated for 2 hours at 37 °C. Peroxidase-labeled peanut agglutinin (2.5 µg/ml) (Sigma Aldrich, St. Louis, MO) was added to each well and incubated for 2 hours. The NA activity levels were determined by using tetramethylbenzidine (eBioscience, San Diego, CA) as a substrate. OD values were read at 490nm.

2.7   Cytokine ELISPOT

To detect interferon (IFN)-γ and interleukin (IL)-4 spot forming cells (SFCs), splenocytes (5×10^5 cells/well) and lung cells (2×10^5 cells/well) were cultured on 96 well plates coated with anti-mouse IFN-γ or IL-4 monoclonal antibodies (BD Biosciences, San Diego, CA) in the presence of M2e peptide (4 µg/ml). The cytokine spots were developed with biotinylated mouse IFN-γ, IL-4 antibodies and alkaline phosphatase labeled streptavidin (BD Pharmingen, San Diego, CA). The spots were visualized with a 3,3'-diaminobenzidine substrate and counted by an ELISpot reader (BioSys, Miami, FL).

2.8   In vivo protection assay of immune sera

To test whether M2e5x VLP immune sera contribute to cross protection, in vivo protection assay was performed as described previously.[34] Briefly, heat inactivated sera at 56 °C for 30 min were diluted and mixed with a lethal dose (10 X LD$_{50}$) of A/California H1N1, rgH5N1, or A/Philippines H3N2. Naïve mice were intranasally infected with a mixture (50 µl) of virus and sera, and the survival rates and body weight changes were daily monitored for 14 days.

2.9   Intracellular cytokine staining and flow cytometry assay

For intracellular cytokine analysis, harvested cells were stimulated with M2e peptides and then stained with fluorescence-labeled anti-mouse CD4 and anti-mouse CD8 antibodies. Subsequently, the cells were made permeable by using the Cytofix/Citoperm kit (BD Biosciences, San Diego, CA) and intracellular cytokines were stained with anti-mouse IFNγ and anti-mouse Granzyme B antibodies. All antibodies were purchased from eBioscience. Stained cells were analyzed using LSR Fortessa (BD Biosciences, San Diego, CA) and FlowJo software (Tree Star).

2.10   Germinal center B cell staining and flow cytometry assay

To determine the germinal center B cell phenotype, cells were harvested from mediastinal lymph nodes (MLN) and then stained with fluorescence-labeled anti-mouse B220, GL7, IgD, and CD19 antibodies. All antibodies were purchased from eBioscience. Stained cells were analyzed using LSR Fortessa (BD Biosciences, San Diego, CA) and FlowJo software (Tree Star).

2.11   Intraperitoneal cell phenotypes

Peritoneal exudates were harvested from WT and C3 KO mice (n=5 each). Isolated peritoneal cells were treated with Fc receptor blocker (anti-CD16/32) and then stained with fluorescence labeled antibodies; CD11b (clone M1/70), CD11c (clone N418), F4/80 (clone BM8), major histocompatibility complex class II (MHC-II) (clone M5/114.15.2), Ly6c (clone HK1.4), and B220 (clone RA3-6B2). All antibodies were purchased from eBioscience. Stained cells were analyzed using LSR Fortessa (BD Biosciences, San Diego, CA) and FlowJo software (Tree Star).

2.12 Statistical analysis

All results are expressed as the mean ± standard error of the mean (SEM). Significant differences among treatments were evaluated by 2-way ANOVA. *P*-values of less than or equal to 0.05 were considered statistically significant.

3   CHAPTER 1. ROLES OF ANTIBODIES TO INFLUENZA A VIRUS HEMAGGLUTININ, NEURAMINIDASE, AND M2E IN CONFERRING CROSS PROTECTION

3.1   Summary

Although neuraminidase (NA) is the second major viral glycoprotein of influenza virus, its immune mechanism as a vaccine target has been less considered. Here we compared the properties of antibodies and the efficacy of cross protection by N1 and N2 NA proteins, inactivated split influenza vaccines (split), and tandem repeat extracellular domain M2 on virus-like particles (M2e5x VLP). Anti-NA immune sera could confer better cross-protection against multiple heterologous influenza viruses correlating with NA inhibition activity compared to split vaccine immune sera. Whereas split vaccine was superior to NA in conferring homologous protection. NA and M2e immune sera each showed comparable survival protection. Protective efficacy by NA immune sera was lower in Fc receptor common γ-chain deficient mice but comparable in C3 complement deficient mice compared to that in wild type mice, suggesting a role of Fc receptor in NA immunity.

3.2 Results

3.2.1 *NA protein vaccination induces IgG antibodies cross-reactive to heterologous virus.*

We compared the relative contributions and roles of serum antibodies specific for NA, inactivated split influenza vaccine (HA), and M2e in conferring cross protection. The groups of mice were intramuscularly immunized with N1 NA protein (5 µg, A/Cal H1N1), N2 NA protein (5 µg, A/Wis H3N2), M2e5x VLP (10 µg), inactivated split influenza vaccines (1 µg, A/PR8 H1N1 or A/Cal H1N1). Reactivity of IgG antibodies in immune sera to different strains of influenza virus was compared among the groups at 2 weeks after boost immunization (Fig. 3.1). N1 NA protein immune sera showed reactivity to the homologous A/Cal H1N1 and heterologous rgH5N1 and A/PR8 H1N1 viruses. A similar pattern was also observed with N2 NA protein immune sera at lower levels of IgG antibodies that bind to homologous A/Wis H3N2 virus and heterologous A/Phil H3N2 virus. However, both N1 and N2 immune sera did not show significant reactivity to different subtypes of influenza virus. M2e5x VLP immune sera exhibited high levels of IgG antibodies reactive to M2e peptide antigens (Fig. 3.1F). A/Cal split vaccine developed more cross-reactive IgG antibodies than A/PR8 split vaccine (Fig. 3.1B, C) although both showed high IgG antibodies specific for homologous virus. Overall, IgG antibodies induced by A/Cal split vaccines were cross-reactive to heterosubtypic influenza viruses but not to M2e. NA protein-induced IgG antibodies could bind to homologous and heterologous viruses within the same NA subtype.
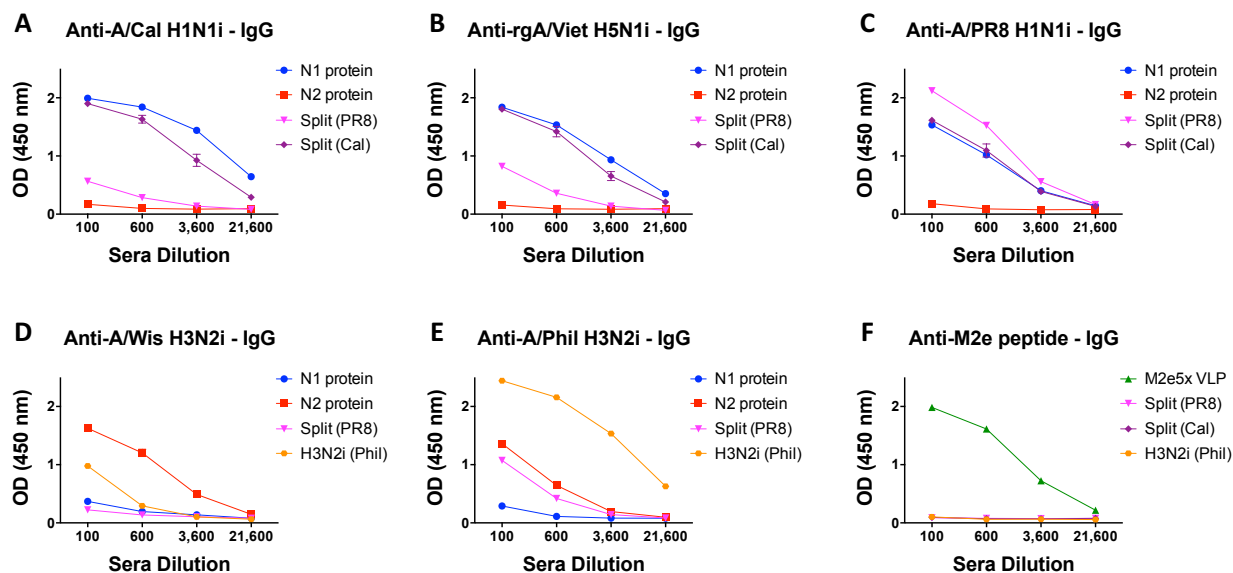
Figure 3.1 Reactivity of vaccine-specific antibodies to different strains of influenza viruses.
Immune sera were collected after boost immunization of BLAB/c mice (n= 5 per group) with N1 NA protein (A/Cal
H1N1), N2 NA protein (A/Wis H3N2), M2e5x VLP, split vaccine (A/PR8 H1N1, A/Cal H1N1), or whole A/Phil
H3N2 virus. Whole inactivated virus particles and M2e peptide were used as ELISA coating antigens: (A) A/Cal
H1N1, (B) rgH5N1, (C) A/PR8 H1N1, (D) A/Wis H3N2, (E) A/Phil H3N2 or (F) M2e peptide. Sera were pooled and
serially diluted.

3.2.2 NA protein vaccination develops NA-inhibiting cross-reactive antibodies.

As a measure of functional antibodies, NA inhibition (NI) activity of immune sera was
determined by a fetuin-based assay (Fig. 3.2). A/Cal N1 NA protein-induced antibodies exhibited
significantly higher levels of NI activity against homologous virus than split vaccine immune sera
(Fig. 3.2A). Also, A/Cal N1 NA immune sera showed low levels of NI activity against
heterologous rgH5N1 and heterosubtypic A/Phil H3N2 virus (Fig. 3.2B & D). N2 NA immune
sera displayed significant levels of NI activity against the homologous virus A/Wis H3N2 and
heterologous A/Phil H3N2 virus (Fig. 3.2C & D). Split A/Cal vaccine immune sera show low NI
activity against homologous but not heterologous virus. Inactivated split influenza vaccines raise
high hemagglutination inhibition titers in a strain specific manner as expected (Fig. 3.2E). These

results indicate that NA protein vaccination induces higher NI activity against both homologous and heterologous viruses than current vaccine platforms of inactivated influenza split virus. NI activity was low or hardly detected in split vaccine immunized mice.
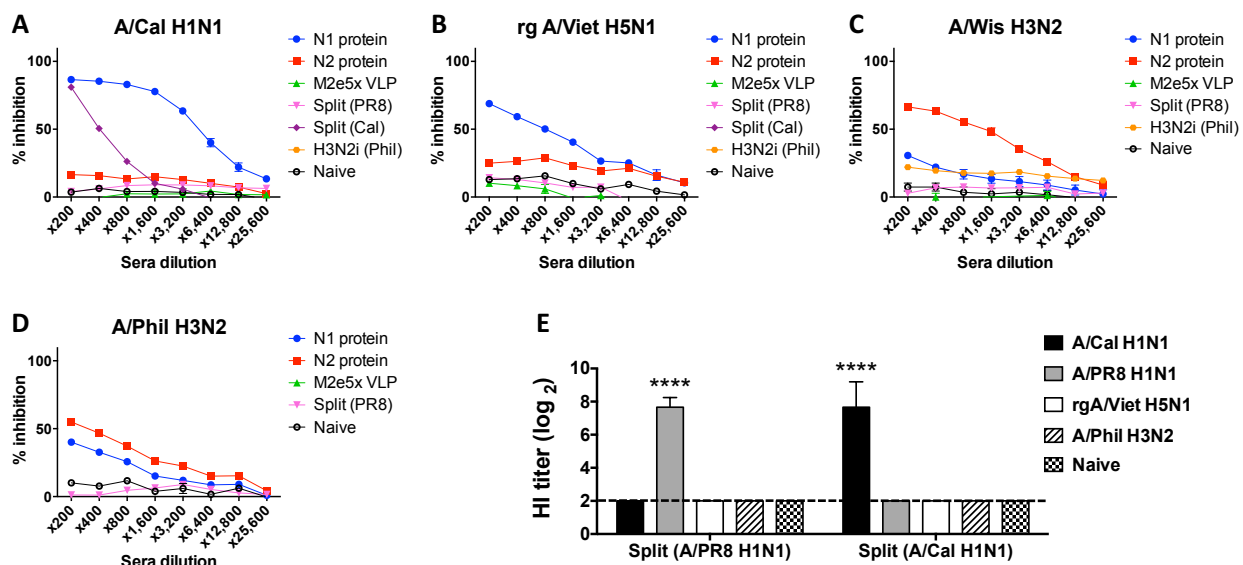
Figure 3.2 NA inhibition activity of vaccine-specific antibodies to different strains of influenza viruses.
Immune sera were collected and pooled after immunization (n=5) with N1 NA (A/Cal), N2 NA (A/Wis), M2e5x VLP, split vaccine (A/PR8, A/Cal), or inactivated A/Phil H3N2 virus. NA inhibition assays were performed against different strains of influenza viruses: (A) A/Cal H1N1, (B) rgH5N1, (C) A/Wis H3N2, or (D) A/Phil H3N2. (E) HI titers against A/Cal H1N1, A/PR8 H1N1, rgH5N1, and A/Phil H3N2 were determined from immune sera of split vaccines (A/PR8 H1N1 and A/Cal H1N1).

3.2.3 *NA protein vaccination is less effective in homologous protection than inactivated split and in heterologous protection compared to M2e5x VLP.*

We investigated protective efficacy in actively vaccinated mice after challenge. As described for Figure 1, mice were immunized with N1 (A/Cal) or N2 (A/Wis) NA proteins, split vaccine (A/Cal), or M2e5x VLP. Immune mice with A/Cal split vaccine developed significant levels of virus-specific serum IgG antibodies (Fig. 3.3A) and they showed high HI activity against the homologous virus strain (Fig. 3.3B). Mice immunized with A/Cal split vaccine did not show body weight loss after challenge with homologous A/Cal H1N1 virus (Fig. 3.3C). Mice that were immunized with A/Cal N1 NA protein displayed significant weight loss (~18%), but all mice in this group survived after challenge whereas all naïve mice died of infection (Fig. 3.3C). Regarding A/Wis N2 NA immunization, a homologous virus challenge mouse model is not available. Thus, we determined efficacy of N2 NA vaccination after a sub-lethal dose challenge with heterologous virus A/Phil H3N2 in comparison with M2e5x VLP vaccination. The A/Wis N2 NA group exhibited lower levels of weight loss (~15%) and a quicker recovery compared to the naïve control (~21%) after A/Phil virus challenge (Fig. 3.3D). Interestingly the mice immunized with M2e5x VLP, a representative cross-protective vaccine, showed better cross protection (~5% weight loss) against A/Phil H3N2 virus than N2 NA protein immunized mice (Fig. 3.3D). Overall, these results suggest that NA protein alone is less effective as a vaccine candidate in homologous protection than HA-based split vaccine and in cross protection compared to M2e5x VLP experimental vaccine.
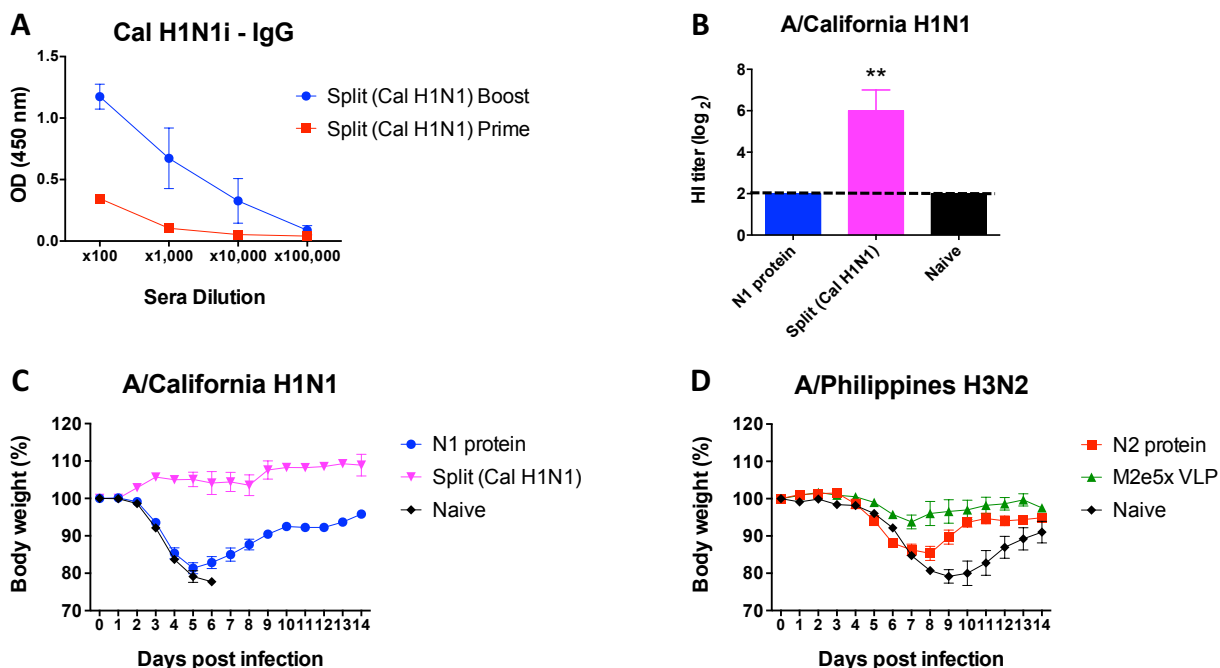
Figure 3.3 Vaccination with NA proteins induces less effective protection.
Mice were prime-boost immunized with N1 (A/Cal) or N2 (A/Wis) NA proteins (5μg), M2e5x VLP (10μg), or A/Cal split vaccine (1μg). (A) Prime and boost IgG levels of A/Cal split vaccine immunized mice. (B) HI titers against A/Cal H1N1 with immune sera of N1 NA protein and split vaccine (A/Cal H1N1). At 4 weeks after boost immunization, mice were challenged with a lethal dose of influenza virus and monitored for body weights. (C) N1 NA protein and split vaccine immunized mice were challenged with a homologous A/Cal virus. (D) N2 NA protein (A/Wis H3N2) and M2e5x VLP immunized groups were challenged with a heterologous virus (A/Phil H3N2). Data represent the mean ± SEM. Statistical significances were evaluated by 2-way ANOVA. **$p<0.01$.

3.2.4 *NA protein immune sera confer better cross protection against heterologous strains.*

We determined the roles of different vaccine immune sera in conferring cross-protection. Naïve mice were infected with a mixture of influenza virus and immune sera collected from each vaccine group, and then daily monitored for weight changes and survival rates for 14 days (Fig. 3.4 A-C). Naïve sera did not confer protection against A/Cal H1N1, rgH5N1, and A/Phil H3N2 viruses as evidenced by severe weight loss (>20%) or no survival rates (Fig. 3.4 A-C). In contrast,

N1 protein immune sera conferred protection against homologous A/Cal H1N1 virus and heterologous rgH5N1 virus although low to moderate weight loss of 5-10% was observed (Fig. 3.4 A, B). A/Cal split vaccine immune sera did not provide cross protection against rgH5N1 virus as shown by severe weight loss similar to that in naïve control sera (Fig. 3.4B). In an additional set of comparing cross protection against A/Phil H3N2 virus, the mice with N2 protein (A/Wis) immune sera showed weight loss of 16% similar to M2e5x VLP immune sera but better protection with a quicker recovery than A/PR8 split vaccine sera exhibiting severe weight loss (~25%) (Fig. 3.4C). Interestingly, combination of M2e5x VLP and N2 NA immune sera resulted in synergistic effects on improving protection against A/Phil virus in naïve mice (Fig. 3.4C). Taken together, these results suggest that NA targeting antibodies confer better cross protection compared to split vaccine-induced antibodies.

3.2.5   *Fc receptor plays a role in NA antibody-mediated protection but C3 is not required.*

We tested whether Fc receptors are involved in NA antibody-mediated protection. Naïve wild type (BALB/c) and Fc receptor common γ-chain knock-out (FcR KO) mice were infected with a mixture of A/Cal H1N1 virus and N1 protein-immune sera (Fig. 3.4D). BALB/c mice showed minimal or no weight loss and were well protected against homologous A/Cal H1N1 virus. However, FcR KO mice that were inoculated with A/Cal H1N1 virus and N1 NA immune sera resulted in substantial weight loss (~12%), but they were still protected compared to the FcR KO mice with naïve serum plus virus inoculation (Fig. 3.4D). These results suggest that Fc receptor contributes to the protection mediated by NA antibodies.

To test whether the complement system plays a role in NA-mediated protection, naïve wild type (C57BL/6) and C3 knock-out (C3 KO) mice were infected with a mixture of A/Cal H1N1

virus and N1 protein-immune sera (Fig. 3.4E). No significant difference in protective efficacy was observed between wild type and C3 KO mice. Both wild type and C3 KO mice that received N1 immune sera and virus exhibited a delay and moderate levels in weight loss (12-15%), compared to naïve serum control groups displaying severe (20-25%) weight loss. It is noted that the C3 KO mice with naïve sera and virus could not fully recover weight loss. C57BL/6 mice showed a trend of lower efficacies in conferring protection than those in BALB/c mice.
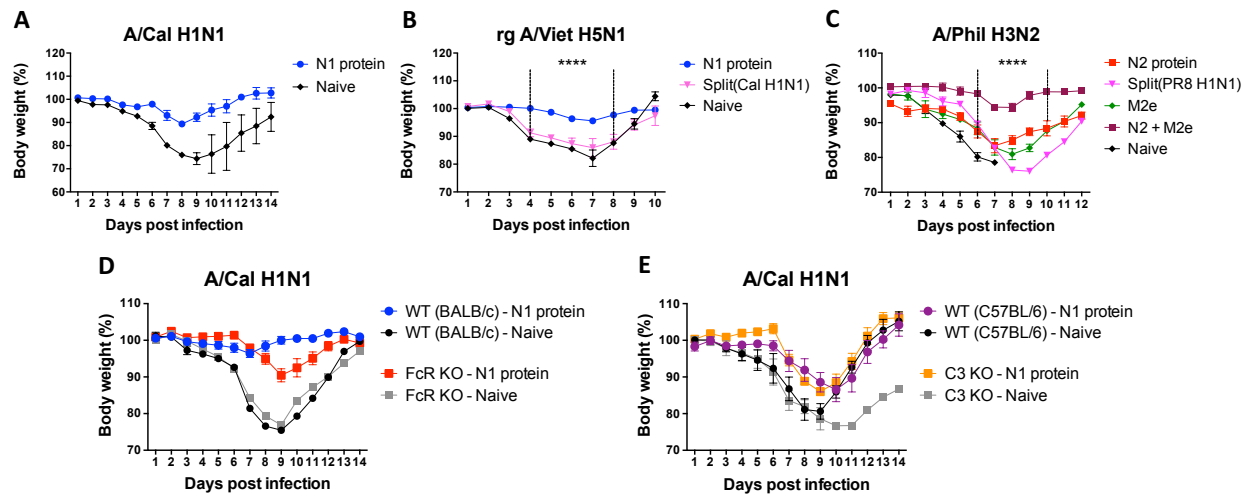
Figure 3.4 Roles of immune sera in conferring protection in naïve mice.
Immune sera were collected and pooled after immunization of BALB/c mice (n=5) with N1 NA (A/Cal), N2 NA (A/Wis), split vaccine (A/Cal or A/PR8 H1N1), M2e5x VLP, or a mixture of N2 NA and M2e5x VLP. Naïve BALB/c mice were intranasally infected with a lethal dose of influenza virus mixed with immune or naïve sera, and monitored for weight changes. Multiple strains of viruses were tested: (A) A/Cal H1N1, (B) rgH5N1 and (C) A/Phil H3N2. (D) BALB/c and Fc receptor knock-out (FcR KO) or (E) C57BL/6 and C3 knock-out (C3 KO) mice (n=4 per each group) were intranasally infected with a lethal dose of influenza virus (A/ Cal) mixed with immune or naïve sera. Data represent the mean ± SEM. Statistical significances were evaluated by 2-way ANOVA. ****$p<0.0001$.

3.3   Discussion

This study compared the efficacy of three different influenza vaccine antigens by using each representative vaccine: split vaccines for HA immunity, NA proteins for NA immunity, and M2e5x VLP for M2e immunity. We evaluated the protective efficacy of each antigen-specific immune sera by determining: (1) their reactivity to different subtypes of influenza A viruses, (2) enzyme inhibition activities to HA and NA, and (3) protection by active immunization or passively administrated antibodies. We found that antibodies to NA proteins confer a broader range of cross protection than HA antibodies. In addition, protection by NA-specific antibodies appears to be mediated by NA inhibition activity and Fc receptors.

Virus neutralizing activity by HA-targeting antibodies is the most effective in conferring protection against the homologous influenza virus. Immunization with split vaccines (A/Cal H1N1, A/PR8 H1N1, A/Phil H3N2) induced significant levels of antibodies binding to heterologous influenza viruses. Nonetheless, these split vaccine immune sera did not show HI activity against heterosubtypic strains and failed to induce cross protection against viral infection with different subtypes, limiting the protection to homologous virus. Based on these results, split vaccines tend to induce mainly HA immunity to homologous virus. NA protein immunization raised antibodies that are cross reactive to different influenza virus strains within the same NA type. In a similar pattern, NA protein immune sera showed high levels of NI activity to homologous and heterologous virus strains. In contrast to HA immunity by split vaccines, NA antibodies contribute to survival protection against homologous and heterologous influenza virus within the same NA subtype viruses, which is consistent with a previous study.[17,60]

HA targeting vaccines are superior to NA vaccines in homologous protection. This is because HI activity of anti-HA antibodies can lead to sterilizing immunity, which cannot be comparable to

infection permissive cross protection. Active immunization with NA protein vaccines induced survival protection against heterologous virus challenge, but its protective efficacy was lower than that of M2e5x VLP which we previously described as for a cross protective vaccine candidate.[61] It is unclear what differences are in the protective mechanisms between NA protein and M2e5x VLP vaccines. T cells induced by M2e5x VLP vaccination were shown to play a role in conferring cross protection.[43] It is possible that in addition to M2e antibodies, T cells induced by M2e5x VLP vaccination contribute to more effective cross protection than NA protein immunization since we observed that N2 NA protein and M2e5x VLP immune sera conferred a similar level of protection against A/Phil H3N2 virus. We hypothesized that non-neutralizing antibody-mediated mechanisms would be involved in the NA antibody-mediated protection, which include antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytolysis (CDC). Fc receptor is known to be required for ADCC via phagocytic cells such as macrophages, natural killer (NK) cells, and neutrophils.[62] C3 component is an essential factor in the complement pathway leading to clearance of antigen-antibody immune complexes via CDC.[6] N1 type NA protein immune sera conferred protection against homologous viral infection in wild type (BALB/c) mice without displaying weight loss whereas a moderate level of body weight loss was observed in FcR KO mice. These results suggest that Fc receptors partially contribute to the protection by NA antibodies. In line with the roles of Fc receptors in mediating protection, HA stalk-specific antibodies were shown to induce phagocytosis of immune complexes in a FcR dependent manner.[63,64] Similarly, Fc receptors were required for M2e immune mediated protection.[57] In contrast, a similar pattern of weight loss was observed in both wild type (C57BL/6) and C3 KO mice, which indicates that C3 is not required for protection by NA antibodies. Significantly more weight loss in C57BL/6 mice than in BALB/c mice might be due to different genetic backgrounds

in these two strains of mice. C57BL/6 mice showed a defect in developing granzyme B-secreting CD8 T cells after influenza virus infection compared to BALB/c mice (unpublished data). It has been reported that C57BL/6 strain is more susceptible to influenza virus infection than BALB/c strain.[65]

To overcome limitations of a single vaccine antigen in conferring cross protection, a multicomponent vaccine strategy was reported. Supplementation of inactivated influenza vaccines with M2e-based antigens resulted in inducing significantly improved cross protection in BALB/c mice.[43] It was reported that addition of NA and M2e vaccines to recombinant HA vaccines confers long-lasting cross protection against primary and secondary influenza virus infections in BALB/c mice.[66] We found that combination of N2 NA and M2e5x VLP immune sera resulted in conferring synergistically improved cross protection compared to each immune serum alone. Our unpublished data support the benefits of multicomponent vaccination that the combination of split, NA protein, and M2e5x VLP vaccines induced antibodies specific to each vaccine antigen, providing significantly improved cross protection in C57BL/6 mice than single component vaccines (data not shown). In addition, we found that the efficacy of cross protection by multicomponent vaccines was significantly lower in either CD4 or CD8 T cell deficient mice compared to that in wild type C57BL/6 mice, suggesting important roles of CD4 and CD8 T cells in cross protection (data not shown). Taken together, findings in this study highlight different roles of HA, NA, and M2e as vaccine antigens and suggest a new possible strategy for improving cross protection.

3.4 Conclusion

This study directly compared the protective efficacy and the properties of antibodies induced by different representative vaccine antigens, which are Hemagglutinin (HA), NA, and the extracellular domain of M2 (M2e). Anti-NA antibodies induced effective protection against multiple heterologous Influenza viruses. These protective immune responses are established by NA inhibition activity of anti-NA antibodies and Fc receptor-mediated antibody-dependent cellular cytotoxicity (ADCC). In contrast, HA immunity showed only strain-specific protection that are mediated by HA inhibition activity of anti-HA antibodies even though anti-HA antibodies were able to bind to heterosubtypic viruses. Taken together, this study suggests a possible vaccine strategy and its immune mechanism that conventional HA-targeting vaccines are supplemented with NA and M2e-targeting vaccines to take advantages from different vaccine targets.

4   CHAPTER 2. CROSS PROTECTIVE EFFICACY OF INFLUENZA VIRUS M2E CONTAINING VIRUS-LIKE PARTICLES IS SUPERIOR TO HEMAGGLUTININ VACCINES AND VARIES DEPENDING ON THE GENETIC BACKGROUNDS OF MICE.

4.1   Summary

Influenza virus M2 extracellular domain (M2e) has been a target for developing cross protective vaccines. However, the efficacy and immune correlates of M2e vaccination are poorly understood in the different host genetic backgrounds in comparison with influenza vaccines. We previously reported the cross protective efficacy of virus-like particle (M2e5x VLP) vaccines containing heterologous tandem M2e repeats (M2e5x) derived from human, swine, and avian influenza viruses. In this study to gain better understanding of cross protective influenza vaccines, we compared immunogenicity and efficacy of M2e5x VLP, H5 hemagglutinin VLP (H5 VLP), and inactivated H3N2 virus (H3N2i) in wild type strains of BALB/c and C57BL/6 mice, and CD4 and CD8 knockout (KO) mice. M2e5x VLP was better than H5 VLP in conferring cross protection whereas H3N2i inactivated virus vaccine provided superior homologous protection. After M2e5x VLP vaccination and challenge, BALB/c mice induced higher IgG responses, lower lung viral loads, and less body weight loss when compared to those in C57BL/6 mice. M2e5x VLP but not H3N2i immune mice after primary challenges developed strong immunity against a secondary heterosubtypic virus as a model of future pandemics. M2e5x VLP and H5 VLP vaccines were able to raise IgG isotypes in CD4 KO mice. T cells were found to contribute to cross protection by playing a role in reducing lung viral loads. In conclusion, M2e5x VLP vaccination induced better cross protection than HA VLP, and its efficacy varied depending on the genetic backgrounds of mice, supporting the important roles of T cells.

4.2   Results

4.2.1   C57BL/6 mice display lower levels of M2e-specific IgG responses after M2e5x VLP vaccination than BALB/c mice.

We determined IgG isotype antibody responses to different influenza vaccine platform antigens in BALB/c and C57BL/6 mice (Fig. 4.1). Groups of mice were i.m. immunized with 10 µg of M2e5x VLP, H5 VLP, or H3N2i. IgG isotypes specific for different influenza virus antigens were determined at 2 weeks after boost immunization. The M2e5x VLP BALB/c mice showed substantially high levels of IgG1, IgG2a, and IgG2b antibodies specific for M2e (Fig. 4.1A-D). The H5 VLP BALB/c mice developed high levels of IgG2a and IgG2b antibodies specific for the homologous rgH5N1 virus antigen. The level of IgG1 antibodies specific rgH5N1 virus were significantly low in both BALB/c and C57BL/6 mice (Fig. 4.1E-H). The H3N2i group in BALB/c mice raised significant levels of IgG1, IgG2a, and IgG2b antibodies specific for the same H3N2 vaccine virus (Fig. 4.1I-L). H5 VLP and H3N2i immune sera showed HA inhibition activity against each antigen specific rgH5N1 and A/Philippines/2/82 (H3N2) virus respectively (Fig. 4.1M, N).

C57BL/6 mice immunized with M2e5x VLP also developed IgG2c and IgG2b isotype antibodies binding to M2e antigens but at lower levels than BALB/c mice (Fig. 4.1A-D). Whereas, comparably high levels of IgG2b antibodies specific for rgH5N1 and H3N2i virus antigens were induced in the H5 VLP and H3N2i C57BL/6 groups (Fig. 4.1E-L).

Overall, both BALB/c and C57BL/6 mice developed differential levels of IgG isotypes depending on the types of antigens and vaccine platforms. M2e5x VLP induced higher IgG1 isotype antibody responses than H5 VLP. Both BALB/c and C57BL/6 mice that were immunized

with H3N2i vaccine developed IgG and class-switched IgG antibody responses at similarly high levels. H5 VLP and M2e5x VLP vaccines showed a tendency of inducing IgG2c isotype antibodies. Meanwhile, H3N2i immunization induced similar levels of class-switched IgG antibodies in both BALB/c and C57BL/6 mice.
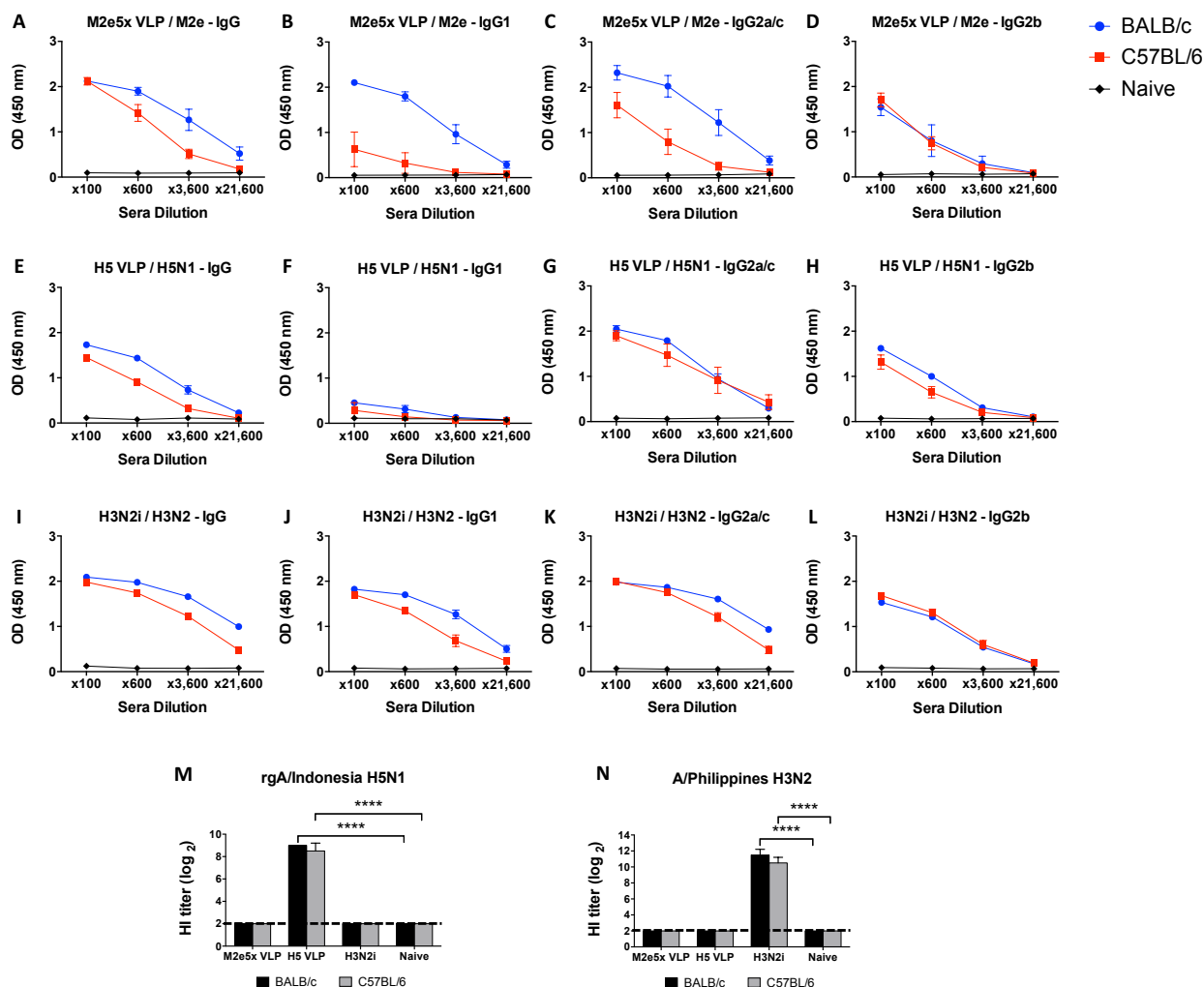
Figure 4.1 IgG isotype antibody responses in BALB/c and C57BL/6 mice after vaccination with different influenza vaccines.
Each group of mice (n=10) was prime and boost i.m. immunized with 10 μg of M2e5x VLP (A-D), H5 VLP (E-H), or inactivated A/Philippines virus (H3N2i) (I-L). Antibody levels of IgG (A, E, and I), IgG1 (B, F, and J), IgG2a in BALB/c mice or IgG2c in C57BL/6 mice (C, G, and K), and IgG2b (D, H, and L) were detected by ELISA. Sera were serially diluted and ELISA were performed by using vaccine specific antigens which are M2e peptide, inactivated A/Indonesia (rgH5N1i), and H3N2i. Error bars indicates mean ± SEM. (M and N) HI titers. HI titers against rgH5N1 (M) and H3N2 (N) were determined from immune sera of M2e5x VLP, H5 VLP, or H3N2i.

4.2.2   M2e5x VLP is more effective in conferring cross protection than H5 VLP.

To determine cross protective efficacy, naïve and vaccinated mice were challenged with H3N2 (A/Philippines/82) virus (Fig. 4.2A). As expected, H3N2i vaccination induced complete protection against the homologous H3N2 virus in both BALB/c and C57BL/6 mice (Fig. 4.2B, E). Also, H5 VLP immunization induced protection against homologous rgH5N1 virus (data not shown). In contrast, H5 VLP vaccination did not induce protection against heterosubtypic H3N2 virus as evidenced by severe body weight loss in BALB/c and C57BL/6 mice similar to naïve control (Fig. 4.2B, E). BALB/c mice immunized with M2e5x VLP showed protection against H3N2 virus challenge despite a slight weight loss (Fig 4.2B). C57BL/6 mice immunized with M2e5x VLP showed significant weight loss (15-18%) compared to the same vaccination of BALB/c mice (Fig 4.2E). Nonetheless, the M2e5x VLP immunized group of C57BL/6 mice showed less weight loss indicating better cross protection than the naïve control or H5 VLP group (Fig 4.2E).

Protective efficacy was further confirmed by lung viral titers at day 7 after H3N2 virus challenge (Fig. 4.2C, F). The naïve and H5 VLP groups showed the highest lung viral titers in a range of 10^6 to 10^7 EID_{50} (per lung/ml) in contrast to the H3N2i group with lung viral clearance below the detection limit. The M2e5x VLP group exhibited approximately 100 and 20 fold lower lung viral titers compared to the H5 VLP group in BALB/c and C57BL/6 mice, respectively. Overall, the results of lung viral titers show a correlation with protection as indicated by weight loss.

4.2.3 *M2e5x VLP-immunized mice that survived primary infection develop immunity against antigenically novel virus infection.*

Outbreaks of pandemics are unpredictable and current vaccination is not effective in preventing pandemics. To address this critical issue, the vaccinated mice that survived the H3N2 virus primary challenge were exposed 7 weeks later to the secondary infection with a lethal dose of heterosubtypic H1N1 virus (A/California/07/2009). H3N2i vaccination did not provide protection against the secondary H1N1 virus, as shown by severe weight loss (over 22% in BALB/c mice) and no survivals (0%) in C57BL/6 mice (Fig. 4.2D, G). The M2e5x VLP group exhibited the best protection and recovery against the secondary H1N1 virus infection in both BALB/c and C57BL/6 mice (Fig. 4.2D, G). BALB/c mice with M2e5x VLP vaccination showed better protection against secondary heterosubtypic virus challenge than the corresponding C57BL/6 mouse group. In a similar trend, naïve BALB/c mice that survived primary H3N2 infection were protected against secondary H1N1 2009 virus (Fig. 4.2D) whereas naïve C57BL/6 mice surviving primary H3N2 infection were not well protected against secondary H1N1 2009 virus (Fig. 4.2G). Taken together, BALB/c mice with vaccination or primary H3N2 virus infection showed stronger immunity during the secondary heterosubtypic H1N1 virus infection than C57BL/6 mice suggesting mouse strain differences in protective efficacy by virus infection or vaccination.
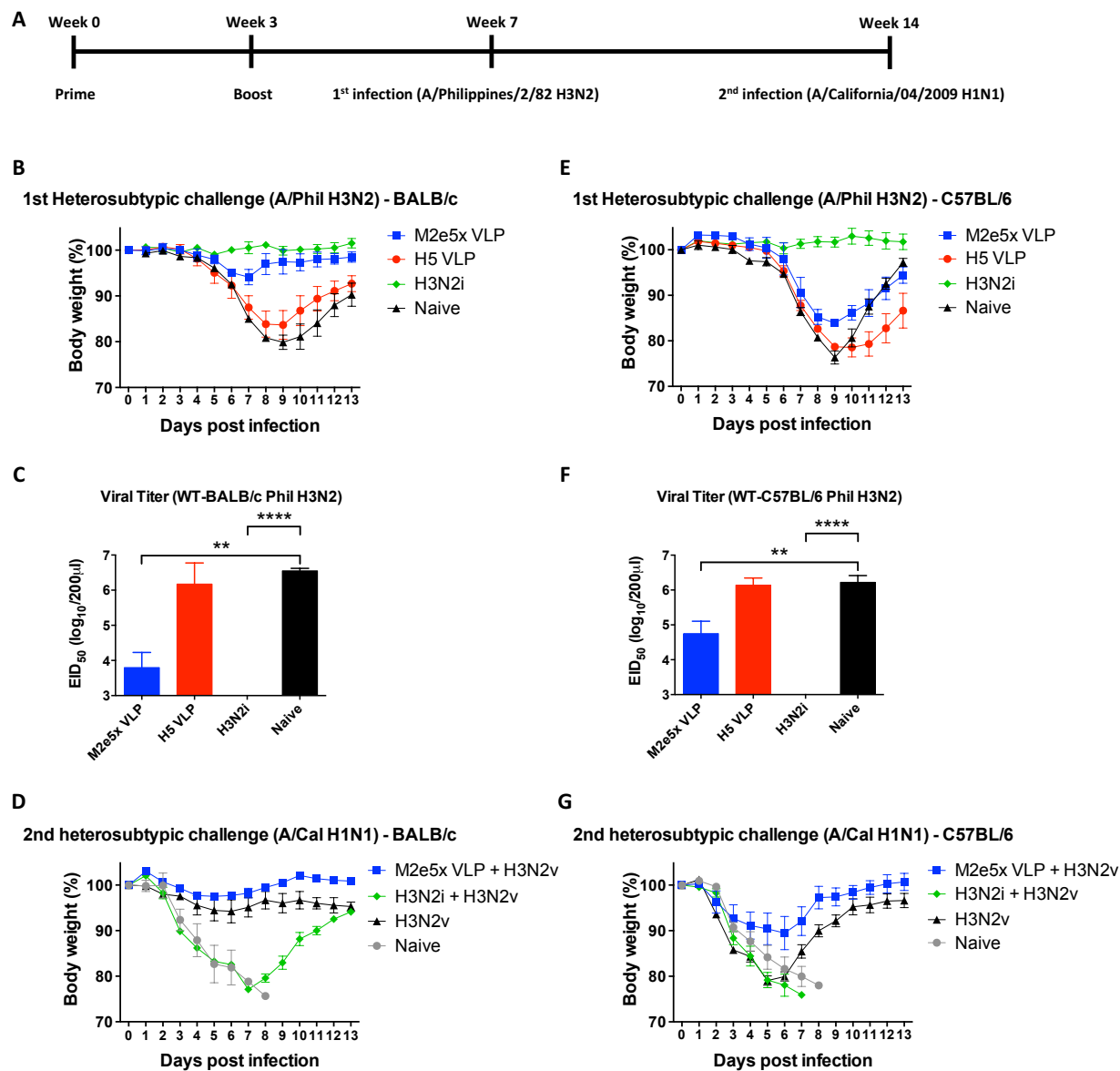
Figure 4.2 Differential efficacy of protection by M2e5x VLP, H5 VLP, or H3N2i after primary H3N2 virus and secondary H1N1 virus challenge in BALB/c and C57BL/6 mice.
A. Time schedule for experiments of vaccination, 1st challenge infection with H3N2 virus, and 2nd challenge infection with H1N1 virus. BALB/c (B and C) and C57BL/6 (E and F) mice were challenged with A/Philippines/2/82 (H3N2) (H3N2v) at 4 weeks after boost with M2e5x VLP, H5 VLP, or H3N2i. Body weights were monitored for 14 days (B and E). Lung viral titers were determined by the egg inoculation assay (C and F). The detection limit of EID_{50} was 1.7 Log10. At 7 weeks after 1st infection with A/Philippines/2/82/ (H3N2), BALB/c (D) and C57BL/6 (G) mice were challenged with a different subtype of influenza virus (A/California/04/2009 H1N1) and body weight changes were monitored for 14 days. H3N2 infection only group was the naïve infection group of B or E. Data represent the mean ± SEM. Statistical significances were evaluated by 2-way ANOVA. **$p<0.01$, ****$p<0.0001$.

4.2.4 BALB/c mice induce higher levels of M2e-specific T cell responses than C57BL/6 mice.

To better understand a difference in the cross protective efficacy between C57BL/6 and BALB/c mice, we determined cellular immune responses by measuring the levels of IFN-γ and IL-4 cytokines secreted into culture supernatants after *in vitro* stimulation with M2e peptides (Fig. 4.3). M2e5x VLP-immunized BALB/c mice showed significantly higher levels of IFN-γ and IL-4 cytokine secreting cells both in the lung and spleen cells collected day 7 post H3N2 challenge compared to M2e5x VLP-immunized C57BL/6 mice (Fig. 4.3). These results indicate the possibility that a genetic background of BALB/c mice is more effective in inducing M2e-specific or virus specific T cell responses than that of C57BL/6 mice.

To further confirm the M2e-specific T cell responses, we carried out intracellular staining and flow cytometry assays for quantification of CD4 and CD8 T cells secreting IFN-γ and granzyme B in lungs and bronchoalveolar lavage fluids (BALF) at 7 days after viral challenge. Significantly higher levels of IFN-γ in CD4 T cells were detected in M2e5x VLP-immunized BALB/c than C57BL/6 mice (Fig. 4.4). In contrast, C57BL/6 mice showed much lower levels of IFN-γ secreting CD4 T cells in lungs and BALF than BALB/c mice (Fig. 4.4). IFN-γ secreting lung CD4 T cells induced by M2e5x VLP vaccination and virus challenge were at a higher level compared to that in naïve mice after challenge (Fig. 4.4D). In BALF, M2e5x VLP immunized C57BL/6 mice showed a lower number of IFN-γ secreting CD4 T cells than the naïve mouse control (Fig. 4.4B). Although the reason for this odd finding is not clear in C57BL/6 mice, this might be due to the delayed recruitment of the effector CD4 T cells to the airway area as a result of better control of lung viral loads. In lungs, C57BL/6 mice immunized with M2e5x VLP showed significantly higher IFN-γ secreting CD4 T cells than the naïve mouse control (Fig. 4.4D) in a

similar pattern with the case in BALB/c mice. The combined IFN-γ secreting CD4 T cells in BALF and lungs were significantly higher in M2e5x VLP-immunized C57BL/6 mice (Fig 4.4C, D). M2e5x VLP vaccination did not increase IFN-γ secretion from antigen-specific CD8 T cells (Supplementary Fig. 4). However, BALB/c mice induced significantly higher levels of granzyme B secreting CD8 T cells than C57BL/6 mice at day 7 after viral infection (Fig. 4.5). In summary, BALB/c mice have a genetic background resulting in higher T cell responses to M2e5x VLP vaccination and influenza viral infection.
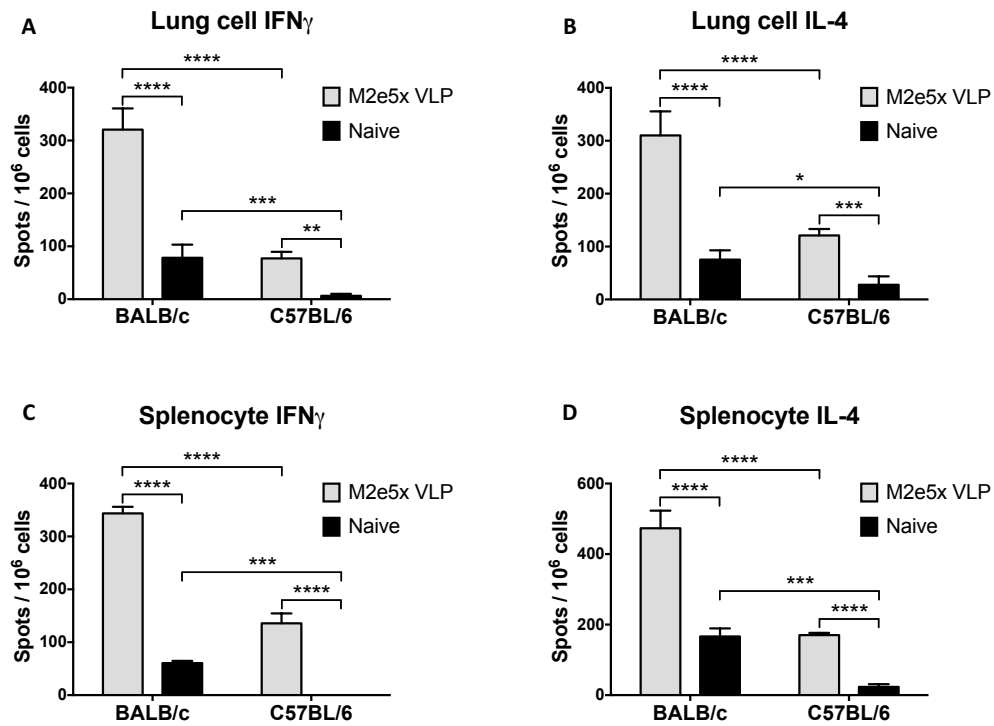
Figure 4.3 Cytokine producing T cell responses by M2e5x VLP immunization in C57BL/6 and BALB/c mice.
A. IFN-γ-secreting cells in lungs. B. IL-4-secreting cells in lungs. C. IFN- γ-secreting cells in splenocytes. D. IL-4-secreting cells in splenocytes. Lung cells and splenocytes were isolated from C57BL/6 and BALB/c mice previously immunized with M2e5x VLP at day 7 post-challenge (A/Philippines/2/82 H3N2). Cytokine-producing cell spots were counted by ELISPOT reader. Data represent the mean ± SEM. Statistical significances were evaluated by 2-way ANOVA. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.
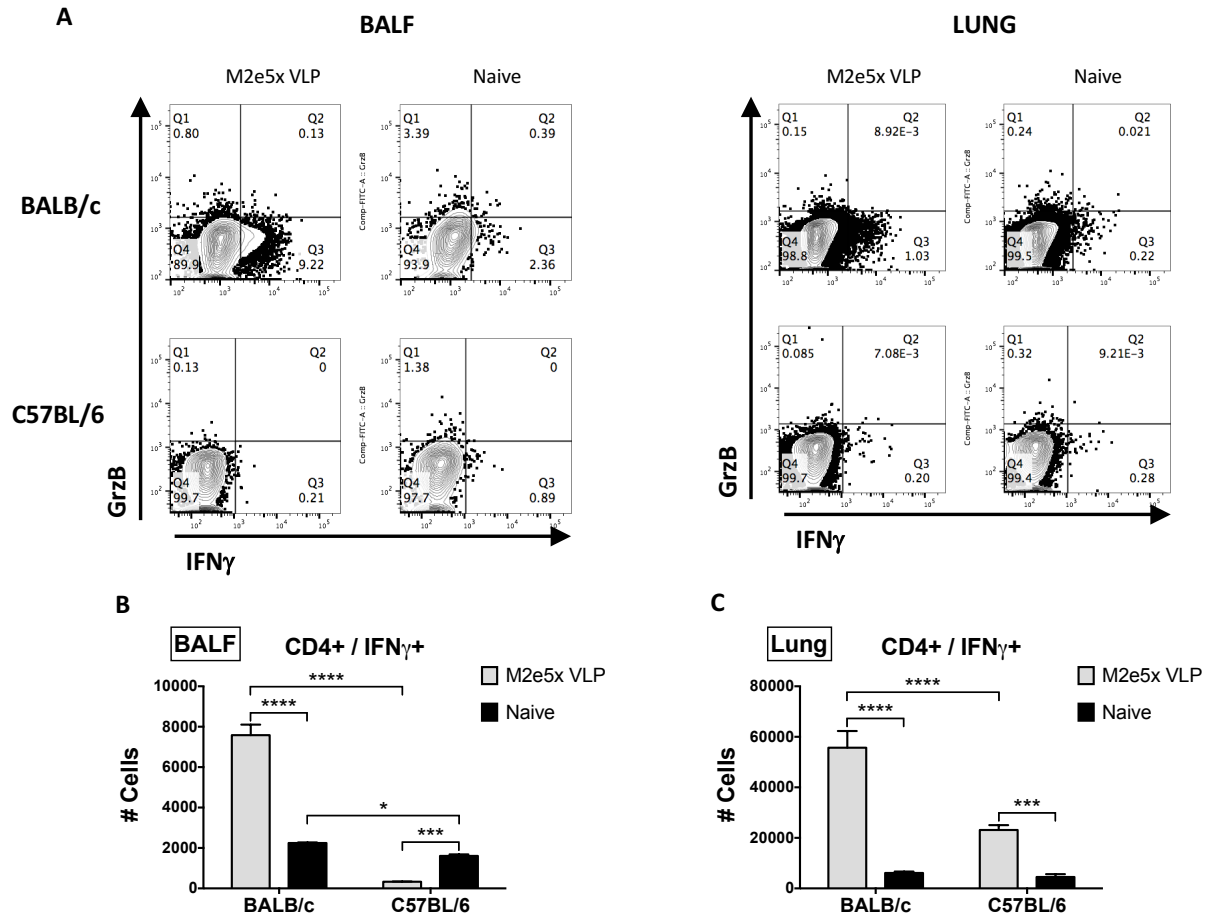
Figure 4.4 Intracellular cytokine staining of CD4^{+} T cells under the condition of M2e5x VLP immunization in C57BL/6 and BALB/c mice.
A. Representative flow cytometry profiles of IFN-γ- and Granzyme B-secreting CD4^{+} T cells in BALF and lungs. B. The cellularity of IFN-γ-secreting CD4^{+} T cells in BALF. C. The cellularity of IFN-γ-secreting CD4^{+} T cells in lungs. The numbers of cells were indicated as the number per mouse (C and D). After gating CD4^{+} cells, IFN-γ^{+} or Granzyme B^{+} cells were measured by flow cytometry of intracellularly stained cells. Data represent the mean ± SEM. Statistical significances were evaluated by 2-way ANOVA. *p<0.05, ***p<0.001, ****p<0.0001.
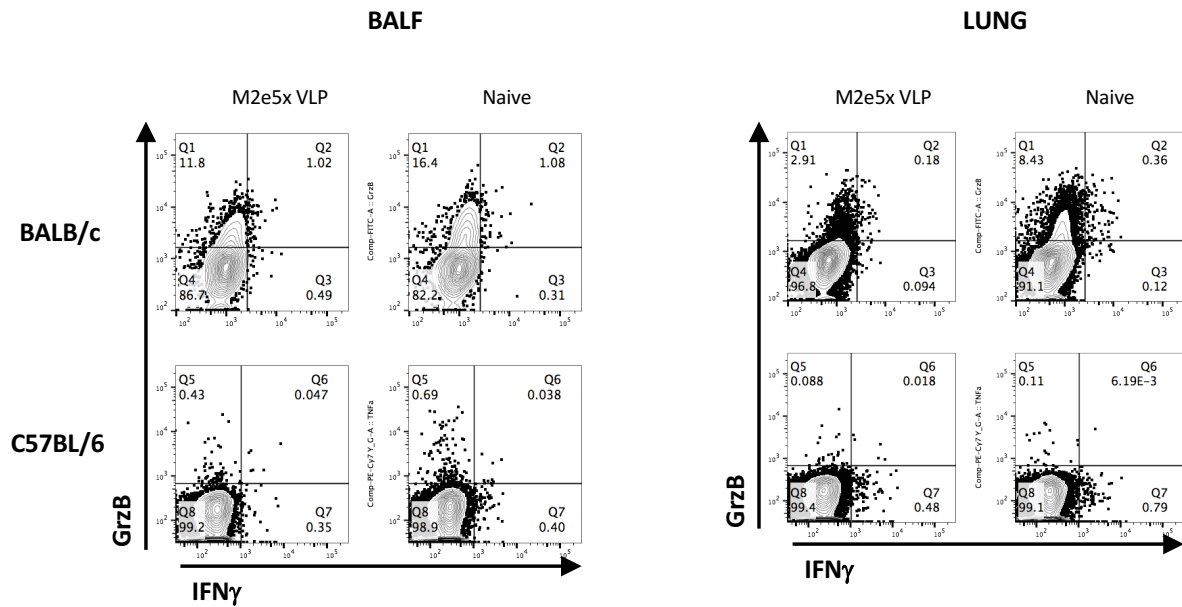
Figure 4.5 Intracellular cytokine staining of CD8^{+} T cells in C57BL/6 and BALB/c mice.
Flow cytometry profiles of IFN-γ- and Granzyme B-secreting CD8^{+} T cells in BALF and lungs. After gating CD8^{+} cells, IFN-γ^{+} or Granzyme B^{+} cells were measured by flow cytometry of intracellularly stained cells.

4.2.5   M2e5x VLP and H5 VLP vaccines induce IgG isotype-switched antibodies in CD4 knockout mice at different levels.

CD4 T cells are known to play critical roles in inducing isotype-switched IgG antibodies.[67] To determine the roles of T cells in inducing IgG isotype-switched antibodies and protective immunity after influenza vaccination, we immunized CD4 knockout (CD4 KO) and CD8 knockout (CD8 KO) mice (Fig. 4.7). Vaccine dose and immunization regimens were the same as described in wild type mice above. CD4 KO mice immunized with M2e5x VLP induced significant levels of M2e-specific IgG antibodies (Fig. 4.6A-D). CD8 KO mice immunized with M2e5x VLP developed similar levels of M2e-specific IgG antibodies (Fig. 4.6A-D). H5 VLP immunization was able to induce rgH5N1 virus-specific IgG antibodies in a similar trend with M2e5x VLP in both T cell KO mice (Fig. 4.6E-H). CD4 KO mice immunized with H3N2i induced significantly lower levels of H3N2 virus-specific IgG, IgG2c, and IgG2b isotype antibodies (Fig. 4.6I-L) than those in wild type C57BL/6 mice (Fig. 4.1I-L). However, H3N2i immunized CD8 KO mice developed similar levels of H3N2 virus-specific IgG antibodies with wild type mice (Fig. 4.6I-L).

4.2.6   T cells contribute to cross protection by M2e5x VLP vaccine.

The roles of CD4 and CD8 T cells in conferring vaccine-induced protection were determined using knockout mouse models (CD4 KO, CD8 KO) after vaccination and challenge (Fig. 4.7). M2e5x VLP vaccinated CD4 KO mice displayed a progressive weight loss to a similar degree as in naïve CD4 KO mice but showed a better recovery at later time points (Fig. 4.7B). A high lung viral titer at day 7 after challenge was observed in the M2e5x VLP CD4KO group, which is similar to the one observed in naïve infection (Fig. 4.7C), suggesting a role of CD4 T cells in M2e-immune mediated cross protection. H3N2i vaccination of CD4 KO induced protection against homologous

virus challenge although there was a substantial lung viral titer in CD4 KO mice (Fig. 4.7C). H3N2i immune sera from CD4 KO mice significantly inhibited the HA activity of homologous virus (Fig. 4.6 M, N). These results suggest that CD4 T cells play a role in preventing severe weight loss and clearing lung viral loads by M2e5x VLP vaccination. As expected from high levels of IgG antibodies, H3N2i vaccinated CD8 KO mice showed no weight loss after homologous H3N2 virus challenge (Fig. 4.7E) similar to the one induced in C57BL/6 mice (Fig. 4.2E). M2e5x VLP vaccinated CD8 KO mice showed similar infection symptoms and high lung viral loads similar to naïve CD8 KO mice against H3N2 virus challenge (Fig. 4.7E, F). Thus, CD8 T cells also play an important role in preventing severe weight loss and in clearing lung viral loads in M2e5x VLP immunized mice.

CD4 KO mice with M2e5x VLP vaccination showed a moderate level of protection against secondary heterosubtypic virus challenge (Fig. 4.7D) in a similar pattern with the corresponding C57BL/6 mouse groups (Fig. 4.2G). CD8 KO mice immunized with M2e5x VLP showed more severe body weight loss compared to CD4 KO and wild type mice (Fig. 4.7G). Taken together, M2e5x VLP-mediated immunity is partially dependent on CD8 T cells during the secondary heterosubtypic virus infection whereas primary infection-mediated immunity is related with both CD4 and CD8 T cells.
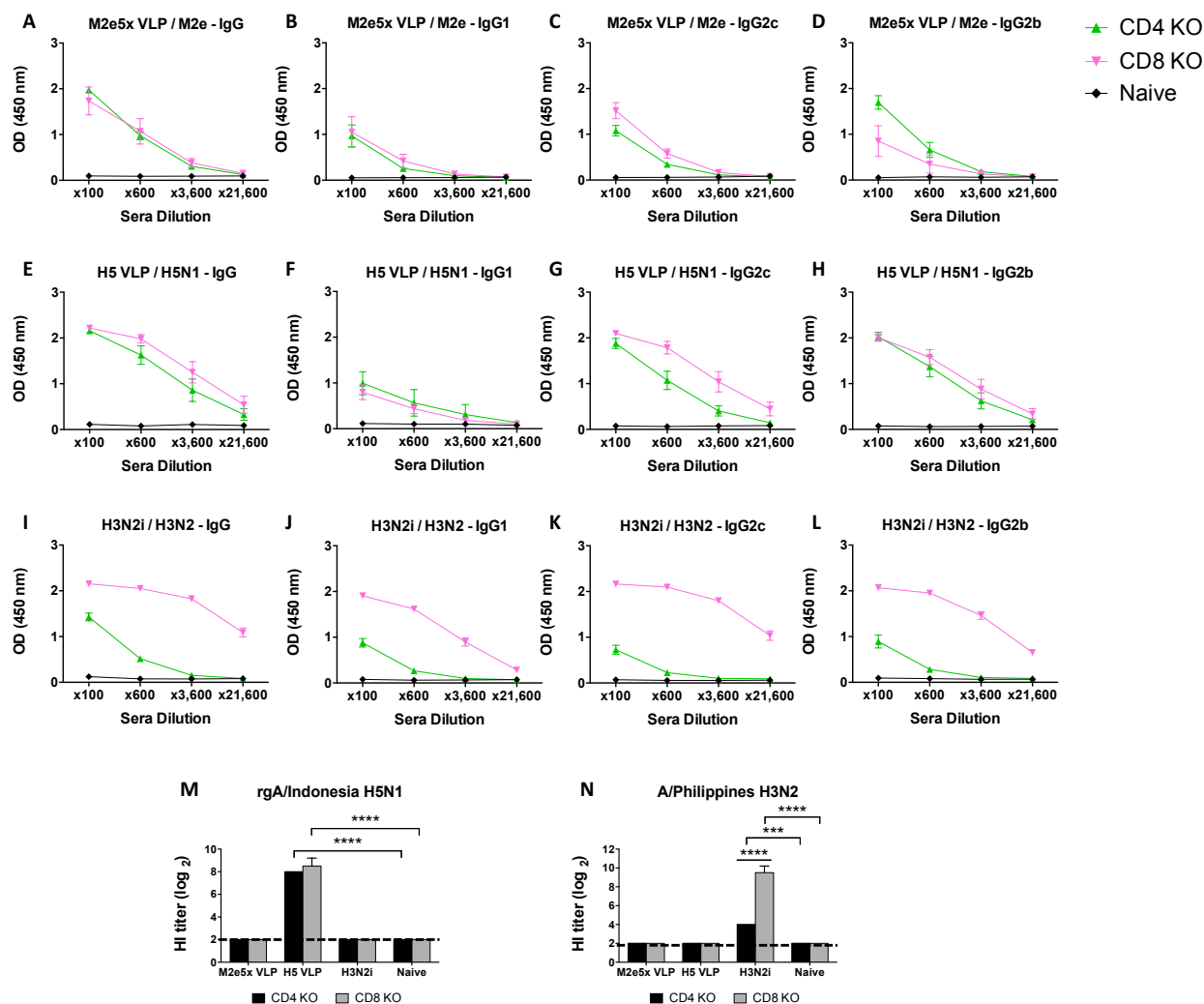
Figure 4.6 IgG isotype antibody responses in CD4 KO and CD8 KO mice after vaccination with different influenza vaccines.
Each group of CD4 KO and CD8 KO mice (n=10) was prime and boost i.m. immunized with 10 µg of M2e5x VLP (A-D), H5 VLP (E-H), or H3N2i (I-L). Antibody levels of IgG (A, E, and I), IgG1 (B, F, and J), IgG2c (C, G, and K), and IgG2b (D, H, and L) were detected by ELISA. The same influenza antigens were used for ELISA as described in Fig. 2. Error bars indicates mean ± SEM. (M and N) HI titers. HI titers against rgH5N1 (M) and H3N2 (N) were determined from immune sera of M2e5x VLP, H5 VLP, or H3N2i.
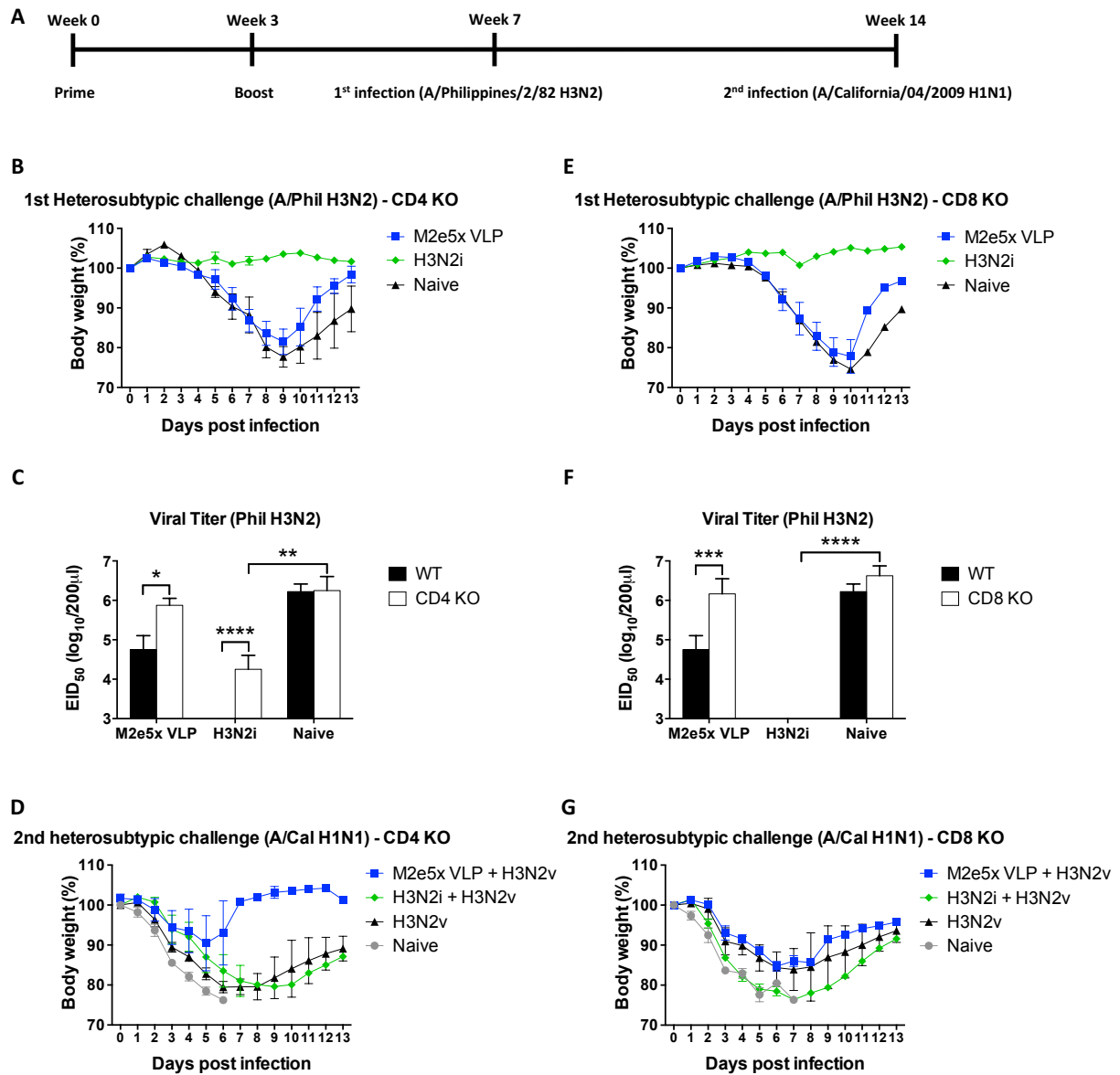
Figure 4.7 Efficacy of protection in CD4 KO and CD8 KO mice by different influenza vaccines after primary H3N2 virus and secondary H1N1 virus challenge.
A. Time schedule for experiments of vaccination, 1st challenge infection with H3N2 virus, and 2nd challenge infection with H1N1 virus. CD4 KO (B, C, and D) and CD8 KO (E, F, and G) mice were challenged with A/Philippines/2/82 (H3N2) (H3N2v) at 4 weeks after boost with M2e5x VLP or H3N2i. Body weights were monitored for 14 days. Lung viral titers were determined by the egg inoculation assay (C and F). The detection limit of EID_{50} was 1.7 Log10. At 7 weeks after 1st infection with A/Philippines/2/82/ (H3N2), CD4 KO (D) and CD8 KO (G) mice were challenged with a different subtype of influenza virus (A/California/04/2009 H1N1) and body weight changes were monitored for 14 days. H3N2 virus infection only group was the naïve infection group of B or E. Data represent the mean ± SEM. Statistical significances were evaluated by 2-way ANOVA. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

4.2.7   M2-specific immune sera play a role in conferring cross protection.

M2e5x VLP was found to induce significant levels of M2e-specific antibodies in C57BL/6, CD4 KO, and CD8 KO mice. Thus, we determined the roles of M2e immune sera in conferring protection independent of T cell immunity as detailed in the methods section. Naïve mice were infected with a mixture of H3N2 virus and immune sera collected from different genotypic M2e5x VLP immune or naïve mice, and then weight changes and survival rates were daily monitored (Fig. 4.8A). M2e5x VLP immune sera from CD4 KO and CD8 KO mice were found to contribute to protection against H3N2 virus at a similar level as observed in M2e5x VLP immune sera from C57BL/6 mice. Consistent with these body weight monitoring results, M2e5x VLP-immune sera from CD4 KO mice representatively showed significantly lower levels of lung viral titers than naïve control sera (Fig. 4.8B). Taken together, these results suggest that M2e-immune sera from different strains and genotypes of mice confer similar levels of protection regardless of genetic backgrounds and T cell immunity, preventing severe weight loss, and increasing survival rates and recovery.
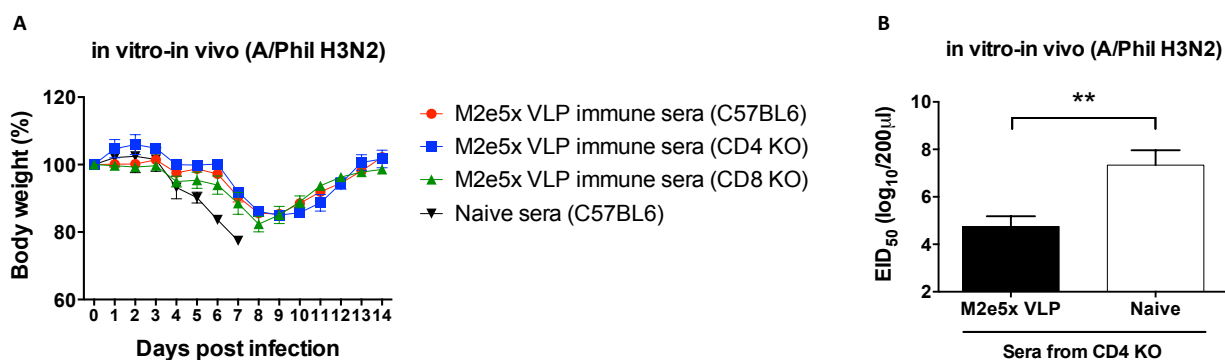
Figure 4.8 M2e antibodies in T cell deficient mice show similar capacity to confer protection in naïve mice. Immune sera collected from immunized C57BL/6, CD4 KO, and CD8 KO mice were incubated with influenza virus (A/Philippines/2/82 H3N2). Naive mice were intranasally infected with a mixture of a lethal dose of influenza virus and immune sera or naïve sera. Body weights were monitored for 14 days (A). Lung viral titers day 7 post infection were determined by the egg inoculation assay (B). The detection limit of EID_{50} was 1.7 Log10.

4.3   Discussion

Most subunit vaccines including cross protective influenza A virus M2 vaccines have been investigated in BALB/c mice.[34,35,39,40,68-70] A limited set of influenza M2 vaccine studies has been performed in C57BL/6 mice.[44,71] In our previous studies[34,68], we have developed an M2e targeting M2e5x VLP vaccine and studied cross protective efficacy mostly in BALB/c mice. In this study of comparing HA-based H5 VLP and inactivated whole influenza virus (H3N2i) vaccines, we investigated heterosubtypic cross protective efficacies of M2e5x VLP in mice with different genetic backgrounds and the roles of T cells in inducing IgG antibodies and protection using knockout mutant mouse models. M2e5x VLP vaccination induced higher levels of IgG antibodies and protective efficacy in BALB/c mice than that in C57BL/6 mice. The efficacy of cross protection by M2e5x VLP was higher than that by H5 VLP but lower compared to homologous protection by H3N2i immunization. M2e5x VLP vaccinated mice that were protected against primary challenge with H3N2 virus have developed future immunity to secondary infection against H1N1 virus, which was not induced in H3N2i immunized mice. M2e5x VLP and H5 VLP vaccines were able to raise substantial amounts of isotype-switched IgG antibodies in CD4 KO mice although antibody levels were lower than those in CD8 KO and C57BL/6 mice. In addition to immune sera containing M2e specific antibodies, both CD4 and CD8 T cells were found to play roles in clearing lung viral loads and in better recovery after M2e5x VLP vaccination and virus challenge.

Efficacy studies in inbred mouse strains might not be predictive in genetically diverse human populations. A previous study reported that no IgG antibodies specific for M2e were induced in C57BL/6 mice that were primed with M2 DNA and boosted with M2 recombinant adenovirus.[71] Also, antibody responses and protection to HA DNA vaccination were reported to be lower in

C57BL/6 mice than those in BALB/c mice.[72] In this study, M2e5x VLP raised similar or lower levels of IgG, IgG2c, and IgG2b isotype antibodies in C57BL/6 mice compared to those in BALB/c mice. Whereas, H5 VLP and H3N2i vaccines developed similar levels of IgG isotype antibodies in C57BL/6 and BALB/c mice. M2e5x VLP and H5 VLP vaccines were immunogenic and able to induce IgG isotype antibodies even in CD4 KO mice, suggesting an alternative pathway of inducing CD4-independent IgG antibodies. H3N2i vaccine appears to require CD4 T helper cells for effective induction of IgG antibodies. Presenting HA proteins on the VLP platform was shown to induce Th1 type antibody responses and enhanced protection compared to soluble form HA protein[56] or split influenza vaccines[73]. In line with these results, H1 VLP was more effective in developing IgG antibodies in CD4 KO mice compared to the same H1N1 strain inactivated split influenza virus vaccine (data not shown). VLP itself appears to grant immunogenic properties to the antigens on it. VLP vaccines were reported to stimulate dendritic cells in vitro and in vivo, and to produce inflammatory cytokines.[74] In addition, VLP-loaded dendritic cells stimulated the induction of T cell responses in vitro.[75] These unique properties of VLP vaccines likely attribute to inducing Th1 type IgG antibodies in BLAB/c and C57BL/6 mice as well as in CD4 KO and CD8 KO mice.

Despite the result that M2e5x VLP had immunogenic properties of inducing IgG antibodies, the protective efficacy varied in genetically different strains and mutant mice. M2e5x VLP showed higher cross protection in BALB/c mice than that in C57BL/6 mice. The efficacy of M2e5xVLP in lowering lung viral replication in CD4 KO and CD8 KO mice was even lower compared to that in C57BL/6 mice. Although H3N2i vaccination induced protection preventing weight loss against homologous primary virus even in CD4 KO mice, H3N2i immune mice were not protective during heterosubtypic secondary virus infection at a later time despite significant viral replication during

the first infection. In contrast, infection permissive M2e5x VLP vaccination after primary challenge developed sufficient immunity in BALB/c mice against secondary heterosubtypic virus, consistent with previous studies in BALB/c mice.[53,66,76] C57BL/6 mice with M2e5x VLP showed lower efficacy of secondary immunity than BALB/c mice, but significantly higher efficacy than naïve C57BL/6 mice with high viral loads during primary infection. High viral replication during primary infection accompanying severe weight loss in naïve C57BL/6 mice was not effective in inducing immunity against secondary heterosubtypic virus infection. This aspect provides evidence of beneficial effects on developing future immunity by inducing cross protective M2e immunity as shown in M2e5x VLP-immunized C57BL/6 mice. The efficacy of future immunity against secondary virus infection was diminished in CD8 KO mice, suggesting that both CD8 T cells and M2e antibodies might be major immune correlates contributing to protection against heterosubtypic virus infection. In addition, this study provides evidence that high viral replication in naïve C57BL/6 mice would not be sufficient for developing immunity against heterosubtypic virus probably due to less efficacy of inducing Granzyme B secreting CD8 T cell responses in C57BL/6 mice compared to those in BALB/c mice.

Several mechanisms have been reported for protection by M2e-immunity. Immune sera containing M2e antibodies regardless of strains of mice conferred similar levels of protection in naïve mice. Thus, M2e antibodies induced in C57BL/6 and CD4 KO mice may have similar capability of protection. Since lung viral loads were higher in CD4 KO and CD8 KO mice than those in C57BL/6 mice in the M2e5x VLP group, it is possible that both CD4 and CD8 T cells play a role of effector functions in lowering viral loads.[77] Alternatively, CD4 T cells may help to sustain the cytotoxic T cell response. Other mechanisms for M2e immune mediate protection include Fc receptor (FcR)[57,68,78], FcR-mediated opsonophagocytosis by macrophages[39], and

natural killer cells [35]. It appears that FcR is a key mediator for conferring cross protection by M2e antibodies. In addition, this study highlights the impacts of host genetic backgrounds and T cell responses in cross protection.

4.4   Conclusion

This study demonstrated that different levels of immune responses are developed depending on the genetic background of the host. M2e5x VLP induced strong cross protective immunity compared to other types of vaccines including H5 VLP and inactivated whole virus and showed unique patterns of immune responses in different wild type mouse strains. BALB/c mice developed higher humoral and cellular responses than C57BL/6 mice by M2e5x VLP, supporting its strong vaccine efficacy in the BALB/c strain. This strain dependent protective efficacy was mediated by different levels of T cell responses by both CD4 and CD8$^+$ T cells. Thus, the results suggest that M2e5x VLP immunization confers better cross protection than HA based vaccines such as H5 VLP and inactivated whole virus, and its efficacy is determined by T cell responses which vary depending on the genetic backgrounds of hosts.

5 CHAPTER 3. COMPLEMENT C3 PLAYS A KEY ROLE IN INDUCING HUMORAL AND CELLULAR IMMUNE RESPONSES AND PROTECTION BY INFLUENZA VIRUS M2 EXTRACELLULAR DOMAIN-BASED VACCINE.

5.1 Summary

The complement pathway is involved in eliminating antigen immune complexes. However, the role of C3 complement system remains largely unknown in influenza virus M2 extracellular (M2e) or hemagglutinin (HA) immune-mediated protection. We found that complement protein C3 was required for effective induction of immune responses to influenza virus M2e or HA vaccination and influenza virus infection, which include M2e-specific isotype-switched antibodies and effector CD4 and CD8 T cells. C3 knockout (C3 KO) mice after active immunization with M2e-based vaccine were not protected although low levels of M2e specific antibodies protective after passive administration with virus. Whereas, C3 KO mice that were immunized with HA-based vaccine were protected against virus challenge. In naïve condition, C3 KO mice showed impaired cellular phenotypes of innate immune cells such as macrophages, monocytes, and dendritic cells. The findings in this study suggest that C3 is required for effective induction of adaptive immune responses after influenza vaccination.

5.2   Results

5.2.1   Induction of IgG antibodies to viral infection is impaired in C3 deficient mice.

To determine the antibody phenotypes as a result of C3 deficiency in naïve mice, we compared natural antibody levels between wild type (WT, C57BL/6) and C3 knock-out (KO) mice (Fig. 5.1A). There were no differences in IgM and IgG isotypes (IgG1, IgG2c, IgG2b) in sera from WT and C3 KO mice in the absence of antigen exposure, indicating no defect in maintaining antigen non-specific natural antibodies in C3 KO mice. However, after virus infection with A/Philippines/2/1982 H3N2 (A/Phil H3N2), C3 KO mice rarely induced IgG antibodies at day 7 post-infection (Fig. 5.1D-G). At a later time-point after viral infection, C3 KO mice could induce moderate levels of H3N2 virus specific IgG antibodies, which were significantly lower than those in WT mice (Fig. 5.1H-K). In line with a defect in inducing virus specific antibodies, C3 KO mice were found to be more susceptible to influenza virus infection compared to WT mice (Fig. 5.1B and C). The sub-lethal dose (1.5 LD_{50}) of A/Phil H3N2 virus for WT mice lead to the 100% lethality in C3 KO mice and a 10-fold lower dose (0.15 LD_{50}) induced a comparable level of weight loss in C3 KO mice (Fig. 5.1B and C). These results suggest that C3 plays a critical role in inducing IgG antibodies and conferring protection in response to viral infection.
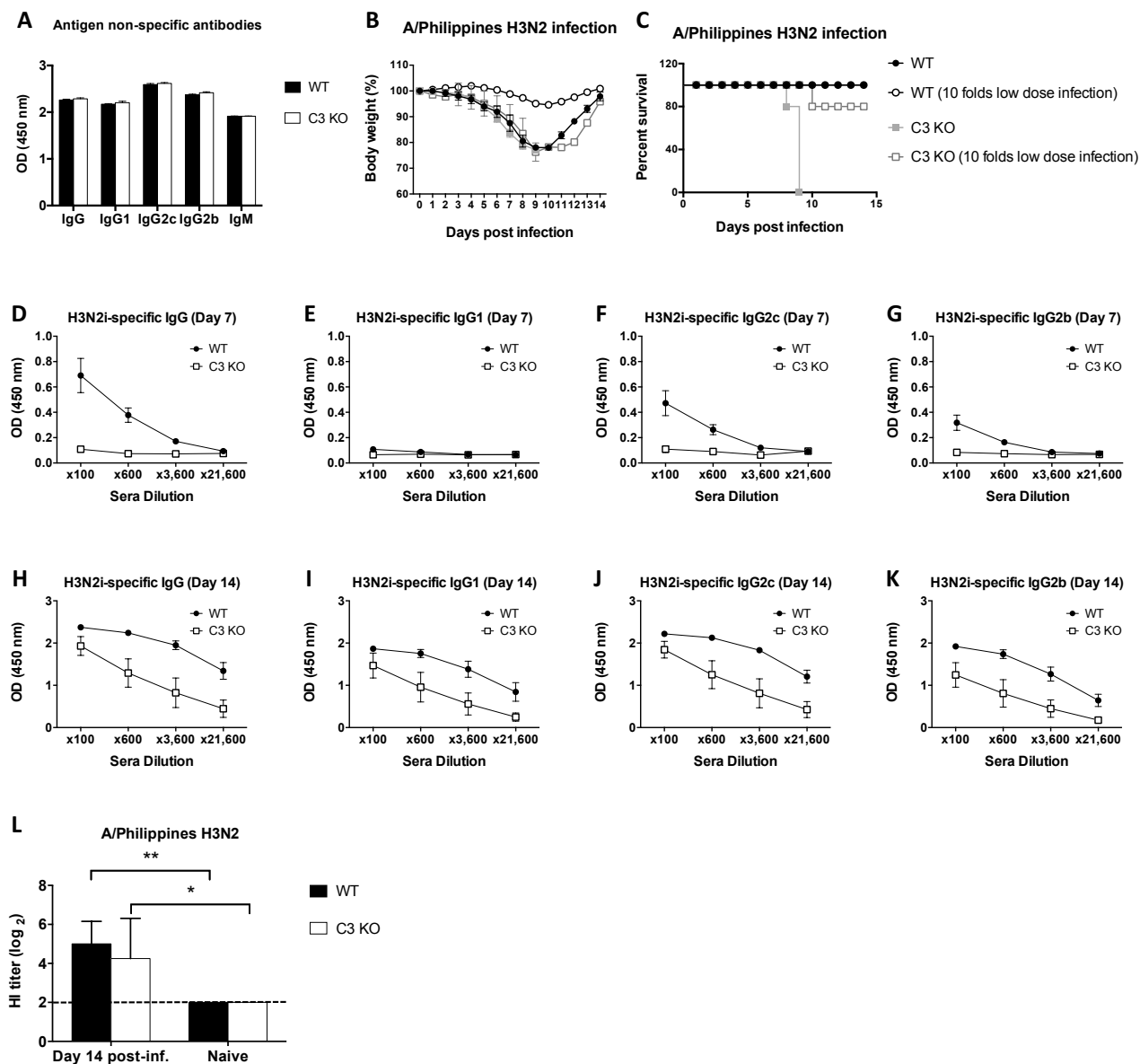
Figure 5.1 C3 KO mice show higher susceptibility to influenza virus infection than WT mice and have defects in virus-specific IgG production after challenge infection.
(A) Total serum IgM, IgG, and isotype-switched IgG antibodies were measured in naïve WT and C3 KO mice. Total natural antibodies were captured using goat anti-mouse IgM, IgG, and isotype-switched IgG antibodies. Each group of mice (n=5) were challenged with 1.5 LD_{50} of influenza virus (A/Philippines/2/82 H3N2). Body weight changes (B) and survival rates (C) were monitored at 14 days after infection. IgG, IgG1, IgG2c, and IgG2b were detected by sera ELISA at 7 days after infection (D-G) and at 14 days after infection (H-K). Sera were serially diluted and ELISA were performed by using the whole inactivated virus (H3N2i), which is the same with challenge virus. Error bars indicates mean ± SEM. L. HI titers against A/Philippines H3N2 were determined from in sera at day 14 post infected. Dotted line indicates the detection limit. Statistical significance was calculated by 2 way ANOVA and a Bonferroni's multiple comparison test. $*p<0.05$, $**p<0.01$ as indicated among the groups.

5.2.2 C3 deficient mice are less effective in inducing M2e-specific IgG responses after M2e5x VLP immunization.

To evaluate the role of C3 in mediating non-neutralizing M2e immunity, we compared IgG antibody levels induced in WT and C3 KO mice after M2e5x VLP vaccination (Fig. 5.2). Groups of mice in WT and C3 KO mice were intramuscularly immunized with 10 μg of M2e5x VLP by a prime boost strategy. At 2 weeks after prime (Fig. 5.2A-D) and boost (Fig. 5.2E-H) immunization, IgG isotype antibodies specific for M2e were measured by ELISA.

Overall, M2e5x VLP-immunized WT mice showed high levels of M2e-specific IgG1 and IgG2c, and IgG2b antibodies both after prime (Fig. 5.2A-D) and boost (Fig. 5.2F-H) immunization. However, the levels of M2e-specific IgG isotypes were significantly lower in C3 KO mice than those in WT mice after prime and boost (Fig. 5.2A-H). These results indicate that C3 protein is important for the production of antigen-specific IgG antibodies after M2e5x VLP vaccination.
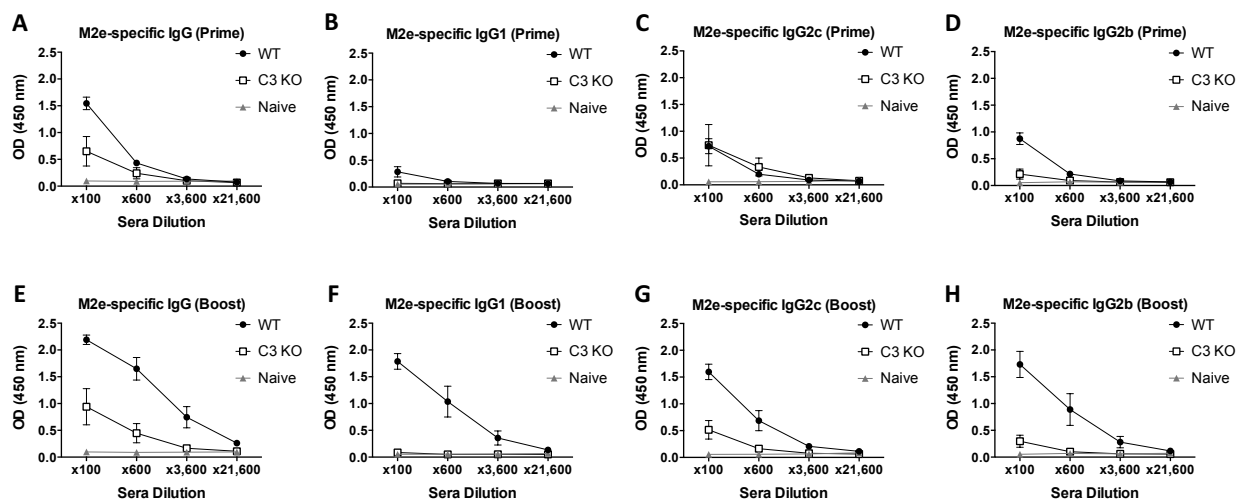
Figure 5.2 C3 KO mice have defects in M2e-specific IgG production.
Each group of mice (n=5) were boost immunized with 10μg of M2e5x VLP at 3 weeks interval. IgG (A), IgG1 (B), IgG2c (C), and IgG2b (D) from prime immune sera and IgG (E), IgG1 (F), IgG2c (G), and IgG2b (H) from boost immune sera were detected by sera ELISA. Sera were serially diluted and ELISA were performed by using vaccine specific antigen, M2e. Error bars indicates mean ± SEM.

5.2.3   M2e5x VLP immune C3 deficient mice are not protected after challenge.

To determine whether the complement system has a role in M2e-mediated cross-protection, the groups of WT and C3 KO mice were prime and boost immunized with M2e5x VLP at an interval of 3 weeks and followed by challenge infection with sub-lethal dose of A/Phil H3N2 virus 3 weeks later (Fig. 5.3). C3 KO mice showed approximately 10-fold higher susceptibility to influenza virus infection, indicating a role of C3 in innate immunity (Fig. 5.1). Thus, we used a 10-fold lower dose to infect C3 KO groups than that of WT groups in order to evaluate the protective efficacy of the vaccine. As expected, WT mice immunized with M2e5x VLP showed a moderate weight loss (10-12%) (Fig. 5.3A and B). Whereas, influenza virus infection resulted in significant weight loss in C3 KO mice immunized with M2e5x VLP, displaying no difference between the immunized and naïve C3 mouse groups (Fig. 5.3C and D). Protective efficacy was further confirmed by lung viral titers at day7 post infection. M2e5x VLP vaccination of WT mice conferred 100-fold lower lung viral titers than the naïve control, but this protection of viral clearance was not observed in vaccinated C3 KO mice (Fig. 5.3E). These results suggest that C3 is an essential immune component in M2e-mediated protection.

A WT - A/Philipplines H3N2 infection

B WT - A/Philipplines H3N2 infection

C C3 KO - A/Philippines H3N2 infection

D C3 KO - A/Philippines H3N2 infection

E Viral Titer (Phil H3N2)
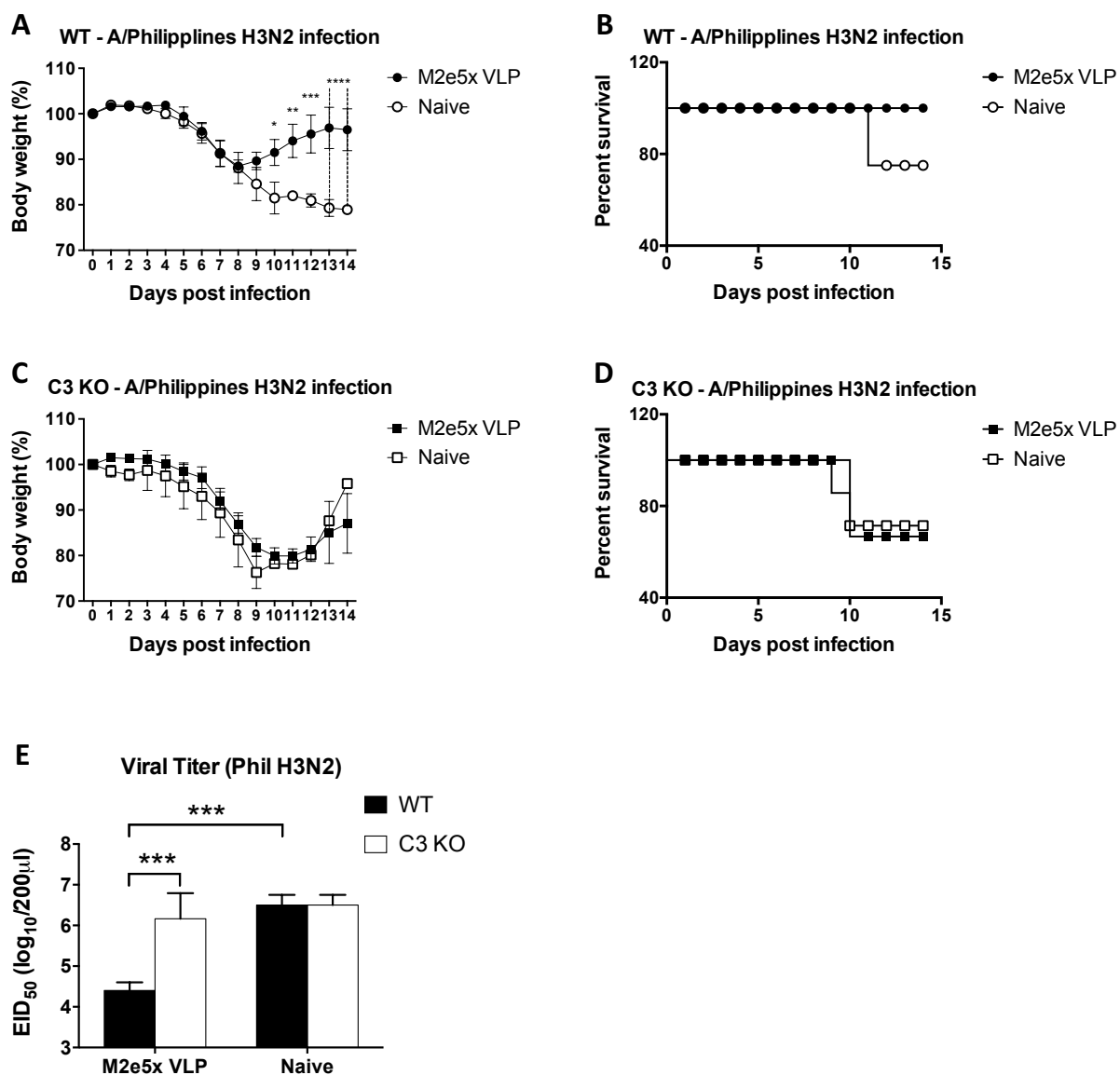
Figure 5.3 Vaccination with M2e5x VLP does not protect C3 KO mice against heterosubtypic virus infection. Each group of mice (n=5) were boost immunized with 10 μg of M2e5x VLP. At 4 weeks after boost immunization, mice were challenged with 1.5 LD_{50} of influenza virus (A/Philippines/2/82 H3N2). Body weight changes (A & C) and survival rates (B & D) were monitored for 14 days. Lung viral titers were determined by the egg inoculation assay at 7 days after challenge. Statistical significance was calculated by 2 way ANOVA and a Bonferroni's multiple comparison test. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$ as indicated among the groups.

5.2.4   C3 KO mice display a defect in generating antibody-secreting and germinal center phenotypic cells.

Developing long-lived antibody-secreting cell responses is important for providing long-term protection. We determined whether C3 would play a role in generating long-lived plasma cells and germinal center phenotypic B cells. *In vitro* antibody production was measured from cultured cells of bone marrow and spleens collected from immune mice at 7 days after influenza virus challenge (Fig. 5.4). As previously reported, both bone marrow cells and splenocytes from the WT group immunized with M2e5x VLP secreted high levels of M2e-specific IgG antibodies, indicating effective development of M2e-specific antibody secreting cells (Fig. 5.4A and B). However, C3 KO mice showed significantly lower production of M2e-specific IgG antibodies compared to WT mice in both bone marrow cells and splenocytes (Fig. 5.4A and B). In addition, C3 deficiency resulted in lower levels of germinal center phenotypic (GL7^{+}) B cells in spleens (Fig. 5.4C and D). These results suggest that C3 plays a key role in the development of antigen specific antibody-secreting cells and germinal center phenotypic B cells.
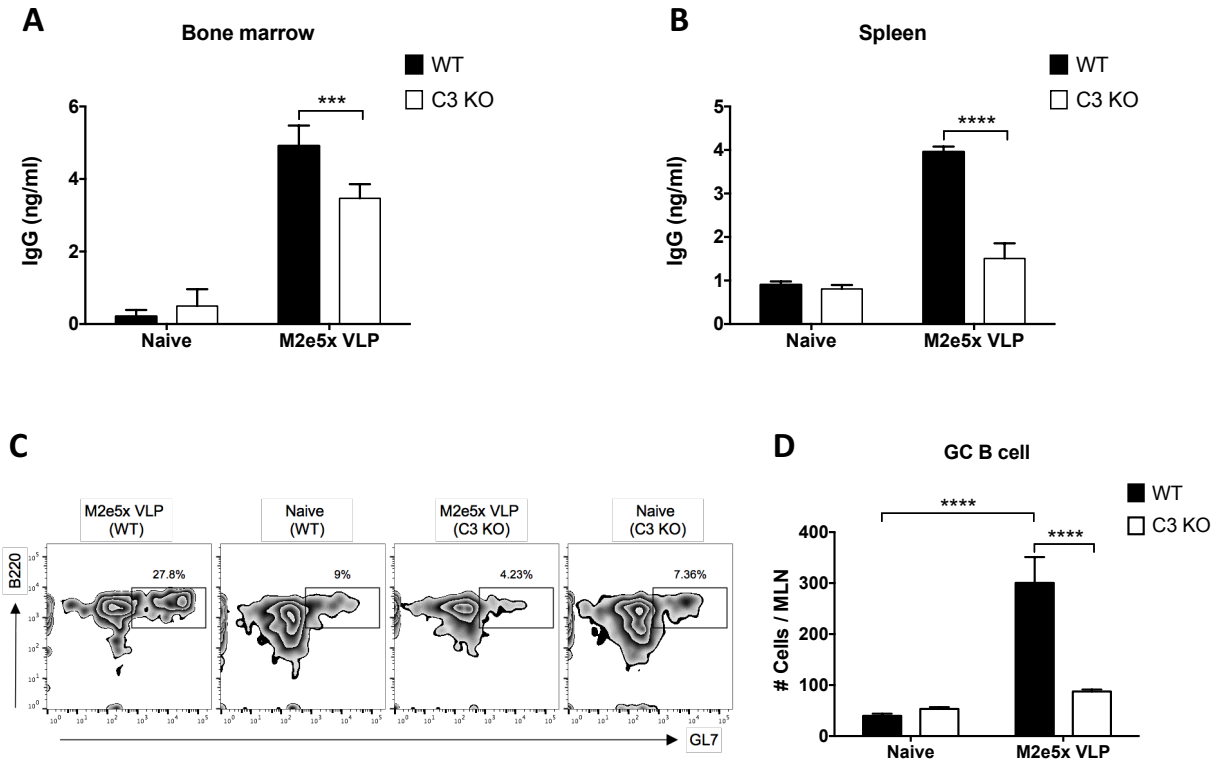
Figure 5.4 Antibody secreting cell responses

(A) IgG production from bone marrow cells of immunized WT and C3 KO mice. (B) IgG production from spleen cells of immunized WT and C3 KO mice. Bone marrow and spleen cells were harvested on day 7 postinfection after 2 months of vaccination. Cells were cultured for 1 day in the presence of M2e peptide antigen. IgG levels were detected by ELISA. (C & D) Germinal center B cells in MLN. MLN cells were harvested, stained with B220, GL7, IgD, and CD19, and analysed by flow cytometry at 7 days after infection. Statistical significance was calculated by 2 way ANOVA and a Bonferroni's multiple comparison test. ***$p<0.001$, ****$p<0.0001$ as indicated among the groups.

5.2.5   C3 is required to develop effective M2e-specific cellular immune responses.

It has been known that T cell responses contribute to broadening cross protective immunity in M2-mediated immune responses.[43,79] Thus, we determined whether C3 would be required for developing M2e-specific T cell immunity. Lung cells and splenocytes were collected at 7 days after challenge and cultured *in vitro*. After stimulation with an M2e peptide, cytokine producing cell spots were counted as an indicator of T cell responses (Fig. 5.5). M2e5x VLP immunized group showed over 7-fold higher levels of IFN-γ and 5-fold higher levels of IL-4 secreting lung cells compared to naïve control group in WT mice as previous reported (Fig. 5.5A and B). However, C3 KO mice failed to develop cytokine producing T cells, showing no difference between M2e5x VLP immunized and naïve groups (Fig. 5.5A and B). A similar pattern was observed in splenocyte cultures showing high levels of IFN-γ and IL-4 secreting lung cells and splenocytes only in WT mice immunized with M2e5x VLP, but not in C3 KO mice (Fig. 5.5C and D).

To investigate further details of M2e-specific T cell phenotypes using intracellular cytokine staining and flow cytometry assays (Fig. 5.6), lung cells and splenocytes were harvested from WT and C3 KO mice previously immunized with M2e5x VLP at 7 days after virus infection. M2e5x VLP immunized WT mice showed significantly high numbers of IFN-γ secreting CD4 and CD8$^+$ T cells in lungs (Fig. 5.6A and B) and spleens (Fig. 5.6C and D). These results suggest that C3 is required to develop effective M2e-specific T cell responses.
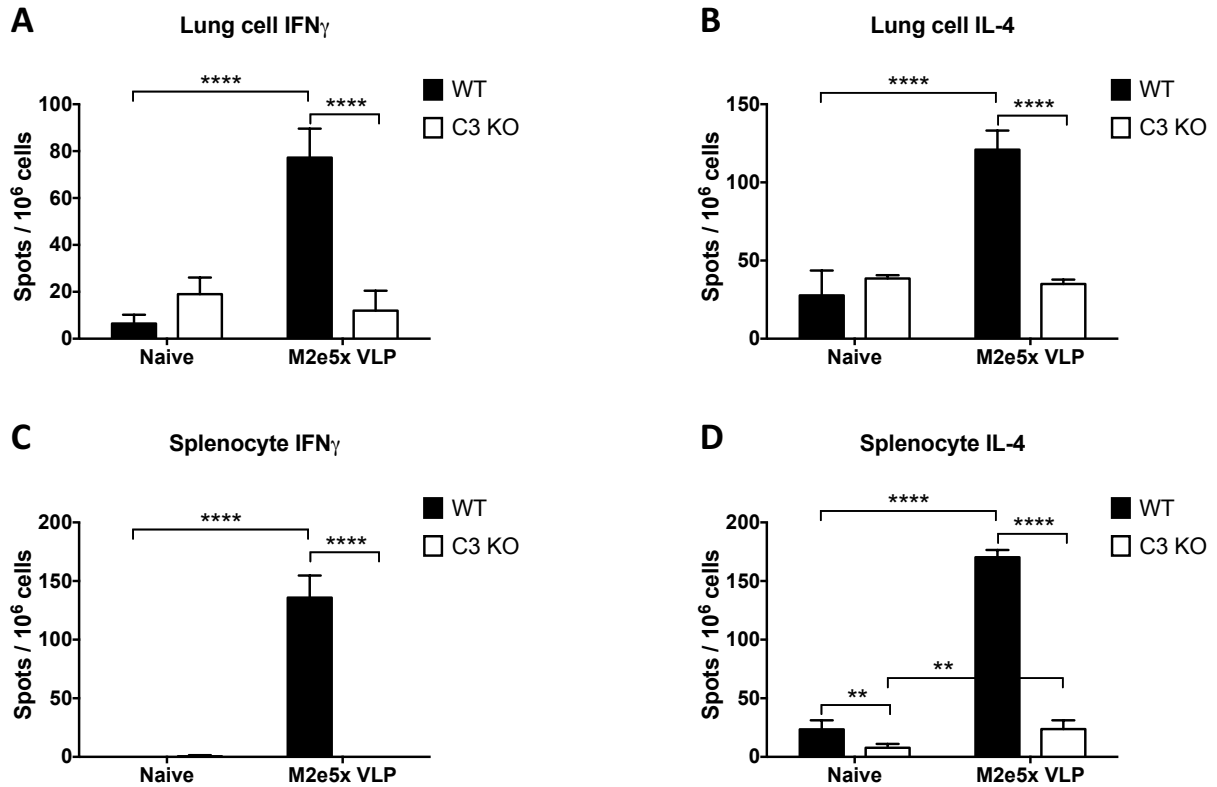
Figure 5.5 M2e-specific cellular immune responses were reduced in C3 KO mice.
(A) IFN-γ-secreting cells in lung cells. (B) IL-4-secreting cells in lung cells. (C) IFN-γ-secreting cells in splenocytes. (D) IL-4-secreting cells in splenocytes. Lung cells and splenocytes were isolated from WT(C57BL/6) and C3 KO mice previously immunized with M2e5x VLP at 7 days post challenge with A/Philippines/2/82 H3N2. Cytokine-producing cell spots were counted by ELISPOT reader. Statistical significance was calculated by 2 way ANOVA and a Bonferroni's multiple comparison test. **$p<0.01$, ****$p<0.0001$ as indicated among the groups.
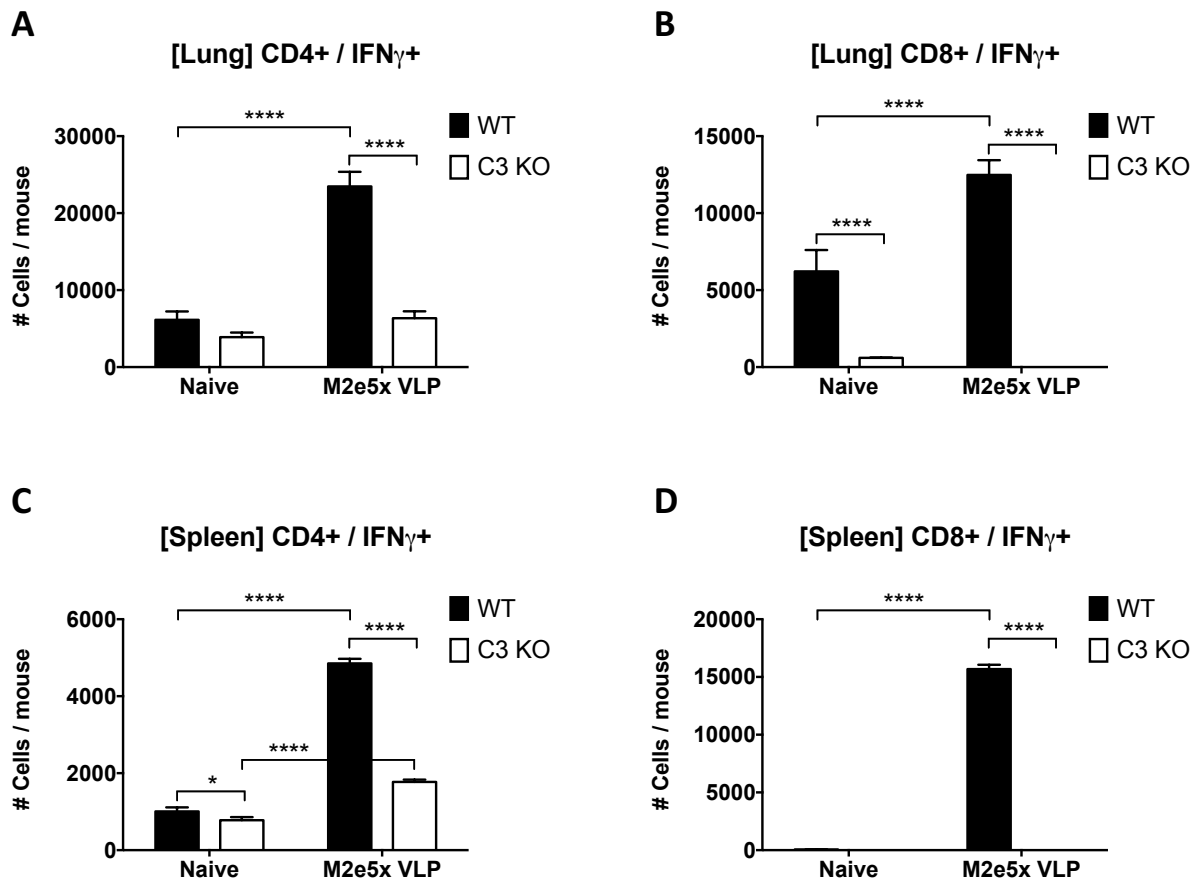
Figure 5.6 M2e-specific interferon-γ (IFN-γ)-secreting CD4+ and CD8+ T cells were significantly decreased in C3 KO mice by M2e5x VLP vaccination.
(A) IFN-γ-secreting CD4+ T cells in lungs. (B) IFN-γ-secreting CD8+ T cells in lungs. (C) IFN-γ-secreting CD4+ T cells in spleens. (D) IFN-γ-secreting CD8+ T cells in spleens. Lung and spleen cells were isolated from WT(C57BL/6) and C3 KO mice previously immunized with M2e5x VLP at 7days post challenge with A/Philippines/2/82 H3N2. Isolated cells were stained with CD45, CD4, CD8α and IFNγ antibodies, and analysed by flow cytometry. Statistical significance was calculated by 2 way ANOVA and a Bonferroni's multiple comparison test. **$p<0.01$, ****$p<0.0001$ as indicated among the groups.

5.2.6   *M2e-specific antibodies induced in C3 KO mice are protective.*

Since M2e5x VLP immunized C3 KO mice were not protected (Fig 5.3), we determined whether M2e-specific antibodies induced in C3 KO mice would have protective capability. Naïve WT mice (BALB/c) were infected with a mixture of heat inactivated M2e5x VLP immune sera and a lethal dose of H3N2 influenza virus and body weights were monitored for 14 days (Fig. 5.7). Naive mice that received WT mouse and C3 KO mouse M2e immune sera together with H3N2 virus showed 19 % and 24 % weight loss respectively and then recovered normal weight. A lower level of M2e specific antibodies in C3 KO mouse immune sera appears to contribute to more weight loss. Naïve mice that received naïve sera and virus did not survive, suggesting that M2e-specific antibodies induced in C3 KO mice have protective capacity.
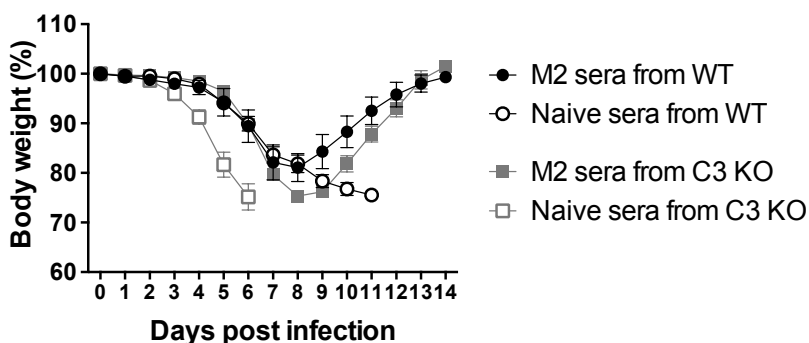
Figure 5.7 C3 KO mice do not have a defect in cross-protective antibody production.
Immune sera collected from M2e5x VLP immunized WT(C57BL/6) and C3 KO mice were incubated with influenza virus (A/Philippines/2/82 H3N2). BALB/c mice were intranasally infected with lethal dose of influenza virus mixed with immune sera or naïve sera. Body weights were monitored for 14 days.

5.2.7 HA VLP vaccinated C3 KO mice induce HA inhibiting antibodies and protection.

Since C3 KO mice with M2e5x VLP could not confer protection, we determined whether HA (A/Indonesia/05/2005) VLP vaccines would induce protection in C3 KO mice. Groups of WT and C3 KO mice were intramuscularly immunized with 10 μg of H5 HA VLP. H5 HA VLP-immunized WT mice showed higher levels of virus-specific IgG1 and IgG2c, and IgG2b antibodies both after prime (Fig. 5.8A-D) and boost (Fig. 5.8E-H) immunization. Although C3 KO mice induced lower amounts of virus-specific IgG isotypes than those in WT mice after H5 HA VLP vaccination, IgG antibody levels were substantially high in C3 KO mice (Fig. 5.8A-H). Consistent with the levels of binding IgG antibodies, immune sera from C3 KO mice showed significant levels of HI titers in a range of 128 to 256 although these levels of HI titers in C3 KO mice were lower than those in WT immune sera (Fig. 5.8K). C3 KO mice immunized with H5 VLP were protected against 1.5 LD_{50} homologous rgH5N1 influenza virus (A/Indonesia/05/2005) without displaying weight loss (Fig. 5.8I and J). These results suggest that neutralizing antibody-mediated protection is independent of C3 roles.
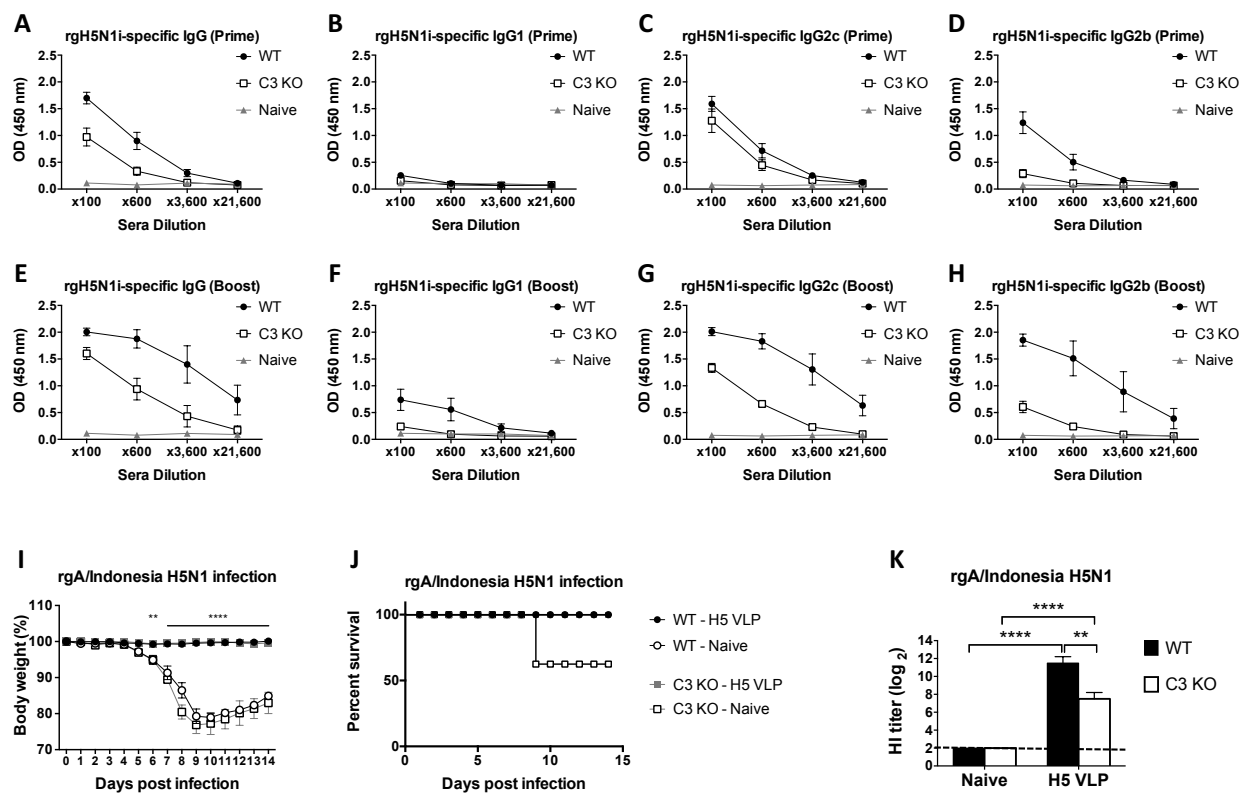
Figure 5.8 C3 KO mice have defects in HA-specific IgG production, but not in HA-mediated homologous protection.
Each group of mice (n=5) were boost immunized with 10μg of H5 VLP. IgG (A), IgG1 (B), IgG2c (C), and IgG2b (D) from prime immune sera and IgG (E), IgG1 (F), IgG2c (G), and IgG2b (H) from boost immune sera were detected by sera ELISA. Sera were serially diluted and ELISA were performed by using vaccine specific antigen, inactivated A/Indonesia (rgH5N1i). Error bars indicates mean ± SEM. Each group of mice (n=5), which were previously boost immunized with 10μg of H5 VLP, were challenged with lethal dose of influenza virus (rgA/Indonesia H5N1). Body weight changes (I) and survival rates (J) were monitored for 14 days. (K) HI titers against rgH5N1 were determined from immune sera of H5 VLP. Dotted line indicates the detection limit. Statistical significance was calculated by 2 way ANOVA and a Bonferroni's multiple comparison test. **$p<0.01$, ****$p<0.0001$ as indicated among the groups.
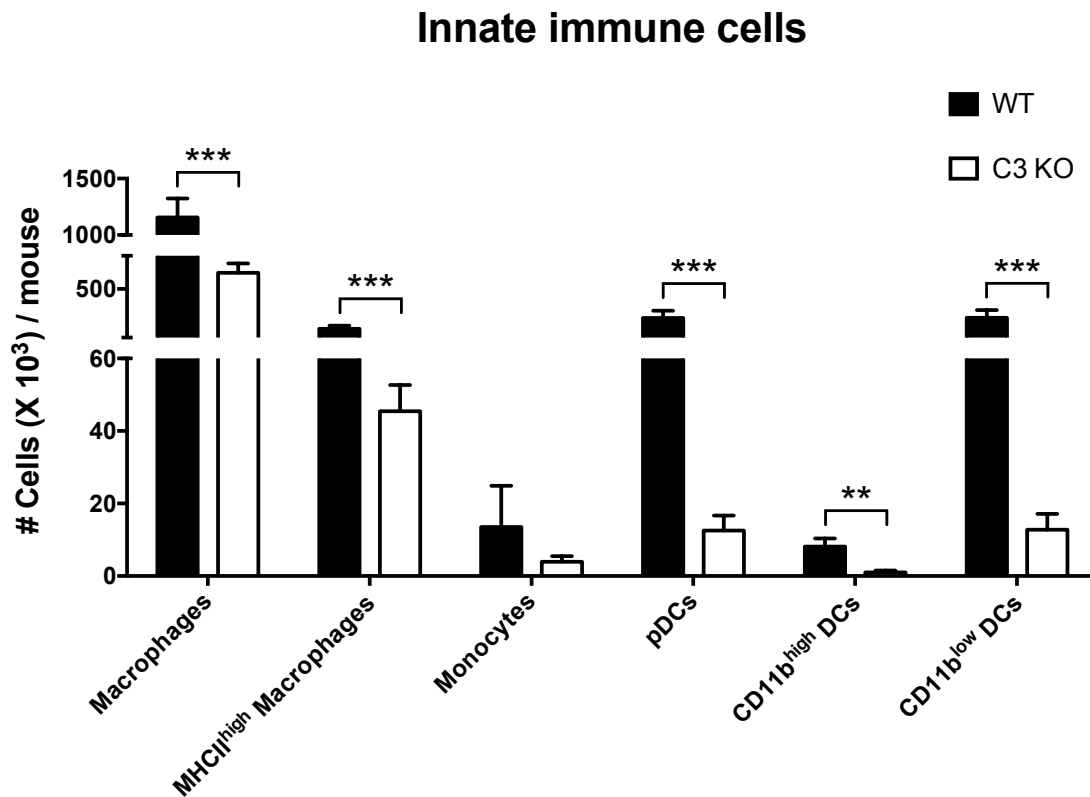
Supplementary Figure 5.9 Cellular phenotypes in WT and C3 KO mice.
Cellularity of different phenotypic cells in peritoneal exudates from WT and C3 KO mice (n=5). Cells in peritoneal exudates were harvested and their phenotypes and cellularity were determined. Macrophages are $CD11b^+$ $F4/80^+$. MHC-II^{high} macrophages are $CD11b^+$ $F4/80^+$ MHC-II^{high}. Monocytes are $CD11b^+$ $Ly6c^{high}$ $F4/80^+$. pDCs are $CD11c^+$ $B220^+$ MHC-II^{high}. CD11b^{high} DCs are $CD11c^+$ $CD11b^{high}$ MHC-II^{high}. CD11b^{low} DCs are $CD11c^+$ $CD11b^{low}$ MHC-II^{high}. Statistical significance was calculated by 2 way ANOVA and a Bonferroni's multiple comparison test. **$p<0.01$ and ***$p<0.001$ as indicated among the groups.

5.3   Discussion

The complement system has been considered to play an essential role in the control of influenza virus infections, including recognition and elimination of virus particles.[41,42,80] It is able to mediate directly viral clearance including neutralization, opsonisation, lysis, and phagocytosis via complement receptors.[81] Moreover, the complement system has been known to modulate the adaptive immune responses via multiple mechanisms.[49] In the present study, we described that the complement system has a critical role in conferring M2e-mediated cross protection through the development of M2e-specific humoral and cellular immune responses.

First, our results explained that complement C3 is essential for inducing M2e-specific IgG isotypes and B cell immunity. Immunization with M2e5x VLP in C3 KO mice failed to fully elicit M2e-specific IgG isotype antibodies (Fig. 5.2) and IgG memory response (Fig. 5.4) compared to wild type control mice. In addition, virus-specific IgG levels were significantly low in C3 KO mice (Fig. 5.1A) even though natural antibody levels were normal in C3 KO mice (Fig. 5.1D). These results suggest that complement C3 has an important role in the formation of humoral memory responses and antibody isotype switching in M2e-mediated immunity.

The role of the complement system in influenza M2e-mediated immunity remains controversial. Previously, passive transfer approaches of M2e-specific antibodies have demonstrated controversial results which explain the role of complement C3. Jegerlehner et al. showed that C3 KO mice passively immunized with M2-HBc immune serum were survived at a similar level with those of WT mice, indicating that complement C3 is not required for M2e antibody-mediated cross protection.[35] In contrast, Wang et al. explained that complement C3 is essential in lung viral clearance of infected mice by passive transfer of anti-M2e monoclonal antibodies.[38] However, it has not been well understood how complement C3 functions in the active

immunization condition of the M2e vaccine. Based on our data, M2e5x VLP immunized C3 KO mice induced moderate levels of IgG isotype antibodies even though those were significantly lower than WT mice. In vivo protection test showed no defect in the cross protective ability of M2e-specific antibody itself generated from C3 KO mice (Fig. 5.7). Although M2e-immune sera from C3 KO mice contain lower amount of M2e-specific antibodies than those from WT mice (Fig. 5.2), immune sera-mediated protection was not affected. A possible explanation is that the amount of M2e-specific antibodies induced in C3 KO mice is enough to confer the protection mediated by direct interaction between antibodies and influenza virus particles. This is supported by our additional data that C3 deficiency does not affect the neutralizing antibody-mediated homologous protection even though the antibody level was lower than in WT condition (Fig. 5.8). Based on these results, we concluded that the more amount of antibodies does not always confer additional effects in the antibody-mediated protection.

The failure to produce the normal amount of M2e-specific antibodies in the C3 deficient condition is able to be explained by reduced number of GC B cells (Fig. 5.4C and D). Two possible mechanisms might be able to explain these failures in M2e-specific IgG induction and GC B cell response in the C3 deficient condition; (1) impaired helper CD4$^+$ T cell priming or (2) abnormal functions of antigen presenting cells. Previous report has demonstrated that C3 deficiency might cause inefficient delivery of viral antigens to professional APCs, leading to impaired priming of influenza virus specific CD4$^+$ helper T cells.[52] Moreover, it was reported that impaired T cell priming by C3 deficiency is linked to the defect in DC migration from the lung to the draining lymph nodes.[82]

In addition to T cell priming, our results demonstrated that complement C3 is necessary for both recruitment and effector function of T cells. The frequency and number of M2e-specific IFN-

γ-producing CD4$^+$ and CD8 T$^+$ cells were reduced at the site of infection in C3 KO mice (Fig. 5.6A and B) as well as spleen (Fig. 5.6C and D). Based on these data, we concluded that complement C3 regulates recruitment into the lungs and antiviral effector functions of M2e-specific both CD4$^+$ and CD8$^+$ T cells. This may expand the precious finding that T cell depletion causes reduced cross protection by M2e targeting vaccine.[43,79]

We additionally investigated how innate immune cells are affected in the complement C3 deficient condition. Our data demonstrated that C3 deficiency leads to significant reductions in innate immune cells in the basal level, including macrophages and major subsets of dendritic cells. This impaired innate immune system by C3 deficiency may cause inefficient delivery of vaccine antigens to antigen presenting cells (APCs) and attenuate T cell priming.

Reduced antigen-specific IgG production in the C3 deficient condition was also observed in a different type of antigens such as live influenza virus and H5 HA VLP vaccine. Homologous protection by the HA targeting vaccine was not affected by the absence of complement C3. Immunized C3 KO mice with H5 HA VLP vaccine did not show weight loss after homologous influenza virus infection. HA-specific neutralizing antibodies induce sterilizing immunity against strain-matched influenza viruses, leading to complete protection.[7] Reduced HI activity of antibodies from C3 KO mice correlates with lower amounts of antibodies compared to WT mice but these HI titers of 128 to 256 would be sufficient for homologous protection independent of C3 (Fig. 5.8K). We previously reported the similar finding in the Fc receptor deficient condition, demonstrating that Fc receptor KO mice immunized with an inactivated virus vaccine (A/PR/8/34 H1N1) showed the HI titer around 128 and good sterilizing homologous protection.[57]

It has been reported that C3 KO mice themselves are highly susceptible to viral infection.[52,83] We confirmed that C3 deficiency results in severe weight loss and rarely induced virus-specific

IgG antibodies after infection with the same dose of influenza virus between WT and C3 KO mice (Fig. 5.1). Natural antibody levels did not show any significant difference in C3 KO mice (Fig. 5.1D). However, C3 KO mice showed significant defects in basal levels of innate immune cells such as macrophages, monocytes, and dendritic cells in their peritoneal exudates (Supplementary Fig. 5.9). Thus, we concluded that high susceptibility of C3 KO mice to viral infection may be due to their defects in innate immune system.

In summary, our results demonstrate that complement protein C3 is required for effective development of adaptive immune responses to influenza virus M2e and HA VLP vaccination as well as virus infection. We found several defects which were caused by C3 deficiency, including: i) reduced M2e-specific IgG isotype antibodies, ii) impaired induction of effector $CD4^+$ and $CD8^+$ T cells, and iii) abnormal innate immune cell phenotypes. Taken together, the data support a hypothesis that complement C3 is important for both M2e-specific humoral and cellular immune responses to be fully induced, conferring effective cross protection against influenza virus infection.

5.4   Conclusion

This study indicated that complement protein C3 is essential in inducing immune responses to influenza virus infection and vaccination, which include M2e-specific isotype-switched antibody production and inducing M2e-specific effector CD4 and CD8 T cells. C3 knock-out (C3 KO) mice showed lower levels of M2e-specific IgG isotype antibodies after M2e vaccination, and no control of lung viral replication and no recovery from weight loss upon challenge infection compared to those in wild type (WT) mice. Whereas, C3 KO mice were protected against homologous virus after immunization with neutralizing antibody inducing hemagglutinin-based vaccine despite lower levels of antibodies than those in WT mice. Those results revealed the critical roles of C3 complement in inducing humoral and cellular immune responses to influenza virus infection and immunization with M2e or HA vaccines. C3 was found to be required for protection by M2e-based but not by HA-based active vaccination as well as for maintaining innate antigen presenting cells. Findings in this study have insight into better understanding the roles of C3 complement in inducing effective adaptive immunity and in non-neutralizing M2e immune mediated protection.

6 CONCLUSIONS

6.1 Cross protective influenza vaccines

Current influenza vaccines are effective in conferring protection against strain-matched influenza viruses when the HA antigenic strains is closely matched with vaccines.[13] This is due to the fact that genetic mutations of the hemagglutinin (HA) are frequently occurred, leading to viral escape from existing immunity.[5,84] Since the immune responses induced by current vaccines are narrow and limited in specific strains, an annual update is required which strains will be most likely circulating and targeted.[12,84] This annual process to produce seasonal influenza vaccines is time-consuming and costly.[5,13] In addition to the economic burden, current vaccines are not appropriate to confer protection against newly emerging pandemic influenza strains which have distinct HA antigenicity and appear at irregular intervals.[5,13] Therefore, several approaches have been tested to develop cross protective influenza vaccines which induce effective immunity to different subtypes of influenza virus strains.[5] For that, several viral antigens, which are highly conserved through most strains of influenza A viruses, have been considered as targets for cross protective influenza vaccines.[12,85] Among influenza viral proteins, multiple conserved targets have been evaluated as possible candidates for cross protective influenza vaccines, including HA stalk domain, the extracellular domain of M2 (M2e), nucleoprotein (NP), matrix protein 1 (M1) and two highly conserved sequences near the NA enzymatic site (Table 1).[12] In this study, three different influenza antigens, which include HA, NA, and M2e, were investigated as candidates for possible cross protective vaccine development (Figure 6.1).

Protein	Targeted site	Proposed mechanisms of protection
Hemagglutinin (HA)	Stem	Inhibition of fusion, maturation of the HA, and viral egress Antibody-dependent cell cytotoxicity (ADCC)
Matrix 2(M2)	Ectodomain of M2 (M2e)	Antibody-dependent cell cytotoxicity (ADCC) Complement-mediated lysis CD4+ or CD8+ T lymphocyte-mediated cytolysis
Nucleoprotein (NP)	T cell and antibody epitopes	Cell lysis by CD8+ cytotoxic T lymphocityes CD4+ T lymphocyte-mediated cytolysis and B cell stimulation
Matrix 1 (M1)	T cell epitopes	Cell lysis by CD8+ cytotoxic T lymphocityes
Neuraminidase (NA)	Conserved sialidase active site	Inhibition of viral spread Antibody-dependent cell cytotoxicity (ADCC)

Table 6.1 Viral targets of universal influenza vaccines.

6.2   Protection mechanisms of the antibody-mediated immunity

The strategy of current influenza vaccination is to induce neutralizing antibodies targeting highly changeable HA, which does not provide effective protection against antigenically mutated viruses and pandemics.[12,85] In the effort to overcome this limitation of current vaccines, new vaccine strategies have been investigated, targeting relatively more conserved viral antigens. Among possible viral antigens, the extracellular domain (M2e) of influenza virus M2 protein has been extensively studied as a vaccine target and utilized in various carrier vehicles and vaccine designs.[6,28,34,86] It has been reported that M2e-specific antibodies induced by M2e targeting vaccines protect hosts by improving the survival rates, reducing lung virus titers or body weight loss against heterosubtypic influenza A virus infection.[18,61,68,87] However, the levels of cross protection by M2e antibody itself are not sufficient, allowing moderate pathological symptoms.[5]

Epidemiologic studies indicated that anti-NA immunity and NA inhibitors prevent severe disease or death by influenza viral infection.[14] NA targeting antibodies do not have viral neutralizing activity, providing infection-permissive protection.[15] Studies have demonstrated that NA immunity induces a broad spectrum of cross protection within the same subtype.[16,17]

Nonetheless, contribution of NA antibodies to cross protection is not well understood yet in comparison with other viral surface antigens.

In the chapter 1, we investigated how NA immune responses contribute to cross protection by comparing with those induced by tandem repeat M2e virus-like particle (M2e5x VLP) and inactivated split virus (as HA) vaccines. NA antibodies were found to be more effective in conferring heterologous cross-protection than strain-specific HA antibodies. The contribution of NA immunity to protection appeared to be limited when compared to M2e immunity. Also, the roles Fc receptors and complement protein C3 in mediating protection by NA antibodies were investigated in mutant mouse models. Protective efficacy by NA-specific antibodies was lower in Fc receptor deficient mice but comparable in complement C3 deficient mice compared to that in the wild type mice. Thus, we concluded that Fc receptor plays a role in passively administered NA antibody-mediated protection but C3 is not required (Figure 6.1).

6.3   The role of the genetic background of hosts in the cross protective immunity

Previously, it has been reported that virus-like particle (VLP) vaccines presenting heterologous tandem repeat M2e (M2e5x VLP) were effective in inducing cross protection against different subtypes of influenza viruses in the absence of adjuvants.[34] Most of previous M2e-based vaccine studies have been carried out in BALB/c mice known to be a high responder.[65,71] No IgG antibodies and T cell responses specific for M2e were induced in C57BL/6 mice that were primed with M2 DNA and boosted with M2 recombinant adenovirus.[71] It is known that M2e5x VLP raises IgG antibodies but provides low efficacy of cross protection in C57BL/6 mice without adjuvants.[44]

Vaccines should be effective in genetically diverse populations. In the chapter 2, to compare different influenza vaccine platforms, we first determined the induction of IgG isotype antibodies

and efficacy of protection in BALB/c and C57BL/6 mice after vaccination with M2e5x VLP, H5 VLP containing H5 subtype HA, or whole inactivated H3N2 virus (H3N2i). Overall, BALB/c and C57BL/6 mice developed different patterns of IgG antibody responses depending on antigen types and vaccine platforms. Especially, M2e-specific IgG antibody levels were significantly higher in BALB/c than C57BL/6 mice, suggesting higher strain dependency of M2e immunity compared to HA based vaccines. In addition, we found that both CD4$^+$ and CD8$^+$ T cells have important roles in M2e immunity and BALB/c mice induce higher levels of M2e-specific T cell responses than C57BL/6 mice (Figure 6.1).

As a model of pandemic in the future, the impact of immunization with different vaccine platforms was analyzed after the primary challenge which was followed by the secondary challenge. We found that BALB/c mice develop stronger secondary heterosubtypic immunity against an antigenically different virus than C57BL/6 mice.

6.4   The cross-protection mechanism of the M2e based vaccine

Possibility of M2 as a cross protective vaccine candidate is based on the observation that passive transfer of an anti-M2 monoclonal antibody into mice significantly reduces influenza virus replication in the lung.[18] Since M2-specific antibodies do not have virus neutralizing activity, anti-M2 antibody-mediated immune protection has been considered to be connected with antibody-dependent cell cytotoxicity (ADCC) or complement-mediated lysis.[35-38] Innate immune cells such as macrophages, dendritic cells, neutrophils, and NK cells lead to lyse antibody-bound infected cells via interaction between Fc receptors (FcR) on their surface and antigens on the infected cells.[13,39,40] Based on these findings, FcR-mediated ADCC can be a possible mechanism of the M2e immunity. The role of NK cell-mediated response is still controversial in M2e immunity. It

has been reported that NK cell-mediated ADCC is important in M2e immunity.[35] However, an arguing study showed NK cell independency in the protection by anti-M2e antibodies.[86] Complement-mediated cell lysis is also a key mechanism for virus elimination.[41,42] In the case of M2e-mediated immunity, it is still controversial whether complement system is required for protection.[35,38] In addition, several studies have shown that M2e-specific T cells play a role in enhancing cross protection against influenza infection.[28,43,44]

In the chapter 3, we investigated the possible roles of C3 in inducing immune responses and protection after live influenza virus infection or immunization with M2e5x VLP or H5 HA VLP vaccines. C3 was found to play an important role in inducing immune responses to live virus infection or VLP vaccination (Figure 6.1). Moreover, we demonstrated a detailed protection mechanism of M2e immunity, suggesting the role of the complement system in developing both humoral and cellular immune responses by M2e vaccination (Figure 6.1). C3 was also found to play a critical role for non-neutralizing immune-mediated protection by M2e5x VLP but not for neutralizing immune-mediated protection by H5 HA VLP (Figure 6.1).
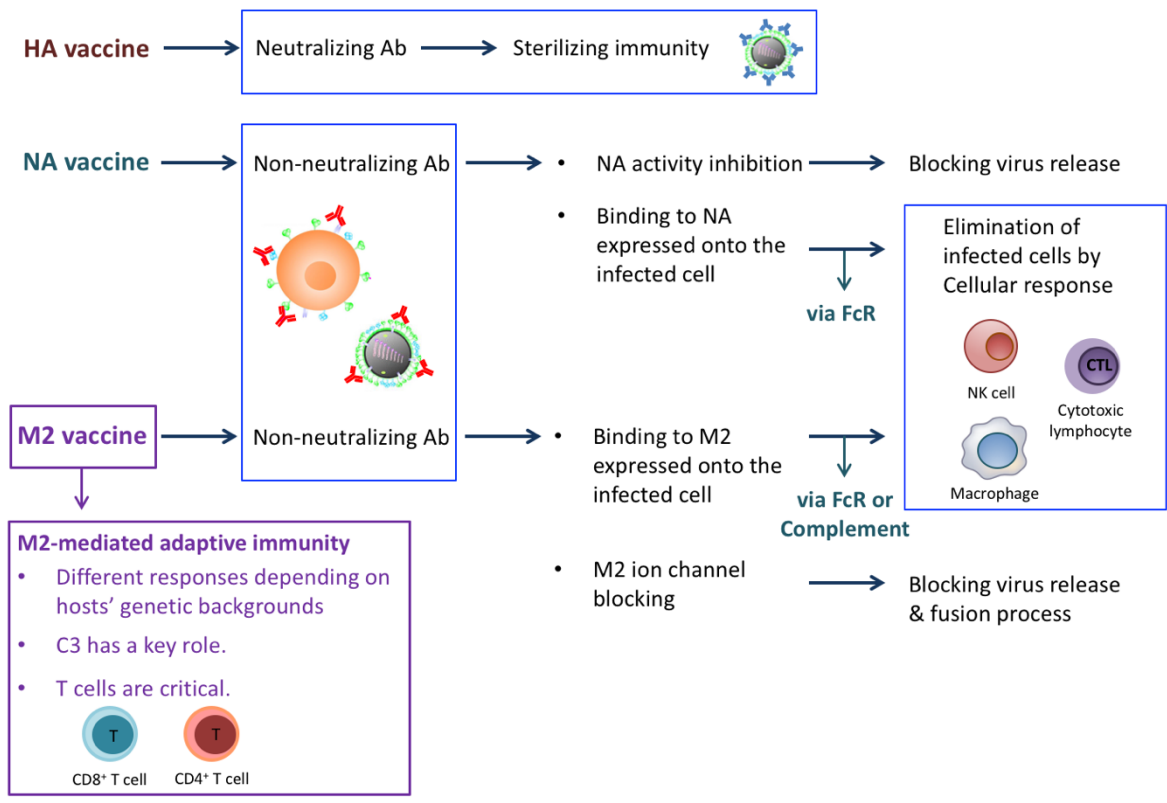
Figure 6.1 Protection mechanisms of influenza vaccines upon different target antigens.

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