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Title: Neuraminidase expressing virus-like particle vaccine provides effective cross protection against influenza virus

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Abstract: Neuraminidase is the second major surface antigen on influenza virus. We investigated the immunogenicity and cross protective efficacy of virus-like particle containing neuraminidase derived from 2009 pandemic H1N1 influenza virus (N1 VLP) in comparison with inactivated split influenza vaccine. Immunization of mice with N1 VLP induced antibody responses specific for virus and cross-reactive neuraminidase inhibition activity whereas an inactivated split vaccine induced strain-specific hemagglutination inhibition activity. N1 VLP-immunized mice developed cross protective immunity against antigenically different influenza viruses, as determined by body weight changes, lung viral titers, infiltrating innate immune cells, and cytokines, and antibody secreting cells, and germinal center B cells. Also, N1 VLP-immune sera provided cross-protection in naïve mice. Immunity by N1 VLP vaccination was not compromised in Fc receptor γ-chain deficient mice. These results suggest that neuraminidase-presenting VLP can be developed as an effective cross-protective vaccine candidate along with current influenza vaccination.

Dear Dr. Terrence M. Tumpey
Editor in Virology

We are resubmitting the revised manuscript (ID: VIRO-19-237) entitled "Neuraminidase expressing virus-like particle vaccine provides effective cross protection against influenza virus" for consideration by VIROLOGY.

We appreciate reviewer's critical comments to improve the quality of manuscript and have addressed these critical comments in the revised manuscript.

In particular, we have carried out additional revision experiments providing new data of SDS-PAGE (Fig. 1C) and NA inhibition activity to A/Phil (H3N2) (Fig. 3C), and quantitation of antigens. These new data are described and provided in the revised manuscript. Detail responses to each review point and concerns are summarized in the rebuttal letter and accordingly incorporated in the revised manuscript. The dose of NA in N1 VLP vaccine and the HA dose in split influenza virus comparison control group were used in a similar dose range (0.2 – 0.3 μg) for immunization of mice in this study. Nonetheless, we are now very careful and conservatively revised and rephrased the conclusions throughout the revised manuscript. Finally, we carefully proof-read and edited the writing to improve the quality of the manuscript. New changes are marked in color in the revised manuscript.

We hope that the revised manuscript will be suitable for publication in the journal, "Virology".

Sincerely,

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***Response to Reviews**

VIRO-19-237

Reviewers' comments:

Reviewer #1: Manuscript Number: VIRO-19-237 Manuscript by Ki-Hye Kim et al. describes experimental studies with NA-based VLPs. The authors show that NA-presenting VLP can be developed as an effective cross-protective vaccine to improve cross-protection against variant influenza viruses. The manuscript is well written with appropriate controls. The authors should explain quantitation of antigens in more detail. In addition, limitations of a mouse model should be discussed.

Response: We appreciate positive comments. In the revised manuscript, quantitation of antigens is explained and provided as addressed in the detail responses below. Mice provide a preferred small animal model for testing early preclinical experimental influenza vaccines and adjuvants. However, mice are not a natural host for influenza virus and immune responses in BALB/c mice might be different from what are expected in humans. Ferrets would be a better animal model for testing advanced preclinical influenza vaccines, which will be a future direction.

This point is discussed in the revised manuscript (page 21).

It is recommended to publish in Virology after addressing the following comments:

1. Last paragraph of introduction - Consider change to: homologous and cross-protection.

Response: This part is corrected in the revised manuscript.

2. Indicate the source of chicken eggs used in this study.

Response: The source (vendor) of chicken eggs used in this study is provided in the revised manuscript (page 5)

3. Explain quantitation of 10 <mu>g/ml. It is not clear if it is total protein or NA only.
Response: We used 10 μg N1 VLP per mouse for immunization and the dose indicate total protein concentration. Thus, we clearly described the vaccine dose used to immunize mice in revised manuscript (page 7).

4. Describe detailed procedure for RDE treatment.

Response: We describe details in the procedure for RDE treatment in revised manuscript (page 8).

5. Indicate species of RBC used in this study.

Response: We provide the species of RBC, which is from chickens. This information is described in the manuscript (page 9).

6. In the characterization of VLPs, show SDS-PAGE image of VLPs used for immunization.

Response: We confirmed the expression level of N1 NA proteins in the VLPs in comparison with controls (A/Cal H1N1 virus and mock VLP) using HCA2 mAb specific to $NA_{222-230}$ by western blot. We included the SDS-PAGE data in figure 1 and described the results in the revised manuscript (pages 7,12).

7. Explain how the amount of HA was determined in the split vaccine from A/PR8.

Response: Previous studies reported the amount of HA in inactivated influenza virus in a range of 25 – 30% of total virus proteins (Hutchinson et al., 2014; Ko et al., 2018; Tumpey et al., 2001). The amount of 0.3 µg HA was estimated to be present in 1 µg Split vaccines, based on previous reports. It would be more appropriate to indicate the total protein dose 1 µg in split virus vaccine immunization, which was in fact used in this study, as we describe in the revised manuscript (pages 6-7, 18).

8. Fig. 3 shows NAI activity against homologous A/Cal (H1N1) virus and heterologous rgH5N1 virus sample. It confirms NA activity inhibition to both viruses containing N1 subtype. Discuss the expected result if H3N2 virus antigens are used in this experiment,or show data.

Response: As suggested, we carried out NA inhibition (NAI) activity assays using A/Phil (H3N2) virus and immune sera. We found that N1 VLP immune sera showed a low level NAI activity against heterosubtypic A/Phil (H3N2), which is higher than sPR8 immune sera. NAI activity against H3N2 virus was lower than those observed against viruses containing N1 subtype (A/Cal and A/PR8). It is likely that a low level of heterosubtypic NAI by N1 VLP immunization might have contributed in part to survival cross protection against heterosubtypic A/Phil (H3N2) influenza virus. We described the cross-reactive NAI activity data in the figure 3 and discussed this data in the revised manuscript (pages 13, 19).

Reviewer #2: The authors studied the immune response induced by the N1 VLP and the protection against the challenge with H1N1, H5N1 and H3N2 viruses provided by N1 VLP. The study demonstrated that the administration of 09pdm N1-based VLP resulted in protection against not only the homologous 09pdm H1N1 virus, but also the heterologous H5N1 virus.

Major concerns:
1. The authors stated that the N1 VLP "showed higher efficacy of cross protection against antigenically different influenza viruses than a current vaccine platform". This is overstated and not justified because 10µg N1 VLP versus 0.3µg split vaccine is simply not a fair comparison.

Response: We agree with the concerns by this reviewer. After further characterization of N1 VLP vaccines (ELISA, Western blot), the amount of NA was estimated to be in a low range of 1 to 3 % of total N1 VLP vaccines, implicating that the NA amount in 10µg N1 VLP would be 0.1 to 0.3µg NA. This NA amount is approximately similar to an estimated HA amount 0.3µg HA in 1 µg total split virus vaccine proteins. Influenza virus is estimated to contain 25% – 30% HA and 1.5% to 8% NA out of total virus proteins (Gravel et al., 2010; Hutchinson et al., 2014; Ko et al., 2018; Tumpey et al., 2001). Nonetheless, we are now very careful and conservatively revised and rephrased the conclusions throughout the revised manuscript (pages 2, 5, 7, 18).

2. In this study the N1 VLP resulted in protection against H3N2 virus challenge, this is very unusual, can the authors comment on the underlying mechanism(s)?

Response: As addressed in response to the reviewer #1 comments, we found that N1 VLP induces a low level of NA-inhibiting cross-reactive antibodies. The levels of NAI activities against A/Cal (H1N1), rgH5N1, and A/Phil (H3N2) induced by N1 VLP appear to be correlated with the efficacy of homo and cross protection as measured by body weight changes and survival rates (Fig. 4D-E). A low level of cross reactive NAI activity might have partially contributed to low efficacy of survival protection against heterosubtypic A/Phil (H3N2) influenza virus. NA antibodies can block the release of infected virus, causing limitation of viral spread, virus aggregation. This is discussed in the revised manuscript (page 19).

Minor comments:
1. Section 2.3 Immunization and viral infection: 1) please specify the age of the FcRϒ KO mice; 2) the authors described that the mice were immunized with N1 VLP at 10µg/ml, what was the absolute quantity of the VLP administered per mouse? 10µg as specified in the legends to Figure 2, Figure 7 and Supplementary Figure S1? 3) what was the volume of VLP and vaccine administered per mouse? 4) how many lethal doses were administered per mouse, only 1 lethal dose?

Response: We have described the age of the FcRr KO mice (6- to 8-week old) in the revised manuscript. The amount of N1 VLP (10 µg per mouse) used for immunization was corrected, as addressed in response to reviewer #1 comments. We provide the information on the lethal dose administered for each challenge virus in the revised manuscript (pages 8, 11).

2. Section 2.5 ELLA: what was the absolute quantity of fetuin was coated onto each well of the plate?

Response: For ELLA, we first diluted 25 µg/ml fetuin with coating buffer, then added 100 µl into 96 well plates, which coated with 25 ng of fetuin per each well. This coating amount was mentioned in the manuscript (page 9).

3. Section 2.9 In vivo protective assay of N1 VLP immune sera:

1) how many lethal doses were administered per mouse, only 1 lethal dose?

Response: We provide the challenge dose information.

2) were the sera diluted before mixing with virus, what volume per mouse was inoculated with the serum/virus mix?

Response: To test the roles of anti-NA sera in conferring protection, heat inactivated immune sera were diluted 2-fold with PBS (25 µl), then mixed with lethal dose (10× LD50) rgH5N1 virus (25 µl). Naïve (WT BALB/c or FcRγ KO) 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. This experimental detail is provided in revised manuscript (page 11).

4. Section 3.4 lines 34-39: "Therefore, these results suggest that N1 VLP platform confer superior cross protection over vaccine against influenza viruses with HA variant strains", the authors are wished to be cautious to claim this as this is not a fair and justified comparison, see also the major concerns about this study; in addition, please measure and report the NI titers of VLP-immunized mouse sera against the NA of A/Phil (H3N2) virus?

Response: As addressed in response to the major concerns, we are now very cautious and rephrased the description of experimental results conservatively in the revised manuscript. “Therefore, these results suggest that N1 VLP platform could confer cross protection against HA variant strains" (page 13). The NAI titers against the NA of A/Phil (H3N2) virus were measured and provided in the revised manuscript (Fig. 3C, page 13).

5.The writing of the manuscript needs to be improved.

Response: We carefully proof-read and edited the writing to improve the quality of the manuscript.

References cited here and are also in the revised manuscript.

Gravel, C., Li, C., Wang, J., Hashem, A.M., Jaentschke, B., Xu, K.W., Lorbetskie, B., Gingras, G., Aubin, Y., Van Domselaar, G., Girard, M., He, R., Li, X., 2010. Qualitative and quantitative analyses of virtually all subtypes of influenza A and B viral neuraminidases using antibodies targeting the universally conserved sequences. Vaccine 28, 5774-5784.

Hutchinson, E.C., Charles, P.D., Hester, S.S., Thomas, B., Trudgian, D., Martinez-Alonso, M., Fodor, E., 2014. Conserved and host-specific features of influenza virion architecture. Nat Commun 5, 4816.

Ko, E.J., Lee, Y., Lee, Y.T., Kim, Y.J., Kim, K.H., Kang, S.M., 2018. MPL and CpG combination adjuvants promote homologous and heterosubtypic cross protection of inactivated split influenza virus vaccine. Antiviral Res 156, 107-115.
Tumpey, T.M., Renshaw, M., Clements, J.D., Katz, J.M., 2001. Mucosal delivery of inactivated influenza vaccine induces B-cell-dependent heterosubtypic cross-protection against lethal influenza A H5N1 virus infection. J Virol 75, 5141-5150.

Neuraminidase expressing virus-like particle vaccine provides effective cross protection against influenza virus

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Key words: Influenza virus, Virus-like particle, Neuraminidase vaccine, Cross protection

Abstract

Neuraminidase is the second major surface antigen on influenza virus. We investigated the immunogenicity and cross protective efficacy of virus-like particle containing neuraminidase derived from 2009 pandemic H1N1 influenza virus (N1 VLP) in comparison with inactivated split influenza vaccine. Immunization of mice with N1 VLP induced antibody responses specific for virus and cross-reactive neuraminidase inhibition activity whereas an inactivated split vaccine induced strain-specific hemagglutination inhibition activity. N1 VLP-immunized mice developed cross protective immunity against antigenically different influenza viruses, as determined by body weight changes, lung viral titers, infiltrating innate immune cells, and cytokines, and antibody secreting cells, and germinal center B cells. Also, N1 VLP-immune sera provided cross-protection in naïve mice. Immunity by N1 VLP vaccination was not compromised in Fc receptor γ-chain deficient mice. These results suggest that neuraminidase-presenting VLP can be developed as an effective cross-protective vaccine candidate along with current influenza vaccination.

1. Introduction

Annual vaccination is recommended to protect seasonal influenza viruses worldwide although its efficacy is highly variable year to year. Hemagglutinin (HA) is a major target for inducing immunity by currently licensed influenza vaccines since antibodies to HA can block the ability of the receptor binding site of HA and thus have neutralizing activity, a protective correlate. However, the antigenicity of HA is highly variable and HA immunity is specific for virus strains with matching HA.

Neuraminidase (NA) is the second major surface protein, playing key roles in the life cycle of influenza virus. NA has an enzymatic function that cleaves terminal sialic acids from glycans, facilitating virus release on the host cell surface (Wohlbold and Krammer, 2014). NA is also suggested to play a role in promoting virus entry into the target host cells (Sakai et al., 2017; Su et al., 2009). Antiviral drugs (oseltamivir, zanamivir) target NA enzymatic activity. Therefore, NA can provide an important vaccine target antigen. Antibodies specific for NA antigens could provide an independent protective correlate alleviating disease in humans (Couch et al., 2013; Memoli et al., 2016; Monto et al., 2015; Murphy et al., 1972). In addition, NA undergoes much slower antigenic changes than HA, suggesting NA as a desirable vaccine target (Kilbourne et al., 1990; Krammer et al., 2018).

Currently, the only HA is required to be quantified in inactivated influenza vaccines while the amount of NA has been found to vary drastically from different manufacturers even if the same vaccine strains were used to make the vaccines (Gravel et al., 2010). HA is known to be immune

dominant and has suppressive effects on inducing NA specific immune responses when both HA and NA antigens are present in the same influenza virus particles as in the inactivated influenza vaccines (Johansson et al., 1987). Co-vaccination with separate HA and NA vaccines was shown to avoid intravirionic antigenic competition, inducing balanced immune responses to both HA and NA antigens (Johansson and Kilbourne, 1993; Johansson et al., 2002).

Different approaches were taken to study the role of NA in protecting against influenza virus, which include purified NA proteins (Brett and Johansson, 2005; Johansson, 1999; Wohlbold et al., 2015), DNA plasmids (Qiu et al., 2006; Sandbulte et al., 2007), and live virus-vectored vaccines expressing NA (Mooney et al., 2017). Vaccination inducing antibodies against NA could suppress viral replication in the lungs and reduce disease severity upon subsequent challenge in animals (Job et al., 2018a; Job et al., 2018b; Liu et al., 2015; Smith et al., 2017; Walz et al., 2018; Wohlbold et al., 2015). However, some limitations might be associated with previous approaches in developing NA vaccines. Specifically, it is laborious and costly to prepare recombinant protein vaccines which often require adjuvants to be included in the vaccination. DNA vaccines are poor in immunogenicity and thus need multiple immunizations, while live vectored vaccines may induce immune responses against the vectors and have safety concerns in humans.

Influenza virus-like particles (VLPs) have distinct advantages over the aforementioned strategies in that they mimic viral structure and morphology, representing one of the novel platforms to develop efficacious vaccines against influenza (Bright et al., 2008). Influenza VLP vaccines (2009 H1N1, H5N1, H7N9) were safe and efficacious in clinical trials (Fries et al., 2013; Khurana et al., 2011; Lopez-Macias et al., 2011). Previous studies demonstrated that vaccination of animals with VLPs containing NA induced high titers of anti-NA antibodies and

protection against viruses with the same subtype NA (Easterbrook et al., 2012; Quan et al., 2012; Smith et al., 2017). However, the roles of NA immunity in cross protection and Fc receptors (FcR) in NA antibody-mediated protection have not been well studied yet. In this study, we constructed influenza VLPs containing NA derived from A/California/2009 (H1N1) virus (N1 VLP) and investigated immunogenicity and efficacy of homologous and cross protection in comparison with inactivated split virus vaccine. N1 VLP was found to induce cross-reactive neuraminidase inhibition activity correlating with cross protection whereas an inactivated split vaccine induced strain-specific hemagglutination inhibition activity. FcR was not required for protection by N1 VLP vaccination or anti-NA immune sera.

2. Materials and Methods

2.1. Animals, viruses, and reagents

BALB/c and Fc receptor (FcR) γ-chain gene knockout (FcRγ KO on the BALB/c genetic background) mice were purchased from Taconic Farms (Hudson, NY) and maintained in the animal facility at Georgia State University (GSU). Animal experiments were performed under the guidelines of the approved IACUC protocol (A18001). Influenza A viruses, A/California/04/09 (A/Cal, H1N1), A/Philippines/2/82 (A/Phil, H3N2), and A/Puerto Rico/8/34 (A/PR8, H1N1) were grown in 10 days old chicken eggs (Hy-Line North America, Mansfield, GA) at 37 °C for 2 days. The reassortant rgH5N1 virus contains HA and NA derived from A/Vietnam/1203/2004 (H5N1) and the remaining backbone genes from A/PR8 (Song et al., 2010). The viruses grown in embryonated eggs were inactivated using formalin at a concentration of 1:4000 (v/v) (Quan et al., 2008). Influenza split vaccine (sCal) derived from the 2009 H1N1 pandemic strain A/Cal/07/2009 was kindly provided by the WHO-certified vaccine manufacturing company (Green Cross, South Korea). The split vaccine of A/PR8 (sPR8) was prepared with inactivated PR8 virus by treating with 1% triton X-100 to disrupt virus particles and dialyzing with phosphate-buffered saline (PBS) as described (Ko et al., 2018). The total protein concentrations of sPR8 and N1 VLP were determined by DC protein assay kit (Bio-rad, Hercules, CA). HCA-2 monoclonal antibody (mAbs) specific for pan NA proteins ($NA_{220\text{-}230}$, LRTQESEC) has been well described in previous studies (Doyle et al., 2013; Gravel et al., 2010).

2.2. Preparation of influenza N1 NA VLP

Virus-like particle expressing NA (N1 VLP) derived from A/Cal (H1N1) was produced in insect cells as previously described (Kim et al., 2018b; Quan et al., 2012; Quan et al., 2010). Briefly, the plasmid pCI with cDNA encoding N1 NA derived from A/Cal (H1N1) virus kindly provided by Dr. Ruben Donis (CDC, Atlanta, GA) and cloned into pFastBac shuttle vector using primers synthesized with forward primer (5'-AAAGAATCCGCCGCC ACCATGA ATCCAAACCAAAAGATAATAACC -3') and reverse primer (5'-AAAAAGCTTTT ACTTGTCAATGGTAAATFF-3'). The cloned pFastBac-NA plasmid was subsequently used to generate recombinant Bacmid DNA containing NA in transformed DH10Bac cells (Invitrogen). Recombinant baculovirus (rBV) expressing N1 NA was generated in Sf9 insect cells transfected with recombinant Bacmid containing NA. N1 VLP was prepared from the culture supernatants from Sf9 cells co-infected with rBVs expressing N1 NA and matrix protein M1 (Quan et al., 2012) by removing cells via centrifugation (3000 rpm, 20 min) and then purified by ultracentrifugation (30,000 rpm, 60 min) (Quan et al., 2012; Quan et al., 2010). Expression and incorporation of N1 NA into VLP were confirmed by ELISA and western blot using anti-rabbit $NA_{222-230}$ mAb, HCA2 (Doyle et al., 2013; Gravel et al., 2010). The amount of NA was estimated to be in a low range of 1% to 3% of total N1 VLP proteins, implicating that the NA amount in 10µg N1 VLP would be approximately 0.2 µg NA. Nanoparticle size distribution of N1 VLP was determined by dynamic light scattering (DLS) with a Malvern Zetasizer Nano ZS (Malvern Instruments, Westborough, MA). Functional NA activity of N1 VLP was estimated by enzyme linked lectin assay (ELLA) as described (Doyle et al., 2013).

2.3. Immunization and viral infection

BALB/c mice (6- to 8-week old) and FcRγ KO mice (6- to 8 week old, n=8-10 per group) were intramuscularly prime-boost immunized with N1 VLP (10 μg total protein per mouse), split vaccines (1 μg total protein per mouse) from A/PR8 (sPR8) or A/Cal (sCal) at weeks 0 and 3. Blood samples were collected at 2 weeks after prime and boost immunization. Immunized mice were challenged with a lethal dose of 3× LD_{50} A/Cal (H1N1), 2.5× LD_{50} rgH5N1, or 1.5× LD_{50} A/Phil (H3N2) at 4 weeks after boost.

2.4. Enzyme-linked immunosorbent assay (ELISA)

IgG antibody responses specific for virus antigens were determined by ELISA in immune sera collected after boost, in bronchoalveolar lavage fluids (BALF) and lung lysates harvested on 7 days post infection (dpi). ELISA coating antigens include inactivated A/Cal (H1N1), A/PR8 (H1N1), and rgH5N1 virus. Serially diluted samples (sera, BALF, lung lysates) were added into a 96-well plate coated with inactivated virus as previously described (Kim et al., 2018a). To determine antibody production *in vitro,* the cells from mediastinal lymph node (MLN) collected at 7 dpi with rgH5N1 virus infection were cultured in the presence of inactivated virus antigens and incubated for 2 days at 37 °C. Antigen-specific antibodies in culture supernatants were determined by ELISA. The levels of cytokines and chemokine were measured from BALF and lung lysates using ELISA kits (eBioscience, San Diego, CA) according to the manufacturer's instructions.

2.5. Neuraminidase inhibition (NAI) analysis by enzyme-linked lectin assay (ELLA)

NA inhibition (NAI) activity against virus in sera was determined by ELLA using a fetuin-based procedure as described (Doyle et al., 2013). Briefly, 96 well plates were coated at 25 ng per each well with fetuin (Sigma-Aldrich) and incubated overnight at 4 °C. After blocking the plates with PBS containing 1% bovine serum albumin (BSA), a mixture of 2-fold serially diluted immune sera and virus with 90% maximum activity was incubated and then added to the fetuin-coated plates, then incubated for 2 hours at 37 °C. Peroxidase-labeled peanut agglutinin (1 µg/ml) was added to the plates for 2 hours at room temperature. NAI activity was measured using tetramethylbenzidine substrate (eBioscience, San Diego, CA) and OD values were read at 490nm. The inhibition percent was calculated using the formula: $100 \times (OD_{\text{virus only control}} - OD_{\text{test sample}})/OD_{\text{virus only control}}$.

2.6. Hemagglutination inhibition (HAI) assay

To determine HAI titers, immune sera were treated with receptor destroying enzymes (RDE, Sigma Aldrich, St. Louis, MO) at 1:3 ratio (sera : RDE) and then incubated for 16 hours at 37 °C as previously described (Ko et al., 2017). The RDE-treated serum samples were inactivated at 56 °C for 30 min, serially 2 fold diluted, and incubated with 4 HA units of A/PR8, A/Cal, or rgH5N1 virus for 30 min, and then admixed with 0.5% chicken red blood cells (RBC) (Lampire Biological Laboratories, Pipersville, PA). The highest serum dilutions interfering with the red spot formation were determined for HAI titers.

2.7. Lung viral titration

Lung viral loads were determined in embryonated chicken eggs. Lung homogenates were serially diluted and injected into the egg allantoic sacs per each dilution of samples and then incubated at 37 °C for 3 days. Highest dilutions displaying hemagglutination activity were measured using RBC to determine viral titers as described (Kim et al., 2018a).

2.8. Flow cytometry

Bronchoalveolar lavage fluids (BALF) and lung tissues were collected at 7 dpi with rgH5N1 virus. BALF cells were harvested by infusing 1 ml of PBS into the trachea using a catheter (Exelint International Co., Los Angeles, CA) to harvest non-adherent cells in the airways as described (Kim et al., 2015). The lung tissues without perfusion were homogenized and spun on 44%/67% Percoll gradients at 2800rpm for 15 min. The lung cells were collected from the layer between 44% and 67%. To determine cell phenotypes in the airways, BAL and lung cells were stained with surface marker antibodies specific for anti-mouse CD11b, CD11c, Ly6c, CD45, F4/80, Siglec F, MHC II (eBioscience or BD Pharmingen, San Diego, CA). MLN cells were collected at 7dpi to determine germinal center (GC) B cells and plasma cells. The MLN cells were stained with specific anti-mouse phenotypic marker antibodies for CD3, CD19, IgD, B220, GL-7, CD138 (eBioscience or BD Pharmingen, San Diego, CA). All samples were analyzed on a Becton-Dickinson LSR-II/Fortessa flow cytometer (BD, San Diego, CA) and analyzed using the Flowjo software (FlowJo V10, Tree Star, Inc., Ashland, OR).

2.9. In vivo protective assay of N1 VLP immune sera

To investigate the roles of N1 VLP immune sera in contributing to cross protection, N1 VLP immune or unvaccinated naïve sera were collected from wild type (WT) BALB/c and FcRγ KO mice, inactivated at 56 °C 30 min, diluted 2-fold with PBS, and mixed with a lethal dose (10× LD_{50}) of rgH5N1 at same volume (25 µl + 25 µl) as previously described (Song et al., 2011). Naïve WT BALB/c and FcRγ KO mice were intranasally infected with a mixture (50 µl) of sera and virus and monitored for the survival rates and body weight changes for 14 days.

2.10. Statistical analysis

All results are presented as the mean ± the standard errors of the mean (SEM). The statistical significance for all experiments was performed by one- or two-way analysis of variance (ANOVA). Prism software (GraphPad Software, Inc., San Diego, CA) was used for all data analysis. The comparison used to generate a P value is indicated by horizontal lines (*; $p<0.05$, **; $p<0.01$, ***; $p<0.001$).

3. Results

3.1. Characterization of influenza VLP expressing neuraminidase (N1 VLP)

The rBV expressing N1 NA protein derived from A/California/04/2009 (A/Cal, H1N1) was confirmed as evidenced by high reactivity of N1 rBV-infected insect cells against HCA-2 mAb specific for NA by cell-surface ELISA (Fig. 1A). N1 NA VLP purified from insect cell culture supernatants also displayed strong reactivity to HCA-2 mAb (Fig. 1B). The NA protein presented on VLPs was also confirmed by western blot at different loading amounts using HCA-2 mAb and estimated to be present at 2-3 folds less than A/Cal virus (Fig. 1C). The particle sizes of NI VLP were an average diameter of 220nm as measured by dynamic light scattering (DLS) using Malvern Zetasizer Nano ZS (Fig. 1D). The functional enzyme activity of NA expressed on the VLP was found to be dependent on the concentrations of N1 NA VLP (Fig. 1E). These results showed that NA displaying on the surface of VLP exhibits NA enzyme activity, implicating native NA conformation.

3.2. N1 VLP vaccine induces Th1-biased IgG2a antibody responses

Groups of mice were intramuscularly immunized with N1 VLP by a prime boost regimen in comparison with influenza split vaccine (sPR8, H1N1). Serum IgG antibodies binding to A/Cal virus were induced after boost immunization at comparable levels in the sPR8 and N1 VLP groups (Fig. 2A). The split sPR8 vaccine group induced T helper type 2 (Th2) IgG1 isotype antibody predominantly (64 folds compared to N1 VLP) whereas the N1 VLP group raised Th1 type IgG2a isotype antibody dominantly (16 folds compared to sPR8) (Fig. 2B, 2C). We further found that split sPR8 vaccine was highly effective in inducing IgG and IgG1 antibodies specific

for the homologous A/PR8 virus antigen (Supplementary Fig. S1). These results suggest that N1 VLP vaccine effectively induce Th1 type IgG2a antibodies compared to split influenza vaccine.

3.3. N1 VLP immunization induces NA-inhibiting cross-reactive antibodies

NA inhibiting (NAI) IgG antibodies prevent release and spread of virus. N1 VLP immune sera exhibited significantly higher levels of NI activity by 11 folds and 16 folds or 4 folds than those of the sPR8 group against A/Cal (H1N1) (Fig. 3A), heterologous rgH5N1 virus (Fig. 3B), and heterosubtypic A/Phil (H3N2) (Fig. 3C) respectively. Split vaccine sPR8 raised high hemagglutination inhibition (HAI) titers against homologous A/PR8 (H1N1) while N1 VLP group did not display HAI activity (Fig. 3D). These results support that N1 VLP is effective in inducing cross reactive NAI antibody responses.

3.4. N1 VLP provides cross protection against HA variant viruses

To investigate cross protective efficacy, the groups of mice immunized with sPR8 or N1 VLP were intranasally challenged with A/Cal (H1N1), heterologous rgH5N1, or heterosubtypic A/Phil (H3N2) (Fig 4). The sPR8 group showed severe weight loss of approximately 20% and 24% against A/Cal (H1N1) and rgH5N1 infections respectively (Fig. 4A-B). In contrast, N1 VLP-immunized mice displayed slight to moderate weight loss of approximately 5% and 10%, and were 100% protected against homologous A/Cal (H1N1) and heterologous rgH5N1 virus lethal challenge respectively (Fig. 4A-B). The mice with sPR8 vaccination displayed high lung viral titers similar to those in unvaccinated naïve mice as determined at 7dpi with rgH5N1 whereas the N1 VLP group showed over 1,000 folds lower lung viral titers (Fig. 4C). Moreover, when we tested cross-protection efficacy against heterosubtypic A/Phil (H3N2), N1 VLP-immunized mice

all survived the virus challenge although the recovery was delayed (Fig. 4D-E). Therefore, these results suggest that N1 VLP platform could confer cross protection against HA variant influenza virus strains.

3.5. N1 VLP vaccination prevents heightened lung inflammation due to heterologous virus infection

To better understand the protective efficacy, we determined whether N1 VLP vaccination would prevent excessive inflammations in the lungs following influenza virus infection. Naïve mice infected with lethal rgH5N1 exhibited highest levels of cytokines (TNF-α, IL-6, IFN-γ) and chemokine (CXCL1/KC) indicating severe inflammation except IL-5 in BALF and lungs (Fig. 5A-E). In comparison, the sPR8-immunized group induced moderate levels of proinflammatory cytokines (TNF-α, IL-6, IFN-γ) and chemokine (CXCL1/KC) (Fig. 5A-C, E), and highest levels of IL-5 cytokine in BALF and lungs (Fig. 5D). In contrast, N1 VLP-immunized mice showed lowest levels of cytokines and chemokines among the virus infection groups (Fig. 5A-E).

Since inflammatory cytokines and chemokines recruit cellular infiltrates during influenza virus infection, innate immune cells were analyzed in the BALF and lungs 7 dpi with rgH5N1 virus (Fig. 5F-H). N1 VLP group showed significantly lower cell numbers of inflammatory monocytes ($CD11b^{+}Ly6c^{hi}F4/80^{+}$), neutrophils ($CD11b^{+}Ly6c^{+}F4/80^{-}$), eosinophils ($CD11b^{+}CD11c^{+}SiglecF^{+}$), and activated dendritic cells (aDCs, $CD45^{+}CD11b^{+}MHCII^{+}$) in the BALF and lungs compared to those in the sPR8 and naïve groups after infection (Fig. 5F-I). In comparison with sPR8 group, N1 VLP-immunized mice exhibited reductions of 5 or 17 folds in monocytes, 6 or 3 folds in neutrophils, 300 or 350 folds in eosinophils in the BALF and lungs respectively post rgH5N1 infection (Fig. 5F-H). Furthermore, histological analysis displayed

diminished immune cell infiltration in the lungs from mice with N1 VLP immunization and heterosubtypic infection whereas the sPR8 and naïve infections groups showed severe infiltrations of immune cells (data not shown). Taken together, these results suggest that N1 VLP confers effective protection in preventing pulmonary inflammation and innate immune cell infiltration after heterologous influenza virus infection.

3.6. N1 VLP immunization induces mucosal IgG and antibody-secreting plasma cells in draining lymph nodes

To investigate the effect of N1 VLP immunization on enhancing humoral immunity, we determined antigen-specific antibody responses from BALF and lung lysates collected at 7dpi with rgH5N1. IgG antibodies specific for A/Cal (H1N1) virus were induced at higher levels in mucosal BALF and lung samples from mice immunized with N1 VLP than those in sPR8 vaccine and naïve mice after infection (Fig. 6A). Furthermore, we determined the secretion of IgG antibodies specific for homologous or heterologous viruses from *in vitro* cultures of MLN cells collected at 7dpi with rgH5N1. Consistent with serum IgG antibody responses, N1 VLP immunized group showed higher levels of IgG and IgG2a, but not IgG1, specific for A/Cal (H1N1) and rgH5N1 viruses than those of sPR8 vaccine and naïve mice (Fig. 6B-C). To better understand effector B cell responses induced by N1 VLP immunization, germinal center (GC) B cells and antibody-secreting plasma cells were determined from MLN cells collected day 7 post infection by flow cytometry. B cells ($IgD^{-}CD19^{+}$), GC B cells ($GL7^{+}B220^{+}IgD^{-}CD19^{+}$), and plasma cells ($CD138^{+}B220^{+/-}IgD^{-}CD19^{+}$) were detected with higher numbers in the MLN from the N1 VLP immunized group when compared with the sPR8 and naïve groups (Fig. 6D-F).

Therefore, these results suggest that N1 VLP immunization induces cross-reactive IgG antibody responses and B cell differentiation into plasma cells.

3.7. N1 VLP is effective in conferring cross protection

We next compared cross protective efficacy from mice immunized with N1 VLP and split vaccine platforms derived from A/Cal (H1N1, sCal) and A/PR8 (H1N1, sPR8) against heterologous influenza virus. The sPR8- and sCal-immunized groups displayed severe and moderate weight loss of approximately 24% and 16%, and survival rates of 75% and 100%, respectively, after rgH5N1 virus infection (Fig. 7A, B). In contrast, N1 VLP-immunized mice induced effective cross protection against heterologous influenza virus infection as evidenced by significantly less body weight loss (~10%) (Fig. 7A-B). These results provide evidence that NA VLP can be relatively effective in conferring cross protection against influenza A viruses with HA antigenic variants.

3.8. N1 VLP vaccination and N1 VLP immune sera confer protection in Fc receptor deficient mice

Next, we determined whether Fc receptors (FcR) would be required for N1 VLP immune serum-mediated protection. Wild type (WT) and FcRγ KO mice were immunized with N1 VLP by a prime boost regimen, then intranasally challenged with A/Cal (H1N1) virus, and daily monitored for 14 days (Fig. 8A-B). FcRγ KO mice with N1 VLP vaccination were well protected, displaying only a slight weight loss in a similar pattern as observed in WT mice with N1 VLP (Fig. 8A-B). Thus, N1 VLP vaccination induces protection in FcRγ KO mice.

To determine the roles of N1 VLP immune sera in conferring cross protection, naïve mice were intranasally inoculated with a mixture of influenza virus and immune sera collected from N1 VLP-immunized or unvaccinated naïve mice and then monitored for weight changes and survival rates for 14 days (Fig. 8 C-D). The naïve mice with unvaccinated sera and virus were not protected against rgH5N1 virus as evidenced by severe weight loss (>24%) and 0% survival rates (Fig. 8C-D). In contrast, naïve mice with N1 VLP immune sera and rgH5N1 virus were protected against rgH5N1 virus with slight weight loss of 5-6% (Fig. 8C-D).

To further investigate the roles of FcR, naïve FcRγ KO mice were inoculated with heterologous rgH5N1 virus and N1 VLP immune sera (Fig. 8E-F). Naïve FcRγ KO mice that received N1 VLP immune sera and rgH5N1 virus were protected, as evidenced by a moderate body weight loss of 7-9% and survival rates of 100% (Fig 8E-F). The cross protection against rgH5N1 mediated by N1 (A/Cal) VLP immune sera was similarly observed in WT (Fig. 8A, B) and FcRγ KO mice despite a delay in recovery in FcRγ KO mice. Taken together, these results suggest that FcRγ is not required for N1 VLP immune sera-mediated protection or protection by N1 VLP vaccination.

4. Discussion

NA is the second major surface antigen on influenza viruses and NA immunity was indicated to be an independent correlate of protection as evidenced by the analysis of human samples (Memoli et al., 2016; Monto et al., 2015). Inactivated split influenza vaccines contain variable contents of NA (Gravel et al., 2010). Vaccination with split vaccines was not effective in inducing antibody responses to NA since HA outcompetes NA in the priming of B and T cell responses when both antigens are close each other on the same virus particles as shown in a mouse model (Johansson et al., 1987). A separate entity of NA vaccines would be desirable. Here we explored the roles of NA VLP immunity in conferring protection against antigenically different influenza viruses in comparison with inactivated influenza split virus, the most common vaccine platform. Influenza virus is estimated to contain approximately 25% – 30% HA and 1.5% to 8% NA out of total virus proteins (Gravel et al., 2010; Hutchinson et al., 2014; Ko et al., 2018; Tumpey et al., 2001), suggesting 0.25 to 0.3 µg HA in 1 µg split virus total protein used to immunize the mice. Although the NA amount in 10µg N1 VLP was estimated to be approximately 0.2 µg NA, N1 NA VLP vaccine was immunogenic, preferentially inducing Th1 type humoral and cellular immune responses in mice with a prime boost regimen. Notably, N1 VLP vaccine was found to be relatively effective in conferring cross protection against influenza viruses with antigenically different HA antigens, compared to HA-based split vaccine inducing strain-specific HAI activity. Protection by N1 VLP vaccination or by immune sera of N1 VLP vaccination was also observed in FcR deficient mice.

NA is known to be less variable and to undergo lower antigenic mutation rates than those of HA (Kilbourne et al., 1990; Sandbulte et al., 2011). The mice that received N1 (A/Cal, H1N1) NA VLP vaccination were well protected against homologous A/Cal (H1N1) virus and

heterologous rgH5N1 within the same N1 subtype, resulting in over 1000 folds lower lung viral loads and minimal weight loss. In contrast, inactivated split (sPR8, H1N1) virus vaccination induced either survival protection against A/Cal (H1N1) virus or no protection against rgH5N1. The levels of IgG antibodies specific for A/Cal virus antigens were observed in sPR8 vaccine immune mice at similar to or higher than those in NA VLP immune mice. This data indicates that virus binding IgG antibody levels induced by split vaccination would not be correlated with cross protection. Transfer of virus-mixed immune sera of NA VLP to naïve mice could confer protection against rgH5N1 virus, without displaying substantial weight loss. The homo and heterologous NA immune-mediated protection observed in this study is consistent with previous studies that anti-NA antibodies can provide cross protection against different antigenic HA viruses within the same NA subtype (Easterbrook et al., 2012; Halbherr et al., 2015; Wan et al., 2013; Wohlbold et al., 2015). The survival protection against heterosubtypic A/Phil (H3N2) virus was observed with N1 VLP immune sera, despite of accompanying significant weight loss.

The underlying mechanisms for homo, heterologous, and heterosubtypic cross protection by NA immunity need to be further studied. Protective NA antibodies can inhibit NA enzymatic function, interfering with virus egress on the infected cells. We found that N1 VLP induces cross-reactive NAI antibodies. Also, the levels of NAI activities against A/Cal (H1N1), rgH5N1, and A/Phil (H3N2) induced by N1 VLP appear to be correlated with the efficacy of homo and cross protection as measured by body weight changes and survival rates. Previous studies reported that plaque sizes are significantly reduced in the presence of NA antibodies at the stages of virus spreading even at low concentrations (Jiang et al., 2016; Wan et al., 2013; Wohlbold et al., 2016; Wohlbold et al., 2017). Most NA inhibiting antibodies are considered to mediate non-neutralizing immunity, thus resulting in infection-permissive protection but significantly

mitigating the severity of disease (Easterbrook et al., 2012; Quan et al., 2011; Wohlbold et al., 2015), consistent with the results in this study. NA was also reported to promote HA-mediated cell fusion and viral infection into the target cells, suggesting a role of NA in viral entry (Sakai et al., 2017; Su et al., 2009). Thus, induction of NAI antibodies would be effective in conferring protection against influenza viruses with homo or closely related heterologous NA as shown in A/Cal (H1N1) and rgH5N1 viruses.

HA stalk-specific antibodies were demonstrated to mediate broad cross protection *in vivo* via Fc receptor interactions (DiLillo et al., 2016; DiLillo et al., 2014) and promoting immune complexes' phagocytosis by neutrophils (Mullarkey et al., 2016). Similarly, *in vivo* cross protection by antibodies to conserved influenza virus M2 extracellular domains (M2e) also requires the engagement of Fc receptors (Kim et al., 2014; Lee et al., 2014; Van den Hoecke et al., 2017) and possibly alveolar macrophages (Song et al., 2011). The requirement of Fc receptors and macrophages for NA antibody-mediated *in vivo* protection remains less well known. It was shown that clodronate-mediated depletion of dendritic and macrophage cells prior to transfer of NA immune sera partially reduced the protective efficacy but resulted in no effects on protective efficacy of neutralizing HA antibodies (Quan et al., 2012). Similarly, *in vivo* protection by immune sera of NA protein vaccination was partially dependent on the Fc receptors (Kim et al., 2017). In this study, similar levels of protection against NA homo A/Cal (H1N1) virus were observed in WT and FcRγ KO mice with NA VLP immune sera. Also, N1 VLP immune sera provided substantial protection in FcRγ KO mice although a moderate delay in recovering weight loss was displayed. It is likely that protection by NA antibodies is less dependent on or does not require the involvement of Fc receptors, in contrast to a critical role of

FcR-mediated protection by M2e or HA stalk antibodies (DiLillo et al., 2016; DiLillo et al., 2014; Kim et al., 2014; Lee et al., 2014; Van den Hoecke et al., 2017).

HA-based vaccines inducing neutralizing antibodies provide the most effective homologous protection, which is superior to M2e- or NA-based vaccines inducing non-neutralizing antibodies (Kim et al., 2017; Lee et al., 2016; Mullarkey et al., 2016). However, when challenged with antigenically different HA viruses as expected, sPR8 vaccine (A/PR8, H1N1) could not confer protection against heterologous (A/Cal, H1N1) and heterosubtypic virus (rgH5N1). In contrast, N1 VLP vaccine was found to be effective in conferring cross protection against these viruses, inducing Th1 type immune responses, and rapidly generating virus specific antibody secreting plasma cells upon challenge. Therefore, developing supplemental vaccines inducing NA immunity independent of HA would provide substantial benefits in conferring cross protection. Priming or supplementing split vaccines with N1 and N2 NA VLP vaccines would provide a promising vaccination strategy conferring cross protection. Mice provide a preferred small animal model for testing preclinical experimental influenza vaccines and adjuvants at early developmental stages. However, mice are not a natural host for influenza virus and immune responses and pathogenesis in BALB/c mice might be different from what are expected in humans. Ferrets would be a better animal model for testing advanced preclinical influenza vaccines, which should be a future direction.

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Figure Legends

Figure 1. Characterization of VLP containing N1 NA (N1 VLP)

(A) The ELISA reactivity to NA mAb (HCA-2) in the M1 (from A/PR8 virus) or N1 NA (from A/Cal, H1N1 virus) rBV-infected insect cells. NA expression on the insect cells infected with M1 or N1 rBVs was determined with anti-NA mAb (HCA-2) by ELISA. (B) The reactivity of N1 VLP to NA mAb (HCA-2) by ELISA. (C) The expression of NA protein on VLPs was determined by western blot probed with rabbit HCA-2 mAb. Influenza N1 VLP (20, 10, 5 μg), inactivated A/Cal (H1N1) virus (10, 5 μg), M2e5x VLP (5 μg) were loaded. (D) Size distribution of N1 VLP as measured by Malvern Zetasizer with dynamic light scattering (DLS). (E) Neuraminidase activity by an enzyme-linked lectin assay. Statistical significance was determined by using one-way ANOVA. Data are representative of individual animal out of two independent experiments. Error bars indicate the means ± SEM. ***, $p<0.001$.

Figure 2. N1 VLP immunization induces Th1-biased IgG2a isotype antibody responses

BALB/c mice (n=8) were immunized with N1 (A/Cal, H1N1) VLP (10 μg) and split vaccine from A/PR8 (H1N1) virus (sPR8, 1 μg total protein) intramuscularly with an interval of 2 weeks and blood samples were collected at week 2 after boost. (A-C) A/Cal (H1N1) virus antigen-specific IgG antibodies were determined by ELISA.

Figure 3. NA inhibition activity is induced in sera from N1 VLP immunized mice

NA inhibition (NAI) activity in boost immune sera was determined against different stains of influenza viruses by an enzyme-linked lectin assay. (A) NAI activity against homologous A/Cal (H1N1) virus. (B) NAI activity against heterologous rgH5N1 virus. Dotted lines indicate the concentration inhibiting 50% of NA enzyme activity. (C) NAI activity against heterosubtypic A/Phil (H3N2) virus. (D) HAI titers against influenza viruses. Statistical significance was determined by using one-way ANOVA. Data (n=4) are representative of individual animal out of two independent experiments. Error bars indicate the means ± SEM. ***, $p<0.001$.

Figure 4. Cross protection induced by mice immunized with N1 VLP

The groups of mice with sPR8 or N1 VLP vaccination (n=4 or 8) were challenged with 3× LD_{50} A/Cal (H1N1), 2.5× LD_{50} rgH5N1, and 1.5× LD_{50} A/Phil (H3N2) intranasally at 4 weeks after boost immunization. Body weight changes were monitored for 14 days. (A) Body weight changes against A/Cal (H1N1) virus. (B) Weight changes after rgH5N1 virus infection. (C) Lung viral titers at 7 dpi with rgH5N1. (D) The mortality against A/Phil (H3N2) virus in N1 VLP immunized mice. Data are representative of individual animal out of two independent experiments. Error bars indicate the means ± SEM. ***, $p<0.001$.

Figure 5. N1 VLP vaccination prevents lung inflammation due to heterologous rgH5N1 virus infection

Inflammatory cytokines, chemokines, and cellular phenotypes were determined in BALF and lung samples collected at 7 dpi with rgH5N1 virus. (A-E) ELISA of cytokines and chemokines in BALF and lungs. (A) TNF-α. (B) IL-6. (C) IFN-γ. (D) IL-5. (E) chemokine CXCL/KC. (F-I) Phenotypes of cellular infiltrates as determined by flow cytometry. (F) Monocytes ($CD11b^+Ly6c^{hi}F4/80^+$). (G) Neutrophils ($CD11b^+Ly6c^+F4/80^-$). (H) Eosinophils ($CD11b^+CD11c^+SiglecF^+$). (I) Activated dendritic cells (aDCs, $CD45^+CD11b^+MHCII^+$). Naïve: unvaccinated mice without virus infection. sPR8: split sPR8 vaccinated mice with rgH5N1 virus infection. N1 VLP: N1 VLP vaccinated mice with rgH5N1 virus infection. Naïve inf: unvaccinated mice with rgH5N1 virus infection. Statistical significance was determined by using one-way and dunnett's multiple comparison test ANOVA. Data (n=4) are representative of individual animal out of two independent experiments. Error bars indicate the means ± SEM. *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$.

Figure 6. Mucosal IgG and antibody-secreting plasma cells in draining lymph nodes after N1 VLP vaccination and rgAH5N1 virus challenge

(A) IgG antibodies specific for A/Cal (H1N1) virus in mucosal BALF and lung samples collected at 7 dpi with rgH5N1 challenge. (B-C) IgG antibody production from *in vitro* cultures with MLN cells collected at 7 dpi. MLN day 2 culture *in vitro*-secreted IgG antibodies specific for A/Cal (H1N1) virus (B) and for rgH5N1 virus (C). (D) Total mature B cells (IgD^-CD19^+ B cells) in MLN cells collected at 7dpi. (E and F) Germinal center B cells ($GL7^+B220^+$ B cells) and plasma cells ($CD138^+B220^{+/-}$ B cells) in MLN cells collected at 7dpi were determined from gated

IgD^{-}CD19^{+}B220^{+} B cells by flow cytometry. Data (n=4) are representative of individual animal out of two independent experiments. Error bars indicate the means ± SEM. *, p<0.05, **, p<0.01, ***, p<0.001.

Figure 7. N1 VLP provides effective cross protection against rgH5N1 virus compared to HA-based split vaccines

Mice (n=4) immunized with split vaccines from A/Cal (H1N1, sCal) or A/PR8 (sPR) in 0.3 μg HA and 10 μg N1 VLP derived from A/Cal (H1N1) were intranasally infected with rgH5N1. (A) Body weigh changes. (B) Survival rates. Statistical significance was determined by using two-way ANOVA. Data are representative of individual animal out of two independent experiments. Error bars indicate the means ± SEM. *, p<0.05, **, p<0.01 in comparison of sCal and N1 VLP; ##, p<0.01, ### p<0.001 in comparison of sPR8 and sCal; p<0.001 in comparison of N1 VLP and sPR8 group.

Figure 8. Roles of FcRγ in providing protection by N1 VLP vaccination or N1 VLP immune sera

(A and B) Protection (weight changes and survival rates) against A/Cal virus in BALB/c and FcRγ KO after N1 VLP vaccination. WT BALB/c and FcRγ KO mice (n=5) that were immunized with N1 VLP were challenged with A/Cal (H1N1) at 4 weeks after boost immunization. (C and D) Protection (weight changes and survival rates) against rgH5N1 virus by N1 VLP immune sera in naive BALB/c mice (n=3). Immune sera collected from N1 VLP-immunized BALB/c mice were incubated with a lethal dose (10× LD_{50}) of rgH5N1 virus. Naïve BALB/c mice were intranasally infected with influenza virus mixed with immune sera or naïve

sera. (E and F) Protection (weight changes and survival rates) against rgH5N1 virus by N1 VLP immune sera in naive FcRγ KO mice (n=3). Naïve FcRγ KO mice were intranasally infected with influenza virus mixed with immune sera or naïve sera. Statistical significance was determined by using tow-way ANOVA. Data are representative of individual animal out of two independent experiments. Error bars indicate the means ± SEM. *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$ in comparison between N1 VLP immunization (or immune sera) and unvaccinated naïve infection (or naïve sera) in BALB/c or FcRγ KO mice.

References:

Brett, I.C., Johansson, B.E., 2005. Immunization against influenza A virus: comparison of conventional inactivated, live-attenuated and recombinant baculovirus produced purified hemagglutinin and neuraminidase vaccines in a murine model system. Virology 339, 273-280.

Bright, R.A., Carter, D.M., Crevar, C.J., Toapanta, F.R., Steckbeck, J.D., Cole, K.S., Kumar, N.M., Pushko, P., Smith, G., Tumpey, T.M., Ross, T.M., 2008. Cross-Clade Protective Immune Responses to Influenza Viruses with H5N1 HA and NA Elicited by an Influenza Virus-Like Particle. PLoS ONE 3, e1501.

Couch, R.B., Atmar, R.L., Franco, L.M., Quarles, J.M., Wells, J., Arden, N., Nino, D., Belmont, J.W., 2013. Antibody correlates and predictors of immunity to naturally occurring influenza in humans and the importance of antibody to the neuraminidase. J Infect Dis 207, 974-981.

DiLillo, D.J., Palese, P., Wilson, P.C., Ravetch, J.V., 2016. Broadly neutralizing anti-influenza antibodies require Fc receptor engagement for in vivo protection. The Journal of clinical investigation 126, 605-610.

DiLillo, D.J., Tan, G.S., Palese, P., Ravetch, J.V., 2014. Broadly neutralizing hemagglutinin stalk-specific antibodies require FcgammaR interactions for protection against influenza virus in vivo. Nature medicine 20, 143-151.

Doyle, T.M., Hashem, A.M., Li, C., Van Domselaar, G., Larocque, L., Wang, J., Smith, D., Cyr, T., Farnsworth, A., He, R., Hurt, A.C., Brown, E.G., Li, X., 2013. Universal anti-neuraminidase antibody inhibiting all influenza A subtypes. Antiviral research 100, 567-574.

Easterbrook, J.D., Schwartzman, L.M., Gao, J., Kash, J.C., Morens, D.M., Couzens, L., Wan, H., Eichelberger, M.C., Taubenberger, J.K., 2012. Protection against a lethal H5N1 influenza challenge by intranasal immunization with virus-like particles containing 2009 pandemic H1N1 neuraminidase in mice. Virology 432, 39-44.

Fries, L.F., Smith, G.E., Glenn, G.M., 2013. A recombinant viruslike particle influenza A (H7N9) vaccine. The New England journal of medicine 369, 2564-2566.

Gravel, C., Li, C., Wang, J., Hashem, A.M., Jaentschke, B., Xu, K.W., Lorbetskie, B., Gingras, G., Aubin, Y., Van Domselaar, G., Girard, M., He, R., Li, X., 2010. Qualitative and quantitative analyses of virtually all subtypes of influenza A and B viral neuraminidases using antibodies targeting the universally conserved sequences. Vaccine 28, 5774-5784.

Halbherr, S.J., Ludersdorfer, T.H., Ricklin, M., Locher, S., Berger Rentsch, M., Summerfield, A., Zimmer, G., 2015. Biological and protective properties of immune sera directed to the influenza virus neuraminidase. Journal of virology 89, 1550-1563.

Hutchinson, E.C., Charles, P.D., Hester, S.S., Thomas, B., Trudgian, D., Martinez-Alonso, M., Fodor, E., 2014. Conserved and host-specific features of influenza virion architecture. Nat Commun 5, 4816.

Jiang, L., Fantoni, G., Couzens, L., Gao, J., Plant, E., Ye, Z., Eichelberger, M.C., Wan, H., 2016. Comparative Efficacy of Monoclonal Antibodies That Bind to Different Epitopes of the 2009 Pandemic H1N1 Influenza Virus Neuraminidase. J Virol 90, 117-128.

Job, E.R., Schotsaert, M., Ibanez, L.I., Smet, A., Ysenbaert, T., Roose, K., Dai, M., de Haan, C.A.M., Kleanthous, H., Vogel, T.U., Saelens, X., 2018a. Antibodies Directed toward Neuraminidase N1 Control Disease in a Mouse Model of Influenza. J Virol 92.

Job, E.R., Ysenbaert, T., Smet, A., Christopoulou, I., Strugnell, T., Oloo, E.O., Oomen, R.P., Kleanthous, H., Vogel, T.U., Saelens, X., 2018b. Broadened immunity against influenza by vaccination with computationally designed influenza virus N1 neuraminidase constructs. NPJ Vaccines 3, 55.

Johansson, B.E., 1999. Immunization with influenza A virus hemagglutinin and neuraminidase produced in recombinant baculovirus results in a balanced and broadened immune response superior to conventional vaccine. Vaccine 17, 2073-2080.

Johansson, B.E., Kilbourne, E.D., 1993. Dissociation of influenza virus hemagglutinin and neuraminidase eliminates their intravirionic antigenic competition. J Virol 67, 5721-5723.

Johansson, B.E., Moran, T.M., Kilbourne, E.D., 1987. Antigen-presenting B cells and helper T cells cooperatively mediate intravirionic antigenic competition between influenza A virus surface glycoproteins. Proc Natl Acad Sci U S A 84, 6869-6873.

Johansson, B.E., Pokorny, B.A., Tiso, V.A., 2002. Supplementation of conventional trivalent influenza vaccine with purified viral N1 and N2 neuraminidases induces a balanced immune response without antigenic competition. Vaccine 20, 1670-1674.

Khurana, S., Wu, J., Verma, N., Verma, S., Raghunandan, R., Manischewitz, J., King, L.R., Kpamegan, E., Pincus, S., Smith, G., Glenn, G., Golding, H., 2011. H5N1 virus-like particle vaccine elicits cross-reactive neutralizing antibodies that preferentially bind to the oligomeric form of influenza virus hemagglutinin in humans. Journal of virology 85, 10945-10954.

Kilbourne, E.D., Johansson, B.E., Grajower, B., 1990. Independent and disparate evolution in nature of influenza A virus hemagglutinin and neuraminidase glycoproteins. Proc Natl Acad Sci U S A 87, 786-790.

Kim, K.H., Kwon, Y.M., Lee, Y.T., Hwang, H.S., Kim, M.C., Ko, E.J., Wang, B.Z., Quan, F.S., Kang, S.M., 2018a. Virus-like particles presenting flagellin exhibit unique adjuvant effects on eliciting T helper type 1 humoral and cellular immune responses to poor immunogenic influenza virus M2e protein vaccine. Virology 524, 172-181.

Kim, K.H., Kwon, Y.M., Lee, Y.T., Kim, M.C., Hwang, H.S., Ko, E.J., Lee, Y., Choi, H.J., Kang, S.M., 2018b. Virus-Like Particles Are a Superior Platform for Presenting M2e Epitopes to Prime Humoral and Cellular Immunity against Influenza Virus. Vaccines (Basel) 6.

Kim, K.H., Lee, Y.T., Hwang, H.S., Kwon, Y.M., Kim, M.C., Ko, E.J., Lee, J.S., Lee, Y., Kang, S.M., 2015. Virus-like particle vaccine containing the F protein of respiratory syncytial virus confers protection without pulmonary disease by modulating specific subsets of dendritic cells and effector T cells. Journal of virology.

Kim, M.C., Lee, Y.N., Ko, E.J., Lee, J.S., Kwon, Y.M., Hwang, H.S., Song, J.M., Song, B.M., Lee, Y.J., Choi, J.G., Kang, H.M., Quan, F.S., Compans, R.W., Kang, S.M., 2014. Supplementation of influenza split vaccines with conserved M2 ectodomains overcomes strain specificity and provides long-term cross protection. Molecular therapy : the journal of the American Society of Gene Therapy 22, 1364-1374.

Kim, Y.J., Ko, E.J., Kim, M.C., Lee, Y.N., Kim, K.H., Jung, Y.J., Kang, S.M., 2017. Roles of antibodies to influenza A virus hemagglutinin, neuraminidase, and M2e in conferring cross protection. Biochem Biophys Res Commun 493, 393-398.

Ko, E.J., Lee, Y., Lee, Y.T., Kim, Y.J., Kim, K.H., Kang, S.M., 2018. MPL and CpG combination adjuvants promote homologous and heterosubtypic cross protection of inactivated split influenza virus vaccine. Antiviral Res 156, 107-115.

Ko, E.J., Lee, Y.T., Kim, K.H., Lee, Y., Jung, Y.J., Kim, M.C., Lee, Y.N., Kang, T., Kang, S.M., 2017. Roles of Aluminum Hydroxide and Monophosphoryl Lipid A Adjuvants in Overcoming CD4+ T Cell Deficiency To Induce Isotype-Switched IgG Antibody Responses and Protection by T-Dependent Influenza Vaccine. Journal of immunology 198, 279-291.

Krammer, F., Fouchier, R.A.M., Eichelberger, M.C., Webby, R.J., Shaw-Saliba, K., Wan, H., Wilson, P.C., Compans, R.W., Skountzou, I., Monto, A.S., 2018. NAction! How Can Neuraminidase-Based Immunity Contribute to Better Influenza Virus Vaccines? MBio 9.

Lee, Y.N., Lee, Y.T., Kim, M.C., Gewirtz, A.T., Kang, S.M., 2016. A Novel Vaccination Strategy Mediating the Induction of Lung-Resident Memory CD8 T Cells Confers Heterosubtypic Immunity against Future Pandemic Influenza Virus. Journal of immunology 196, 2637-2645.

Lee, Y.N., Lee, Y.T., Kim, M.C., Hwang, H.S., Lee, J.S., Kim, K.H., Kang, S.M., 2014. Fc receptor is not required for inducing antibodies but plays a critical role in conferring protection after influenza M2 vaccination. Immunology 143, 300-309.

Liu, W.C., Lin, C.Y., Tsou, Y.T., Jan, J.T., Wu, S.C., 2015. Cross-Reactive Neuraminidase-Inhibiting Antibodies Elicited by Immunization with Recombinant Neuraminidase Proteins of H5N1 and Pandemic H1N1 Influenza A Viruses. Journal of virology 89, 7224-7234.

Lopez-Macias, C., Ferat-Osorio, E., Tenorio-Calvo, A., Isibasi, A., Talavera, J., Arteaga-Ruiz, O., Arriaga-Pizano, L., Hickman, S.P., Allende, M., Lenhard, K., Pincus, S., Connolly, K., Raghunandan, R., Smith, G., Glenn, G., 2011. Safety and immunogenicity of a virus-like particle pandemic influenza A (H1N1) 2009 vaccine in a blinded, randomized, placebo-controlled trial of adults in Mexico. Vaccine 29, 7826-7834.

Memoli, M.J., Shaw, P.A., Han, A., Czajkowski, L., Reed, S., Athota, R., Bristol, T., Fargis, S., Risos, K., Powers, J.H., Davey, R.T., Jr., Taubenberger, J.K., 2016. Evaluation of Antihemagglutinin and Antineuraminidase Antibodies as Correlates of Protection in an Influenza A/H1N1 Virus Healthy Human Challenge Model. MBio 7, e00417-00416.

Monto, A.S., Petrie, J.G., Cross, R.T., Johnson, E., Liu, M., Zhong, W., Levine, M., Katz, J.M., Ohmit, S.E., 2015. Antibody to Influenza Virus Neuraminidase: An Independent Correlate of Protection. The Journal of infectious diseases 212, 1191-1199.

Mooney, A.J., Gabbard, J.D., Li, Z., Dlugolenski, D.A., Johnson, S.K., Tripp, R.A., He, B., Tompkins, S.M., 2017. Vaccination with Recombinant Parainfluenza Virus 5 Expressing Neuraminidase Protects against Homologous and Heterologous Influenza Virus Challenge. J Virol 91.

Mullarkey, C.E., Bailey, M.J., Golubeva, D.A., Tan, G.S., Nachbagauer, R., He, W., Novakowski, K.E., Bowdish, D.M., Miller, M.S., Palese, P., 2016. Broadly Neutralizing Hemagglutinin Stalk-Specific Antibodies Induce Potent Phagocytosis of Immune Complexes by Neutrophils in an Fc-Dependent Manner. MBio 7.

Murphy, B.R., Kasel, J.A., Chanock, R.M., 1972. Association of serum anti-neuraminidase antibody with resistance to influenza in man. N Engl J Med 286, 1329-1332.

Qiu, M., Fang, F., Chen, Y., Wang, H., Chen, Q., Chang, H., Wang, F., Wang, H., Zhang, R., Chen, Z., 2006. Protection against avian influenza H9N2 virus challenge by immunization with hemagglutinin- or neuraminidase-expressing DNA in BALB/c mice. Biochem Biophys Res Commun 343, 1124-1131.

Quan, F.S., Compans, R.W., Nguyen, H.H., Kang, S.M., 2008. Induction of heterosubtypic immunity to influenza virus by intranasal immunization. J Virol 82, 1350-1359.

Quan, F.S., Kim, M.C., Lee, B.J., Song, J.M., Compans, R.W., Kang, S.M., 2012. Influenza M1 VLPs containing neuraminidase induce heterosubtypic cross-protection. Virology 430, 127-135.

Quan, F.S., Li, Z.N., Kim, M.C., Yang, D., Compans, R.W., Steinhauer, D.A., Kang, S.M., 2011. Immunogenicity of low-pH treated whole viral influenza vaccine. Virology 417, 196-202.
Quan, F.S., Vunnava, A., Compans, R.W., Kang, S.M., 2010. Virus-Like Particle Vaccine Protects against 2009 H1N1 Pandemic Influenza Virus in Mice. PLoS One 5, e9161.
Sakai, T., Nishimura, S.I., Naito, T., Saito, M., 2017. Influenza A virus hemagglutinin and neuraminidase act as novel motile machinery. Scientific reports 7, 45043.
Sandbulte, M.R., Jimenez, G.S., Boon, A.C., Smith, L.R., Treanor, J.J., Webby, R.J., 2007. Cross-Reactive Neuraminidase Antibodies Afford Partial Protection against H5N1 in Mice and Are Present in Unexposed Humans. PLoS Med 4, e59.
Sandbulte, M.R., Westgeest, K.B., Gao, J., Xu, X., Klimov, A.I., Russell, C.A., Burke, D.F., Smith, D.J., Fouchier, R.A., Eichelberger, M.C., 2011. Discordant antigenic drift of neuraminidase and hemagglutinin in H1N1 and H3N2 influenza viruses. Proc Natl Acad Sci U S A 108, 20748-20753.
Smith, G.E., Sun, X., Bai, Y., Liu, Y.V., Massare, M.J., Pearce, M.B., Belser, J.A., Maines, T.R., Creager, H.M., Glenn, G.M., Flyer, D., Pushko, P., Levine, M.Z., Tumpey, T.M., 2017. Neuraminidase-based recombinant virus-like particles protect against lethal avian influenza A(H5N1) virus infection in ferrets. Virology 509, 90-97.
Song, J.M., Kim, Y.C., Barlow, P.G., Hossain, M.J., Park, K.M., Donis, R.O., Prausnitz, M.R., Compans, R.W., Kang, S.M., 2010. Improved protection against avian influenza H5N1 virus by a single vaccination with virus-like particles in skin using microneedles. Antiviral Res 88, 244-247.
Song, J.M., Van Rooijen, N., Bozja, J., Compans, R.W., Kang, S.M., 2011. Vaccination inducing broad and improved cross protection against multiple subtypes of influenza A virus. Proc Natl Acad Sci U S A 108, 757-761.
Su, B., Wurtzer, S., Rameix-Welti, M.A., Dwyer, D., van der Werf, S., Naffakh, N., Clavel, F., Labrosse, B., 2009. Enhancement of the influenza A hemagglutinin (HA)-mediated cell-cell fusion and virus entry by the viral neuraminidase (NA). PLoS One 4, e8495.
Tumpey, T.M., Renshaw, M., Clements, J.D., Katz, J.M., 2001. Mucosal delivery of inactivated influenza vaccine induces B-cell-dependent heterosubtypic cross-protection against lethal influenza A H5N1 virus infection. J Virol 75, 5141-5150.
Van den Hoecke, S., Ehrhardt, K., Kolpe, A., El Bakkouri, K., Deng, L., Grootaert, H., Schoonooghe, S., Smet, A., Bentahir, M., Roose, K., Schotsaert, M., Schepens, B., Callewaert, N., Nimmerjahn, F., Staeheli, P., Hengel, H., Saelens, X., 2017. Hierarchical and Redundant Roles of Activating FcgammaRs in Protection against Influenza Disease by M2e-Specific IgG1 and IgG2a Antibodies. J Virol 91.
Walz, L., Kays, S.K., Zimmer, G., von Messling, V., 2018. Neuraminidase-Inhibiting Antibody Titers Correlate with Protection from Heterologous Influenza Virus Strains of the Same Neuraminidase Subtype. J Virol 92.
Wan, H., Gao, J., Xu, K., Chen, H., Couzens, L.K., Rivers, K.H., Easterbrook, J.D., Yang, K., Zhong, L., Rajabi, M., Ye, J., Sultana, I., Wan, X.F., Liu, X., Perez, D.R., Taubenberger, J.K., Eichelberger, M.C., 2013. Molecular basis for broad neuraminidase immunity: conserved epitopes in seasonal and pandemic H1N1 as well as H5N1 influenza viruses. J Virol 87, 9290-9300.
Wohlbold, T.J., Chromikova, V., Tan, G.S., Meade, P., Amanat, F., Comella, P., Hirsh, A., Krammer, F., 2016. Hemagglutinin Stalk- and Neuraminidase-Specific Monoclonal Antibodies Protect against Lethal H10N8 Influenza Virus Infection in Mice. J Virol 90, 851-861.
Wohlbold, T.J., Krammer, F., 2014. In the shadow of hemagglutinin: a growing interest in influenza viral neuraminidase and its role as a vaccine antigen. Viruses 6, 2465-2494.
Wohlbold, T.J., Nachbagauer, R., Xu, H., Tan, G.S., Hirsh, A., Brokstad, K.A., Cox, R.J., Palese, P., Krammer, F., 2015. Vaccination with adjuvanted recombinant neuraminidase induces broad heterologous, but not heterosubtypic, cross-protection against influenza virus infection in mice. MBio 6, e02556.
Wohlbold, T.J., Podolsky, K.A., Chromikova, V., Kirkpatrick, E., Falconieri, V., Meade, P., Amanat, F., Tan, J., tenOever, B.R., Tan, G.S., Subramaniam, S., Palese, P., Krammer, F., 2017. Broadly protective murine

monoclonal antibodies against influenza B virus target highly conserved neuraminidase epitopes. Nat Microbiol 2, 1415-1424.

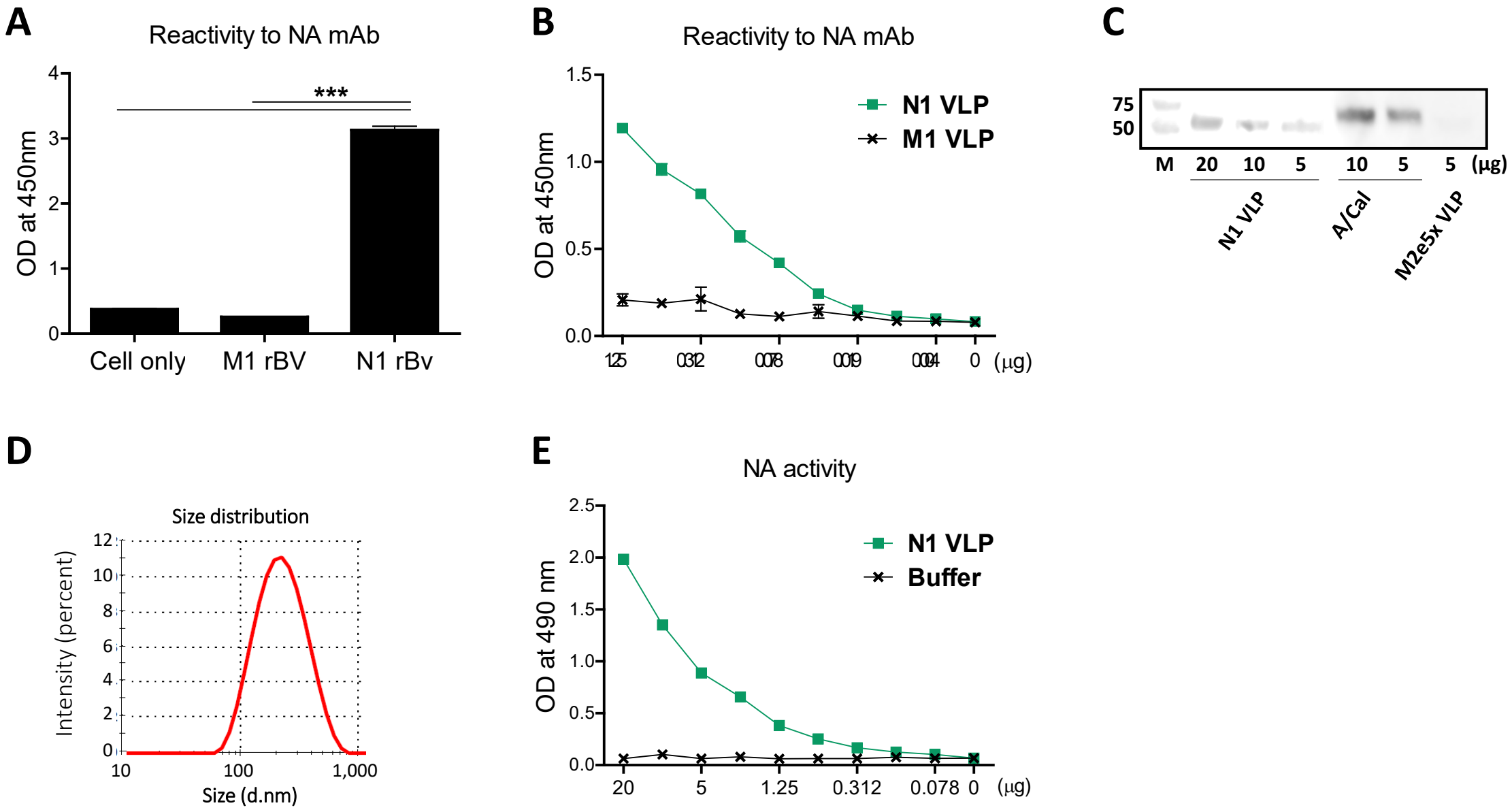

Figure 2

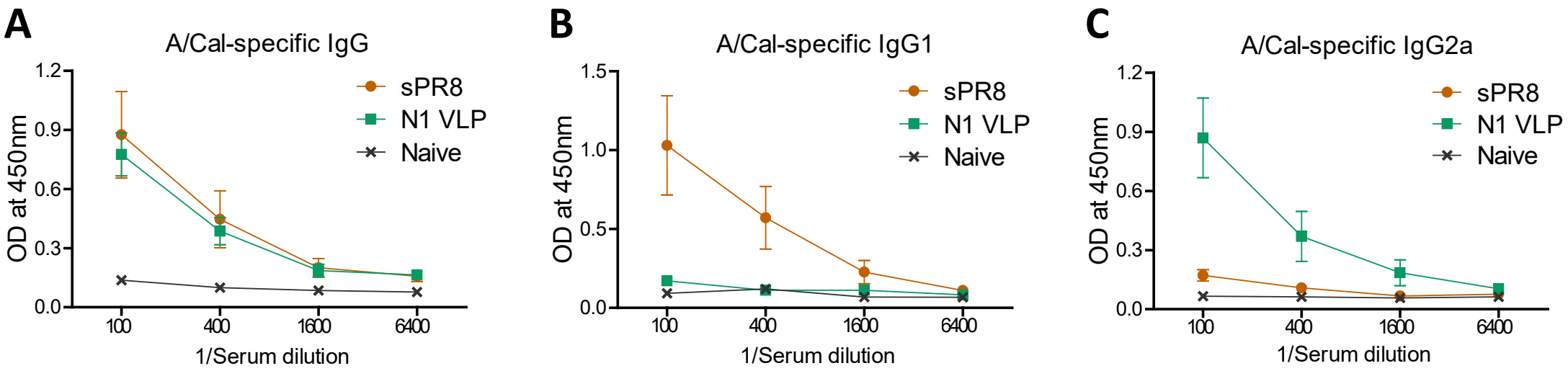
A
A/Cal-specific IgG
OD at 450nm
1.2
0.9
0.6
0.3
0.0
100
400
1600
6400
1/Serum dilution
sPR8
N1 VLP
Naive
B
A/Cal-specific IgG1
OD at 450nm
1.5
1.0
0.5
0.0
100
400
1600
6400
1/Serum dilution
sPR8
N1 VLP
Naive
C
A/Cal-specific IgG2a
OD at 450nm
1.2
0.9
0.6
0.3
0.0
100
400
1600
6400
1/Serum dilution
sPR8
N1 VLP
Naive

Figure 3

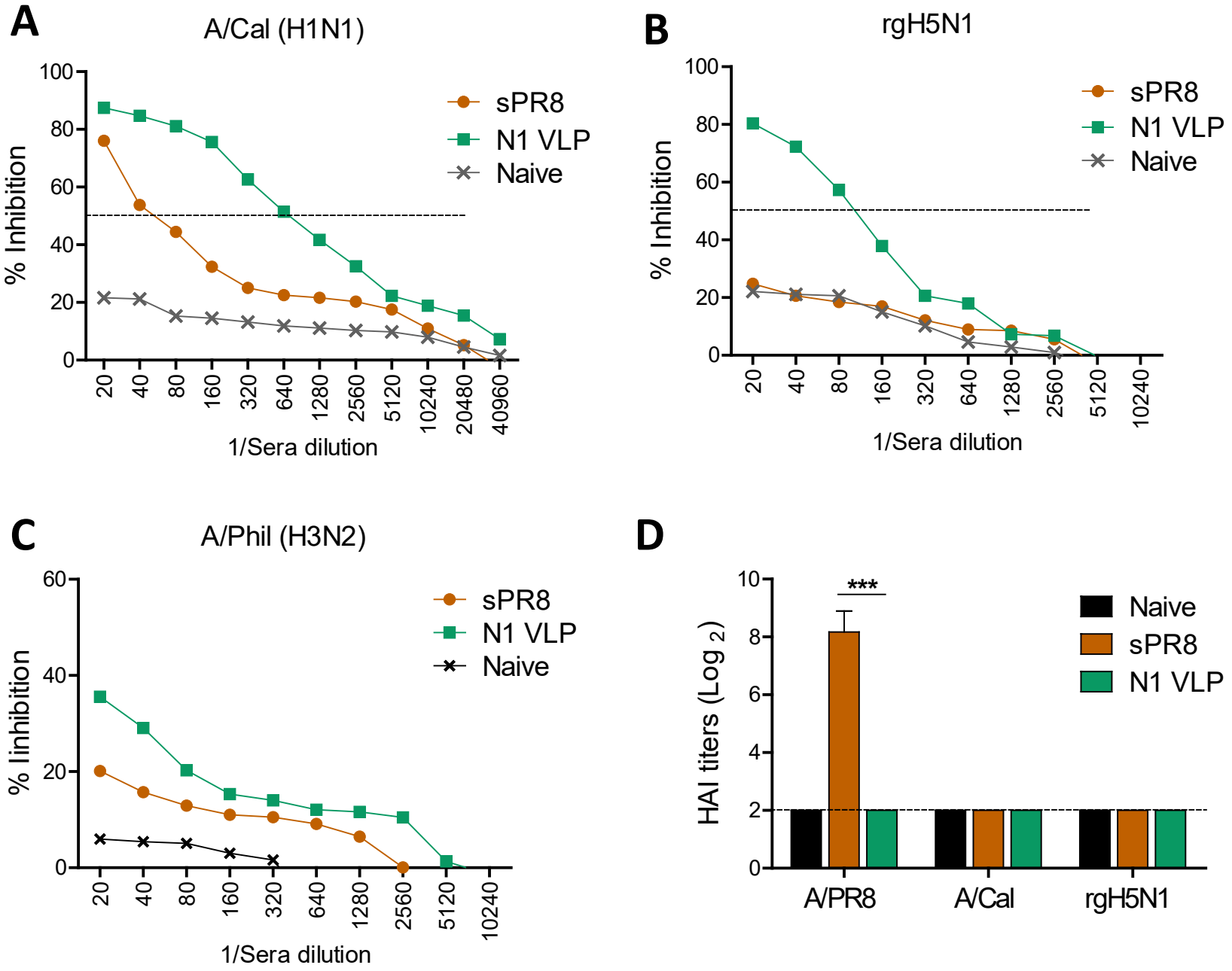
A
A/Cal (H1N1)
% Inhibition
100
80
60
40
20
0
20
40
80
160
320
640
1280
2560
5120
10240
20480
40960
1/Sera dilution
sPR8
N1 VLP
Naive
B
rgH5N1
% Inhibition
1/Sera dilution
sPR8
N1 VLP
Naive
C
A/Phil (H3N2)
% Iinhibition
1/Sera dilution
sPR8
N1 VLP
Naive
D
HAI titers (Log 2)
***
Naive
sPR8
N1 VLP
A/PR8
A/Cal
rgH5N1

Figure 4

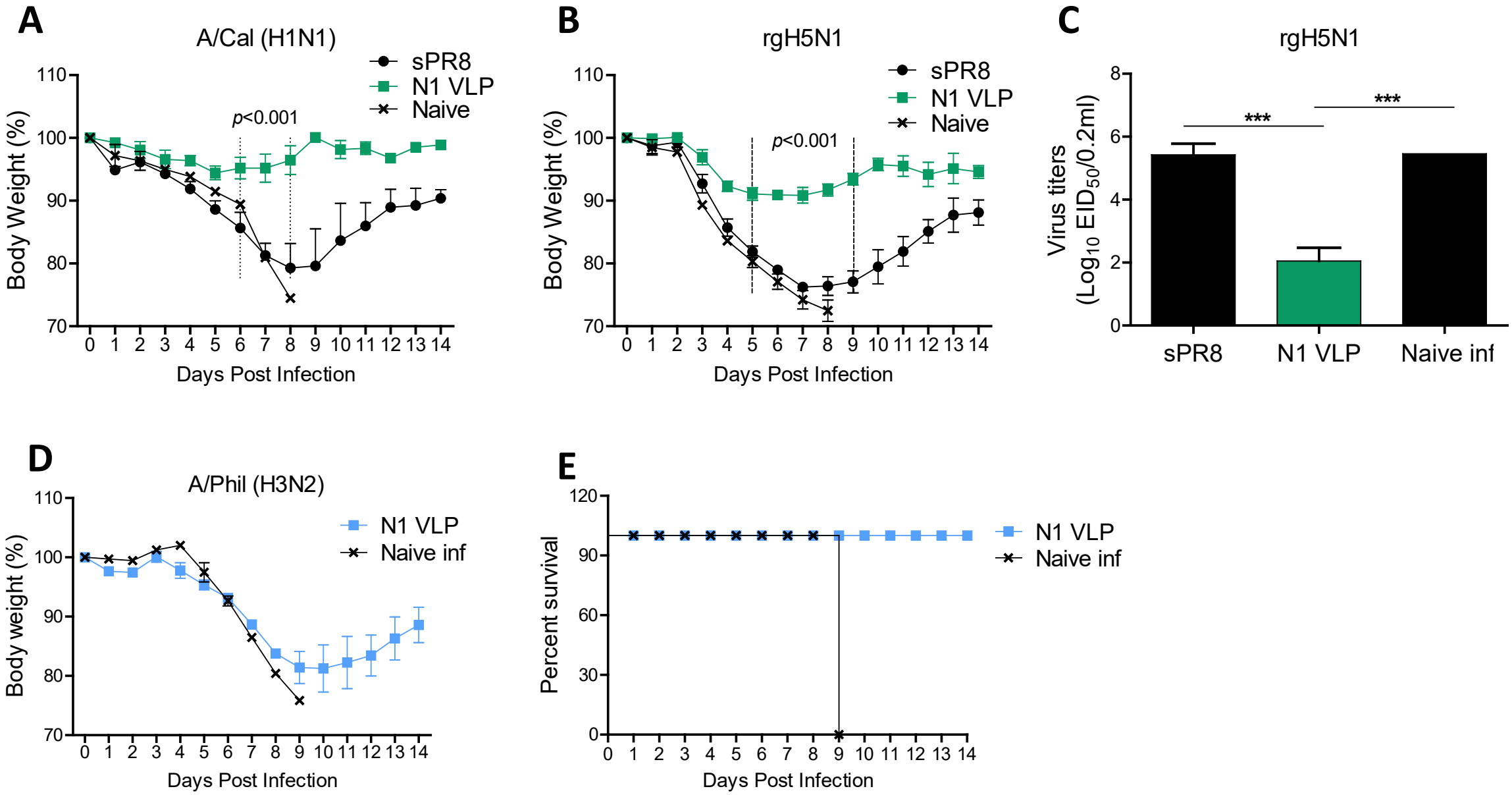
A
A/Cal (H1N1)
sPR8
N1 VLP
Naive
p<0.001
Body Weight (%)
Days Post Infection
B
rgH5N1
sPR8
N1 VLP
Naive
p<0.001
Body Weight (%)
Days Post Infection
C
rgH5N1
***
***
Virus titers
(Log10 EID50/0.2ml)
sPR8
N1 VLP
Naive inf
D
A/Phil (H3N2)
N1 VLP
Naive inf
Body weight (%)
Days Post Infection
E
N1 VLP
Naive inf
Percent survival
Days Post Infection

Figure 5

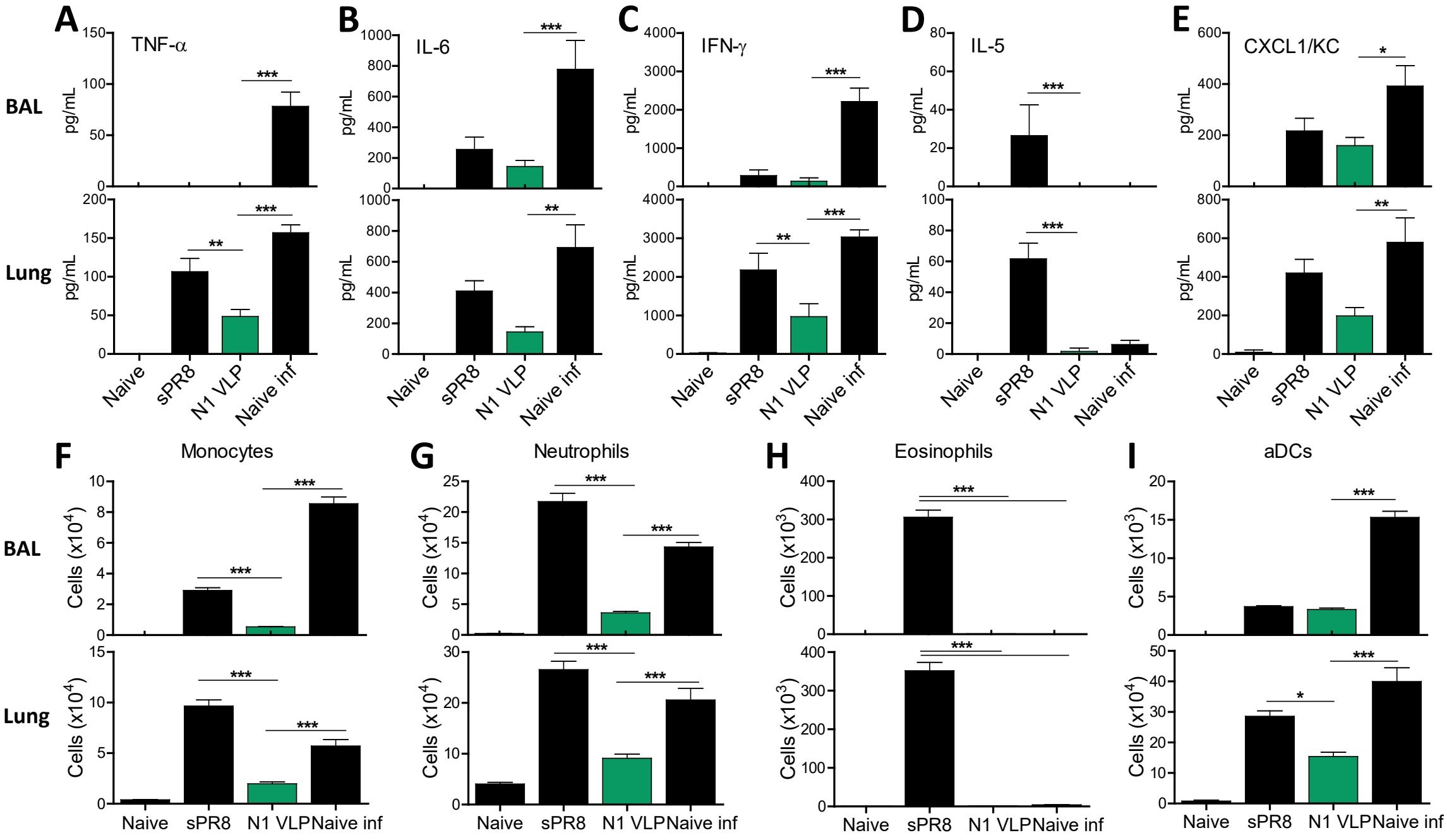
A
TNF-α
B
IL-6
C
IFN-γ
D
IL-5
E
CXCL1/KC
BAL
Lung
pg/mL
Naive
sPR8
N1 VLP
Naive inf
F
Monocytes
G
Neutrophils
H
Eosinophils
I
aDCs
Cells (x10^4)
Cells (x10^3)

Figure 6

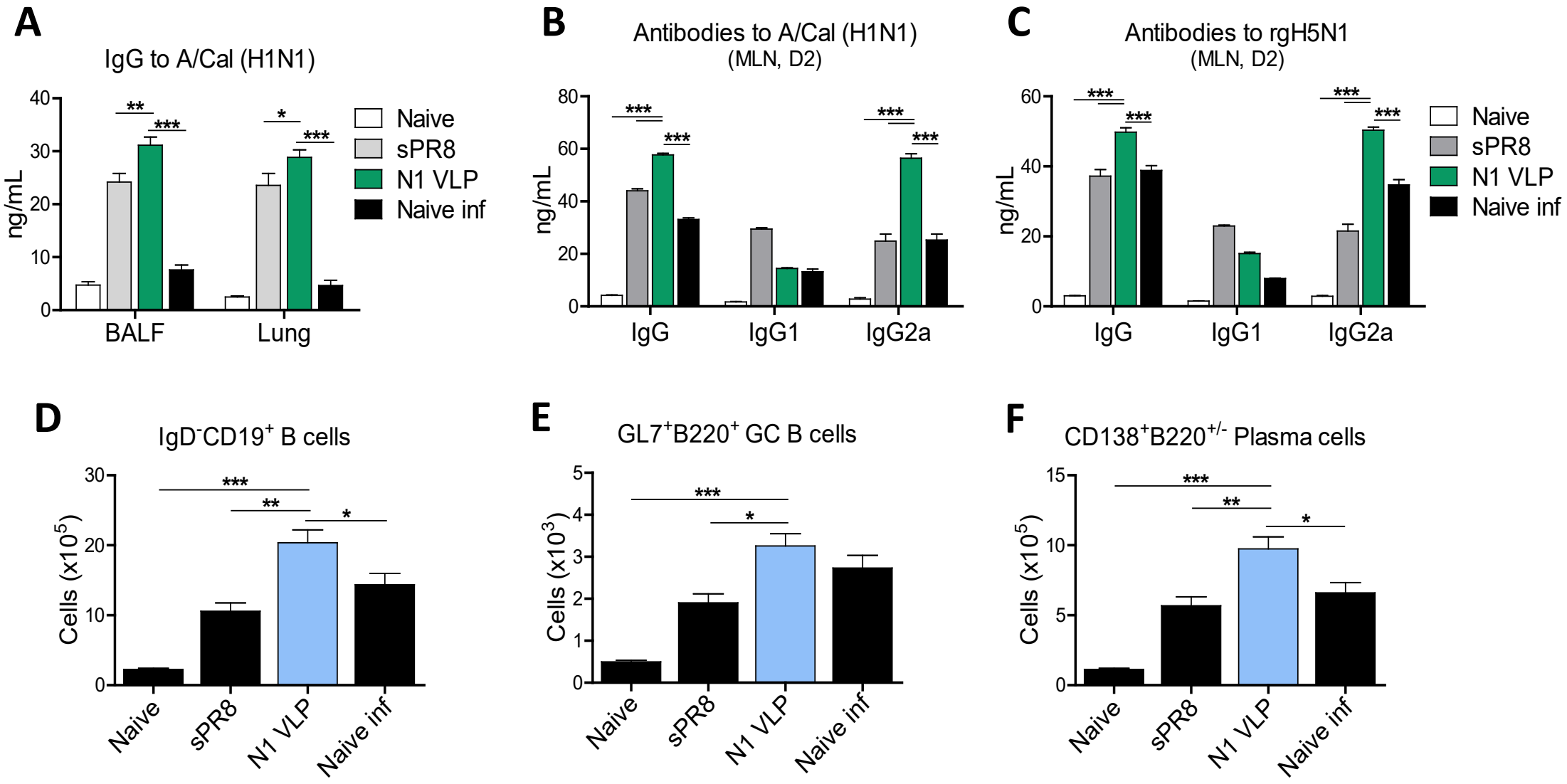
A
IgG to A/Cal (H1N1)
ng/mL
40
30
20
10
0
**
***
*
***
BALF
Lung
Naive
sPR8
N1 VLP
Naive inf
B
Antibodies to A/Cal (H1N1)
(MLN, D2)
ng/mL
80
60
40
20
0
***
***
***
***
IgG
IgG1
IgG2a
C
Antibodies to rgH5N1
(MLN, D2)
ng/mL
60
40
20
0
***
***
***
***
IgG
IgG1
IgG2a
Naive
sPR8
N1 VLP
Naive inf
D
IgD-CD19+ B cells
Cells (x10^5)
30
20
10
0
***
**
*
Naive
sPR8
N1 VLP
Naive inf
E
GL7+B220+ GC B cells
Cells (x10^3)
5
4
3
2
1
0
***
*
Naive
sPR8
N1 VLP
Naive inf
F
CD138+B220+/- Plasma cells
Cells (x10^5)
15
10
5
0
***
**
*
Naive
sPR8
N1 VLP
Naive inf

Figure 7

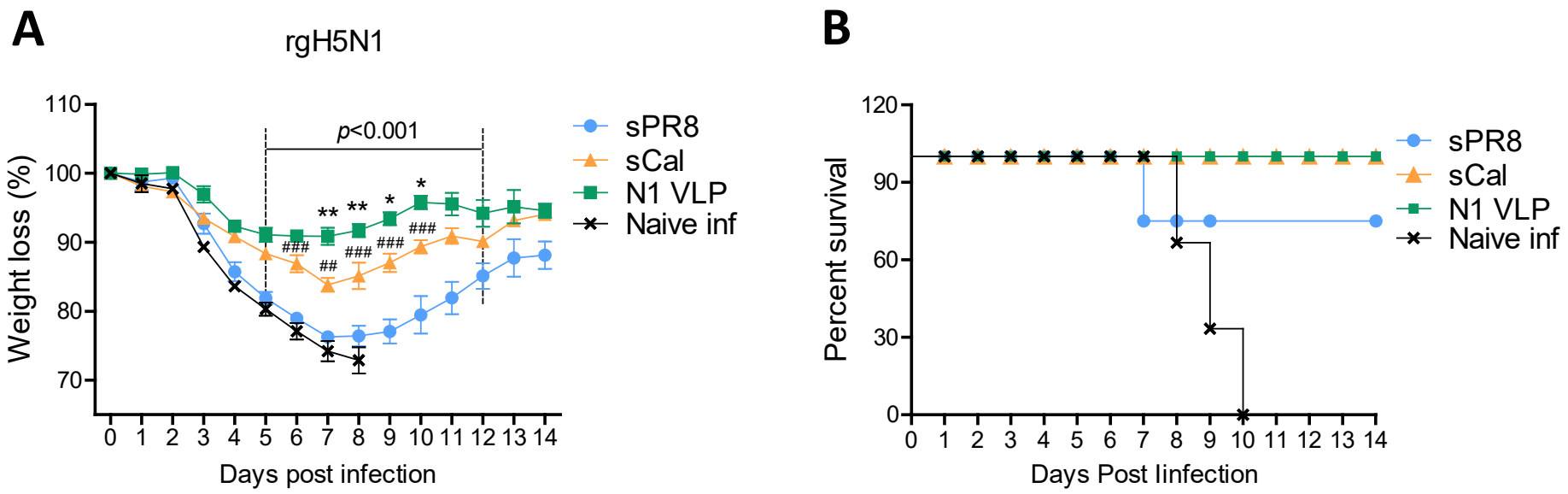
A
rgH5N1
Weight loss (%)
110
100
90
80
70
p<0.001
**
**
*
*
###
##
###
###
###
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Days post infection
sPR8
sCal
N1 VLP
Naive inf
B
Percent survival
120
90
60
30
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Days Post Iinfection
sPR8
sCal
N1 VLP
Naive inf

Figure 8

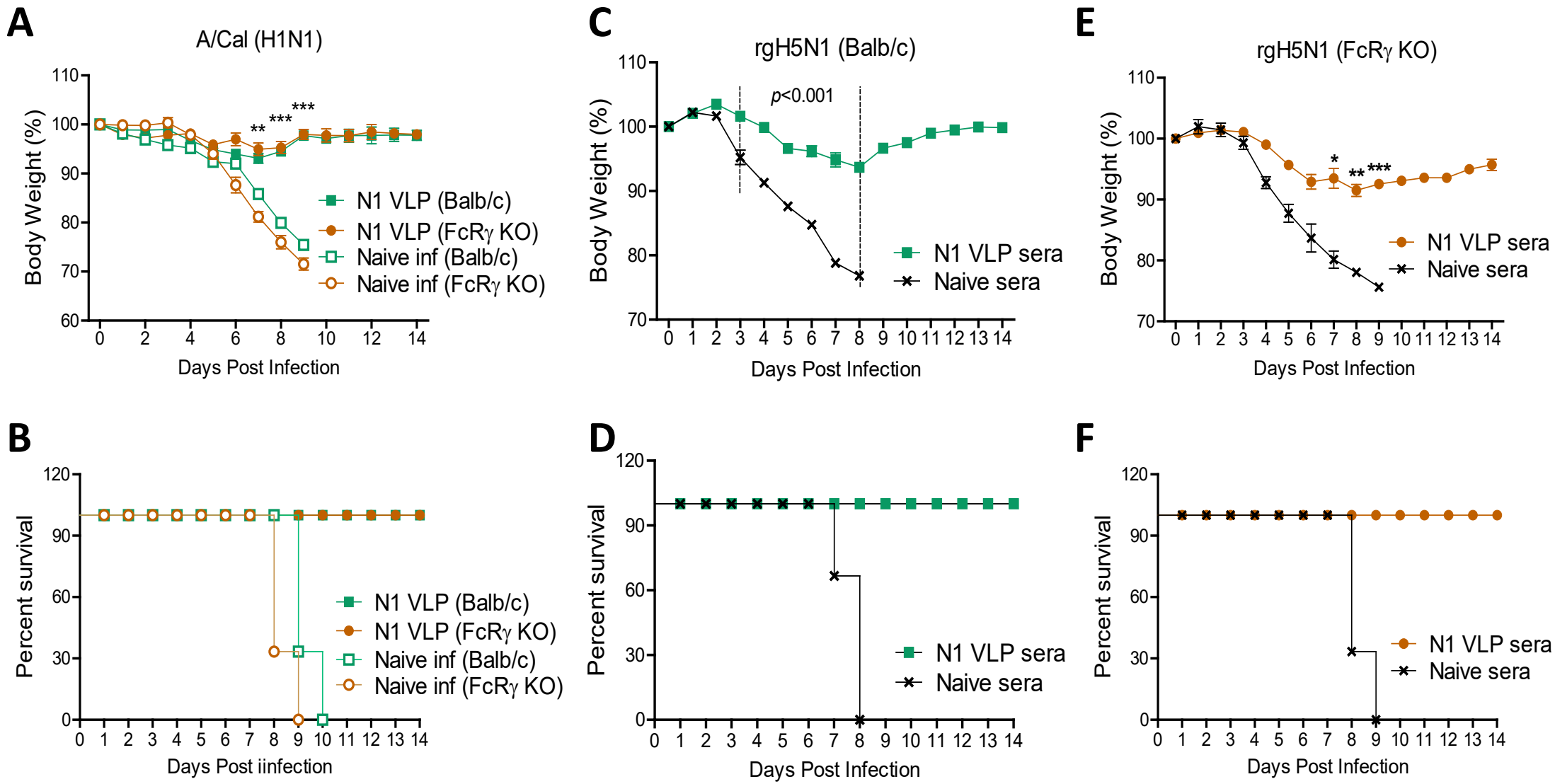
A
A/Cal (H1N1)
Body Weight (%)
Days Post Infection
N1 VLP (Balb/c)
N1 VLP (FcRγ KO)
Naive inf (Balb/c)
Naive inf (FcRγ KO)
**
***
***
B
Percent survival
Days Post iinfection
N1 VLP (Balb/c)
N1 VLP (FcRγ KO)
Naive inf (Balb/c)
Naive inf (FcRγ KO)
C
rgH5N1 (Balb/c)
p<0.001
Body Weight (%)
Days Post Infection
N1 VLP sera
Naive sera
D
Percent survival
Days Post Infection
N1 VLP sera
Naive sera
E
rgH5N1 (FcRγ KO)
Body Weight (%)
Days Post Infection
*
**
***
N1 VLP sera
Naive sera
F
Percent survival
Days Post Infection
N1 VLP sera
Naive sera

Figure 1

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Figure 1

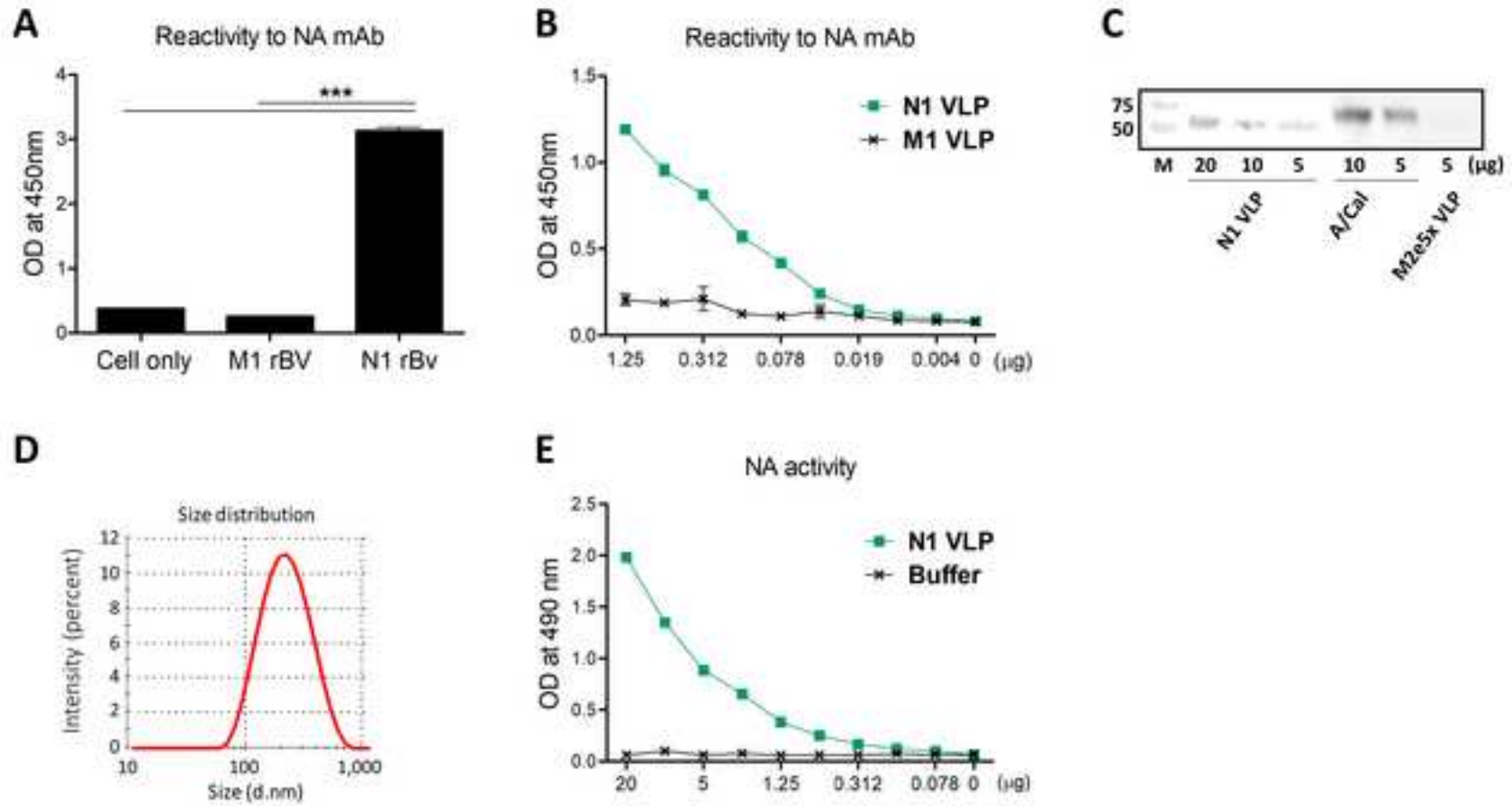

Figure 2

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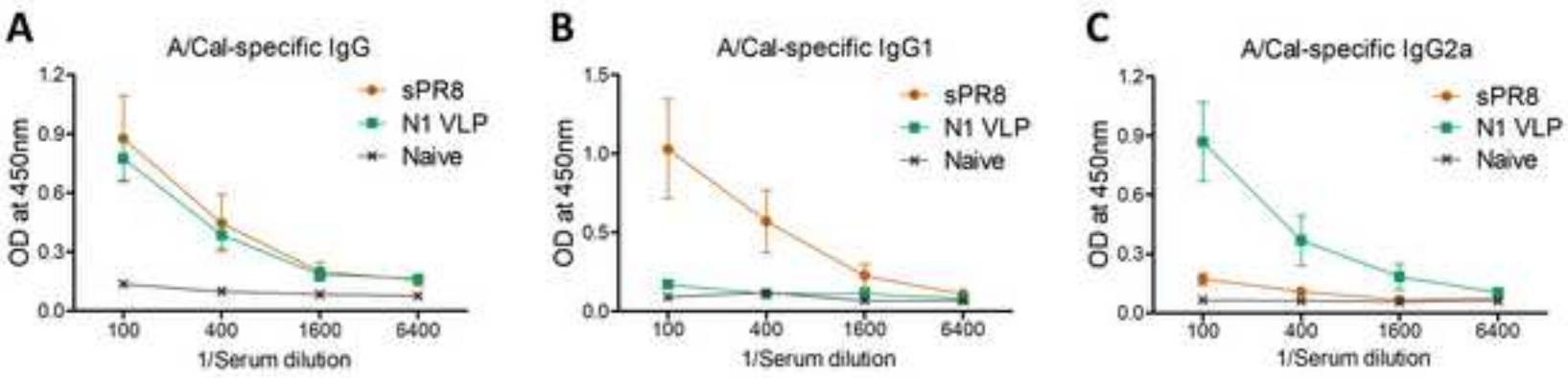

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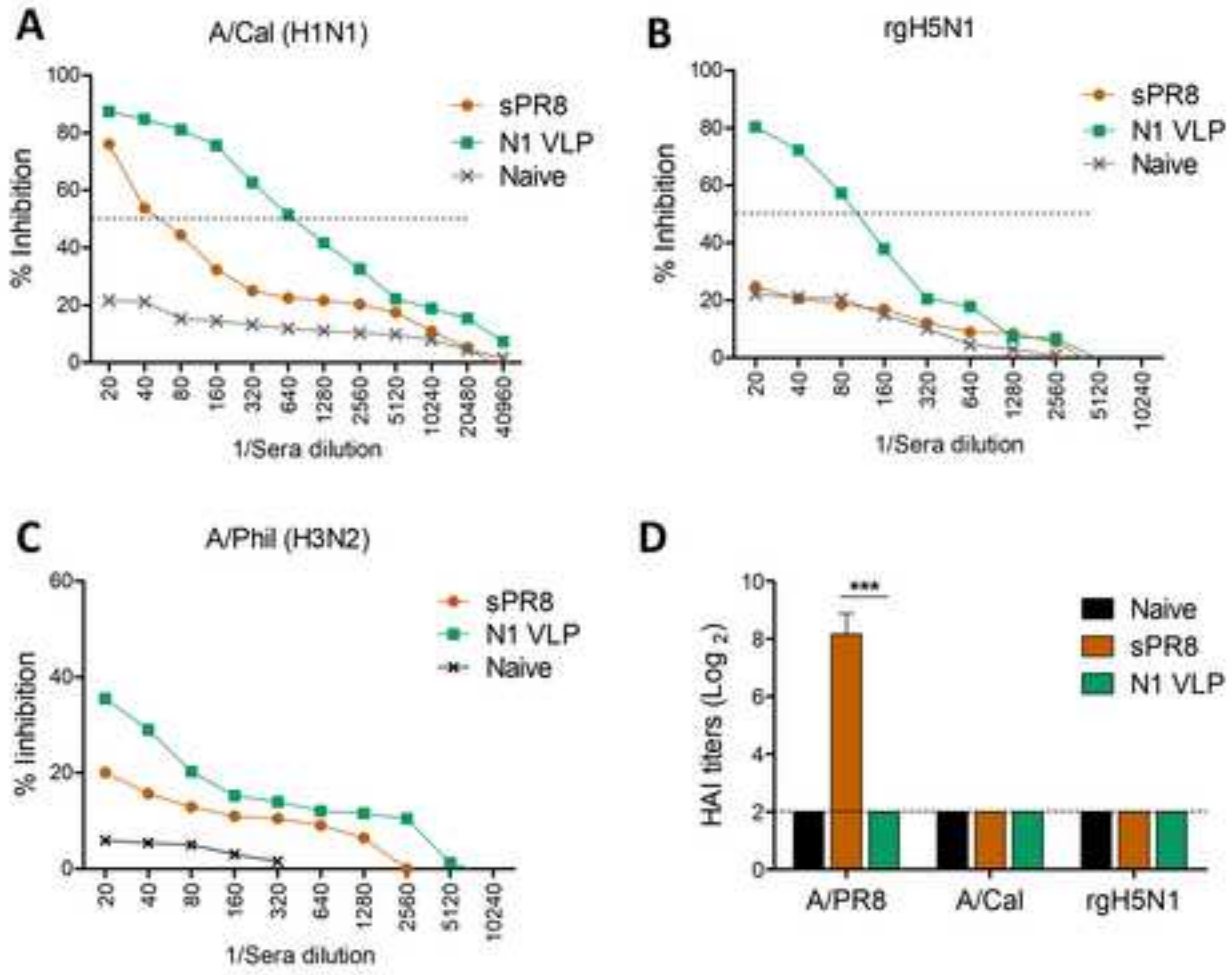

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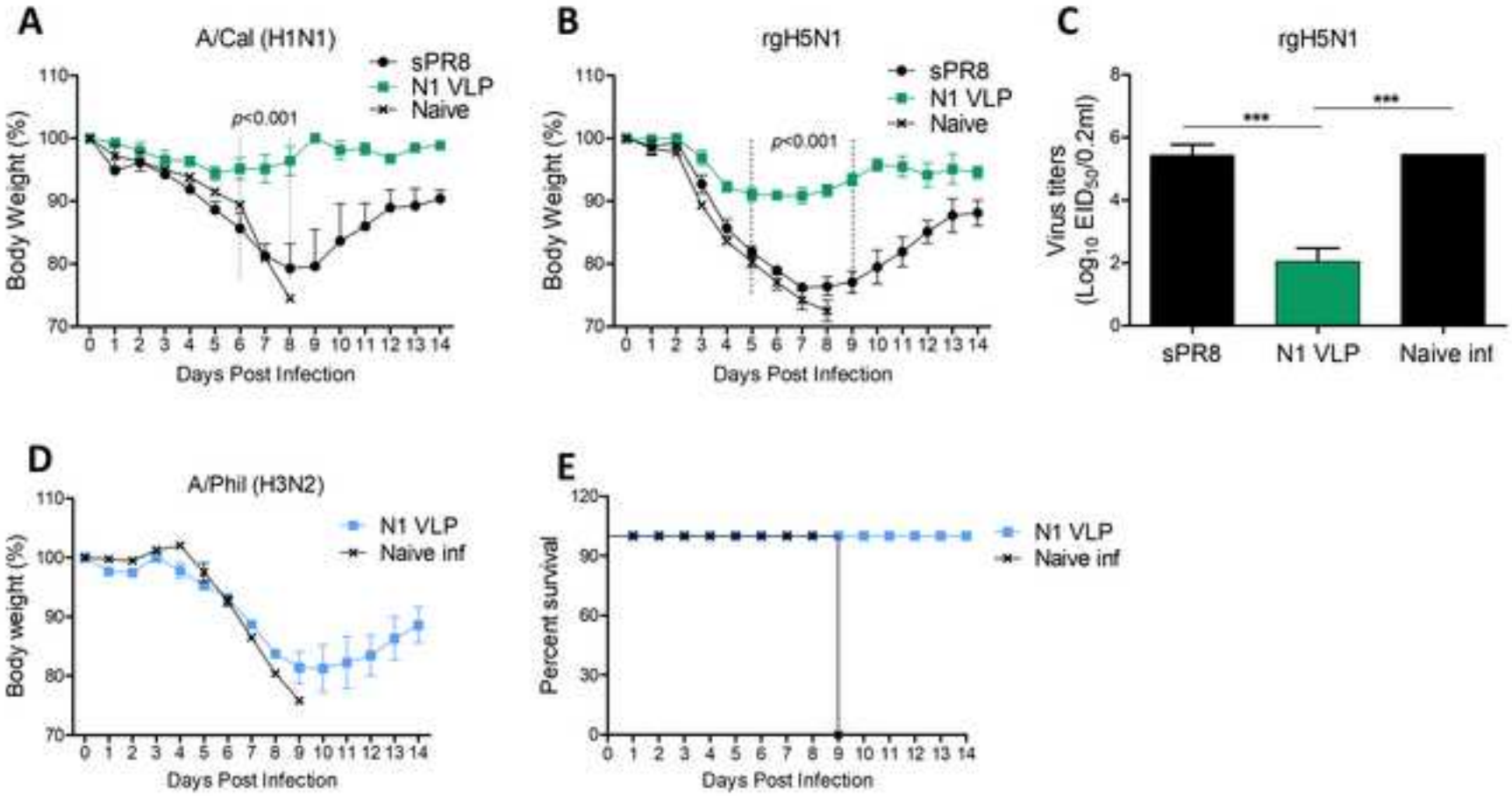

Figure 5

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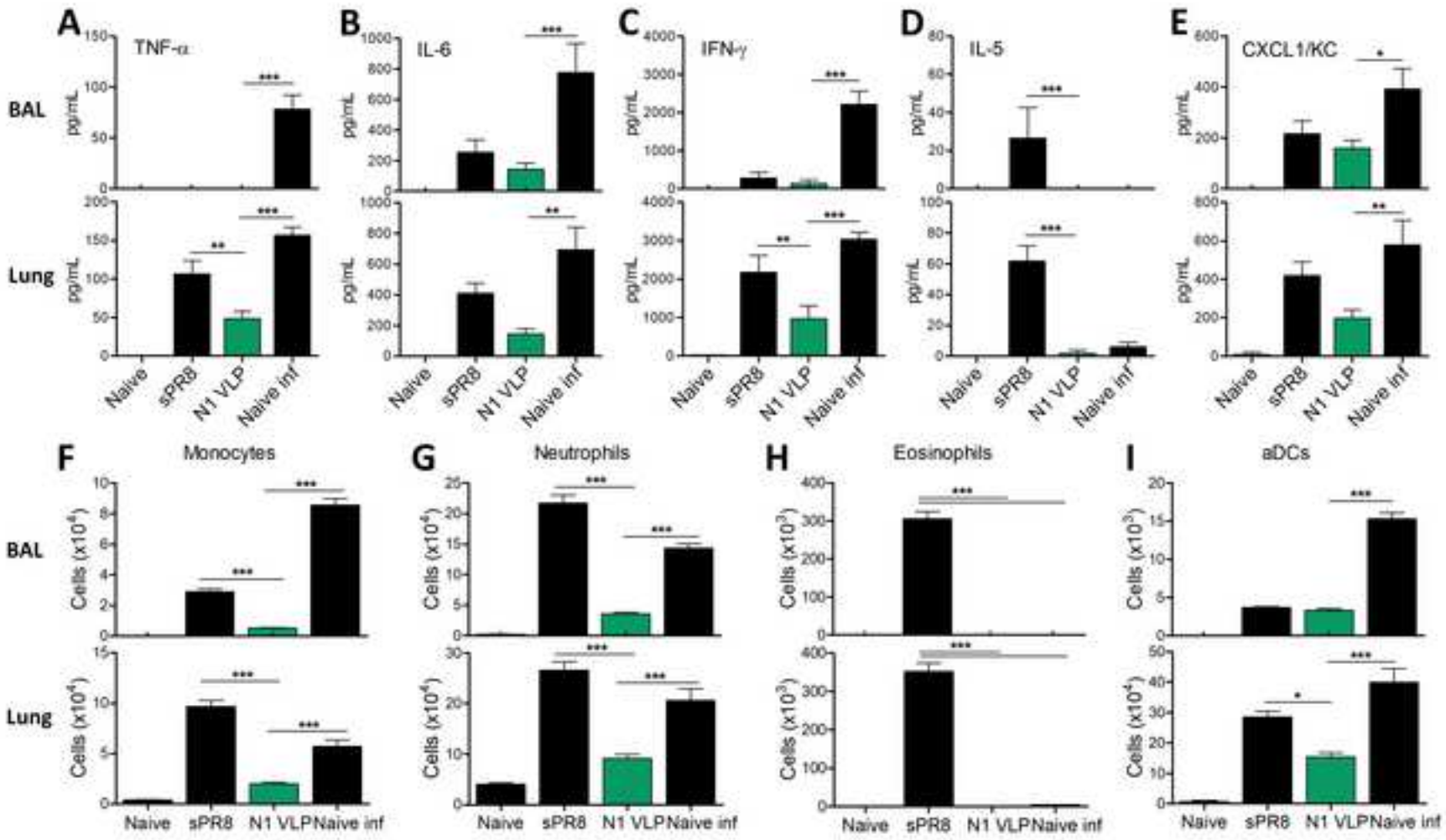

Figure 6

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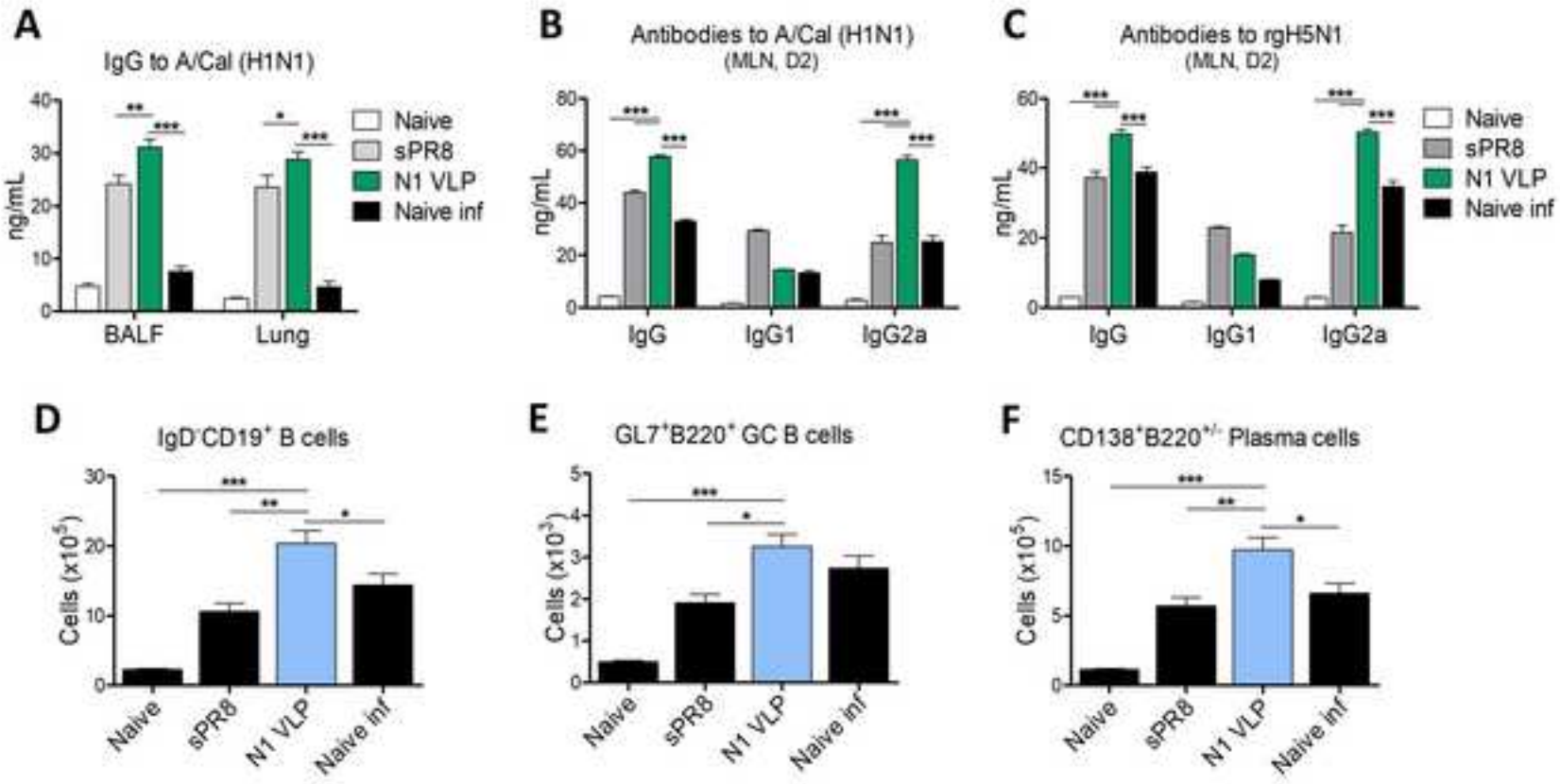

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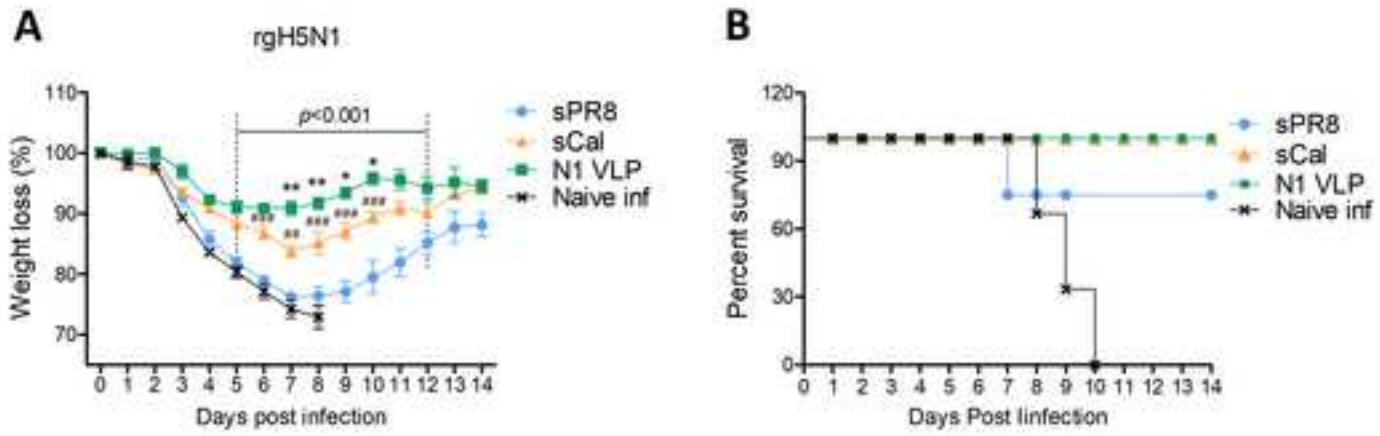

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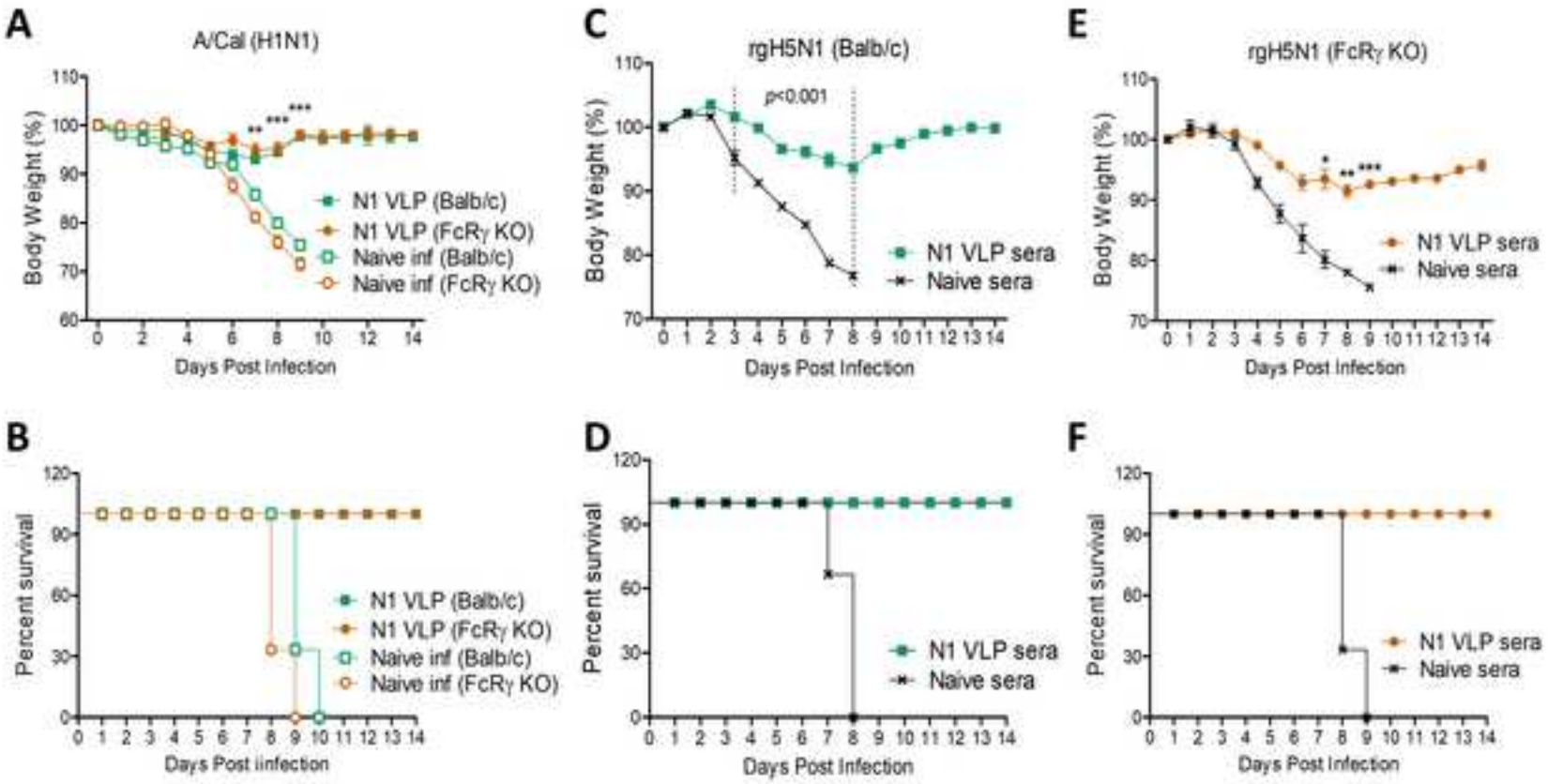

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