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Poor protective potential of influenza nucleoprotein antibodies despite wide prevalence

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Abstract

Humans are exposed to influenza virus through periodic infections. Due to these repeated exposures, human populations commonly have elevated antibody titres targeting the conserved internal influenza virus nucleoprotein (NP). Despite the presence of anti-NP antibodies, humans are acutely susceptible to drifted influenza viruses with antigenically different surface proteins and the protective potential of human NP antibodies is unclear. In this study, high levels of anti-NP antibody and NP-specific B cells were detected in both adult humans and influenza-infected mice, confirming that NP is a major target of humoral immunity. Through sorting single B cells from influenza-exposed human adults, we generated a panel of 11 anti-NP monoclonal antibodies (mAbs). The majority of anti-NP human mAbs generated were capable of engaging cellular Fc receptors and bound NP on the surface of influenza-infected cell lines *in vitro*, suggesting that anti-NP mAbs have the potential to mediate downstream Fc effector functions such as antibody-dependent cellular cytotoxicity and antibody-dependent phagocytosis. However, human anti-NP mAbs were not protective *in vivo* when passively transferred into a murine influenza challenge model. Future *in vivo* studies examining the synergistic effect of anti-NP mAbs infused with other influenza-specific mAbs are warranted.

Introduction

Most studies of antibody-based protection against influenza virus have focussed on responses targeting the viral surface proteins hemagglutinin (HA) and neuraminidase (NA). Neutralising anti-HA antibodies inhibit influenza virus entry into host cells by blocking receptor binding and viral fusion with the cellular membrane. Anti-HA antibodies are a known correlate of protection against the acquisition of influenza infection and are elicited by seasonal influenza vaccination.[1, 2] Anti-NA antibodies prevent progeny virions from budding out of influenza-infected host cells and decrease influenza disease severity.[3-5] Both anti-HA and anti-NA antibodies can also mediate effector functions through the antibody Fc region including antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent phagocytosis (ADP), which have protective value demonstrated in murine models of influenza.[6, 7]

Unfortunately, antibody responses targeting HA, and to a lesser extent NA, suffer from poor protective breadth due to antigenic diversity within these viral glycoproteins.[8, 9] The highly mutable nature of influenza surface glycoproteins leads to loss of antibody recognition and

the emergence of drifted or shifted antigenic variants that evade prevailing immunity and reinitiate cycles of infection and disease.[10] There is, therefore, interest in viral targets with potential to mediate broader and more durable immune protection, including antibodies against more highly conserved influenza proteins such as the viral nucleoprotein (NP)[11-16] and matrix 2 protein (M2).[17, 18]

Human adults, who are repeatedly exposed to influenza virus via infection and/or vaccination, typically have high titres of circulating anti-NP antibodies.[16, 19, 20] Yet, the protective potential of these antibodies is unclear. NP is not present on the surface of the virion but has been reported on the surface of influenza-infected respiratory epithelial cell lines *in vitro*,[11, 21, 22] suggesting that antibody-mediated killing of infected cells by ADCC (or other mechanisms like complement activation) may be able to contribute to viral clearance *in vivo* and thereby moderate disease severity. Supporting this, previous studies show that anti-NP antibodies can cross-link human FcγRIIIa and activate natural killer (NK) cells *ex vivo*.[16] Further, human adults with low H7N9 anti-HA and anti-NA titres can possess high anti-NP antibody titres that correlate with killing of H7N9-infected cells *in vitro*.[13]

In murine models of influenza, immunisation with recombinant NP (rNP), or alternatively passive transfer of immune serum from NP-immunised animals, can provide protection from heterosubtypic influenza virus challenge.[12, 14, 15] In contrast, rNP immunisation does not protect against lethal influenza virus challenge in ferrets.[23] The protective effect of anti-NP IgG was reduced in FcγR deficient mice, suggesting that the engagement of cellular FcγRs is critical for NP antibody function *in vivo*.[14]

Given the protective potential observed in mice, high sequence conservation of NP and the prevalence of high serum titres of anti-NP antibodies, the question remains why are human populations acutely susceptible to influenza following only minor antigenic changes to HA? Here we evaluated NP-specific antibody and B cell immunity in humans, and in experimentally infected mice. We show that humans and infected mice generate high levels of NP-specific humoral immunity. We then generated and characterised 11 human anti-NP monoclonal antibodies (mAbs) *in vitro*. We found that most anti-NP human mAbs were capable of engaging Fc receptors and bound NP on the surface of influenza-infected cell lines, indicating that anti-NP mAbs have the potential to mediate Fc functions such as ADCC

and ADP. However, human anti-NP mAbs were not protective *in vivo* in a passive transfer mouse model, which may explain why humans remain susceptible to influenza despite the wide prevalence of anti-NP antibody.

Results

NP-specific serum antibody and memory B cells are widely prevalent in adult humans

Humans are exposed to diverse strains and subtypes of influenza virus through periodic infection and/or immunisation. We examined NP- and HA-specific humoral immune responses to A/California/07/2009 (H1N1pdm09), the H1N1 virus circulating in the human population since the 2009 pandemic, in a cohort of 20 adult blood donors. In line with previous reports, anti-NP IgG titres (mean = 1748) were readily detectable in all donors at higher levels than anti-HA IgG titres (mean = 616.5) from the same strain of virus (P = 0.0001; Figure 1a).

We also quantified the frequency and specificity of IgG^+ memory B cells using H1N1pdm09 HA and NP flow cytometric probes (gating Supplementary figure 1). Both HA- and NP-specific IgG^+ memory B cell populations were detectable in most human donors at comparable frequencies (~0.15% of total IgD^- IgG^+ memory B cells; Figure 1b & Supplementary figure 2). These results indicate that prior infection of humans with influenza generates a robust humoral and B cell immune response targeting the influenza NP.

Influenza NP is highly immunogenic during a primary influenza infection in mice

The anti-NP humoral immune response in humans likely arises from periodic influenza virus infection. Supporting this, we found that NP is highly immunogenic in experimentally infected mice eliciting robust serum antibody responses (Figure 2). Anti-NP antibody titres were 25-40 times higher than anti-HA antibodies against the infecting strain (P = 0.0022; mean anti-NP titre: day 14 post-infection = 256,000 and day 28 post-infection =1,024,000 vs mean anti-HA titre: day 14 post-infection = 6,400 and mean day 28 post-infection = 38,400).

NP-specific GC and memory B cell populations were readily detectable in the spleen and lung-draining lymph nodes (MLNs) of mice after primary influenza infection (gating Supplementary figure 3), typically at or above the frequency of HA-specific B cells (P < 0.01; Figure 3 and Supplementary figures 4 & 6).

Isolation of anti-NP human mAbs

We next generated a panel of recombinant human mAbs from single-cell sorted NP-specific B cells from PBMCs from three of the adult blood donors (known to have populations of NP-specific memory B cells; donors marked with an asterisk in Figure 1b). PBMCs were stained, followed by sorting of single B cells and BCR-sequencing as previously described.[24] From 252 sorted B cells, productive heavy and light chains were recovered from 149. On the basis of similarities in variable (V) gene usage, complementarity-determining region 3 (CDR-H3) sequence length and CDR-H3 amino acid sequence, we identified 16 unique clonal lineages. These lineages represent clonally expanded families found within a single donor as shown in Supplementary table 1, which contains the BCR sequences recovered from the three donors including V gene family designation and V gene somatic mutation. A random selection of 16 clonally expanded BCR sequences were chosen for cloning and recombinant expression, with a total of 11 (out of 16) anti-NP mAbs that could be expressed (highlighted in Supplementary table 1). The anti-NP mAbs were named based on the donor that the BCR sequence originated from, with three anti-NP mAbs from donor N11 (N11-B10, N11-C06 and N11-E08), six anti-NP mAbs from donor N16 (N16-A12, N16-B03, N16-C01, N16-H07, N16-F11and N16-03) and two anti-NP mAbs from donor N19 (N19-E10 and N19-R12). The anti-NP mAbs were tested by ELISA for binding to NP (n = 11) and all 11 of the mAbs bound to rNP from the H1N1pdm09 virus, exhibiting a range of half maximal effective concentrations (EC50s) from 0.018 to 13.3 μg mL^{-1} (Figure 4a).

Anti-NP mAbs are capable of engaging human FcγRIIIa and FcγRIIa

We have previously shown that binding to recombinant human FcγRIIa and FcγRIIIa dimers correlates with immune cell activation, ADP and ADCC *in vitro*.[25-28] Anti-NP antibodies in polyclonal serum can also cross-link FcγRs and activate NK cells.[16] To test the capacity of the human anti-NP mAbs to bind FcγRs, recombinant soluble FcγRIIIa and FcγRIIa dimer ELISAs were performed. Eight of the 11 anti-NP mAbs (N11-B10, N11-C06, N16-A12, N16-B03, N16-C01, N16-F11, N16-H07 and N19-E10) demonstrated the ability to bind both human FcγRIIIa and FcγRIIa dimers (Figure 4b and c). The eight anti-NP mAbs capable of engaging FcγRIIIa and FcγRIIa in the FcγR dimer ELISA all had EC50s between 0.01 μg mL^{-1} and 1 μg mL^{-1} (Figure 4d). In contrast, three anti-NP mAbs did not demonstrate detectable FcγRIIIa or FcγRIIa dimer binding: N11-E08, N16-E03 and N19-R12. The anti-

NP mAbs that were not capable of binding human FcγRIIIa and FcγRIIa had EC50s greater than 1μg mL^{-1}.

Anti-NP mAbs can bind to NP on the surface of live influenza-infected A549 cells

We next assessed whether the human anti-NP mAbs could bind to NP on the surface of influenza-infected cells since this would be required for the initiation of ADCC or complement-dependent cellular cytotoxicity (CDCC) (gating Supplementary figure 5). There was no detectable binding of anti-NP mAbs to NP on the surface of influenza-infected A549 cells at 4h post-infection (Figure 5a). However, at both 8h and 16h post-infection eight anti-NP mAbs (N11-B10, N11-C06, N16-A12, N16-B03, N16-C01, N16-F11, N16-H07 and N19-E10) showed detectable binding to NP on the surface of live, influenza-infected A549 cells (Figure 5b and c). Three human anti-NP mAbs (N11-E08, N16-E03 and N19-R12) did not demonstrate any detectable binding to NP on the surface of influenza-infected A549 cells at 8h or 16h post-infection. This trend mirrors what we observed in the FcγR dimer ELISAs. Interestingly, two anti-NP mAbs, N11-C06 and N16-B03, demonstrated greater binding to influenza-infected A549 cells than the control HA stem-binding mAb CR9114 (Figure 5b and 5c).

Anti-NP mAbs prolonged survival but did not protect mice against influenza virus challenge

Based on high binding to influenza-infected A549 cells, two human anti-NP mAbs (N11-C06 and N16-B03) were selected for passive infusion assays. Mice passively infused with N11-C06 and N16-B03 succumbed to influenza virus challenge 7 days-post infection, which was similar to mice treated with the negative control mAb PGT121 (Figure 6). Mice infused with the HA-specific control neutralising mAb (HV-B10) were fully protected from mortality and had minimal weight loss following influenza virus challenge. In a murine model, human anti-NP mAbs were unable to provide protection from mortality following lethal influenza virus challenge.

Discussion

Human populations are exposed to different strains and subtypes of influenza virus through natural infections and/or vaccinations, resulting in the generation of antibodies against the conserved influenza NP. The majority of antibody-based influenza virus protection studies

focus solely on responses targeting the surface glycoproteins (HA and NA); therefore, the protective potential of human anti-NP antibodies remains largely uncharacterised.

Healthy blood donors uniformly have NP-specific memory B cells and anti-NP IgG targeting the H1N1pdm09 influenza virus, a virus which has circulated in the human population for over a decade. Detection of NP-specific humoral immunity in healthy donors supports previous findings that high titres of anti-NP antibodies are common in human adults.[16, 19, 20] Periodic influenza virus infections, in combination with the high sequence conservation of NP, likely leads to expanded NP-specific memory B cell populations and high levels of anti-NP antibodies over the lifetime of influenza-exposed humans.

To examine the immune response to a primary influenza virus infection, NP-specific B cells and IgG were measured in a mouse model. Influenza-infected mice also generate a strong B cell and antibody response targeting the NP of the infecting virus, which generally exceeded the response mounted against the HA glycoprotein. Prior work by Tan *et al.* showed that NP-specific GC B cells were at high frequencies, relative to HA-specific B cells, in the murine lung, spleen and MLNs following influenza virus infection.[29] Interestingly, murine anti-NP polyclonal antibodies conferred a degree of heterosubtypic protection in mice following influenza virus infection, rNP immunisation and passive transfer of NP-immune sera.[12, 14, 15] Curiously, the protection afforded by anti-NP antibodies in mice is dependent upon both CD8 T cells and FcγRs.[14] It has been speculated that immune complexes containing NP and anti-NP antibodies may stimulate antigen presentation to CD8 T cells. The exact mechanism is yet to be elucidated,[30] but may be relatively specific to murine antibodies since ferrets are not protected from lethal influenza challenge following rNP immunisation.[23] Future studies should examine the antibody isotype of GC and memory phenotype B cells in influenza virus-infected mice to improve our understanding of the humoral immune response to NP during primary infection.

Given that high titres of anti-NP antibodies do not appear to provide heterosubtypic influenza protection in humans (as they do in mice), a panel of 11 human anti-NP mAbs were generated to assess protective potential. Most NP-specific mAbs can bind FcγRIIIa and FcγRIIa, suggesting that human anti-NP mAbs are capable of engaging FcγRs and mediating downstream Fc effector functions like ADCC, ADP and complement activation. A range of

231 unique FcγR binding capacities were observed for the different anti-NP mAbs, which
232 indicates that the mAbs may be binding to distinct epitopes on the influenza virus NP.
233 Further investigation to determine the NP epitopes being bound by the anti-NP mAbs is
234 warranted. Polyclonal anti-NP antibodies in human sera have previously been reported to
235 cross-link FcγRIIIa, activate NK cells and correlate with antibody-dependent killing of
236 influenza-infected cells *in vitro*.[13, 16, 31] A limitation of this study is that anti-NP mAbs were
237 not tested for binding across all human FcγRs, and future studies should assess the capacity
238 of anti-NP antibodies to bind high affinity (FcγRI) and inhibitory (FcγRIIb) human FcγRs.
239
240 Fc-mediated effector functions like ADCC and CDCC require anti-NP antibodies to bind
241 directly to viral antigens on the surface of influenza-infected cells. NP is located inside the
242 influenza virion, but several groups report that NP is also found on the surface of influenza-
243 infected cell lines *in vitro*.[11, 21, 22] The presence of viral NP on the surface of influenza-
244 infected cells may be the result of active expression or a passive association between
245 extracellular NP and the cell surface.[22] NP-specific serum antibodies as well as anti-NP
246 mAbs have been shown to bind to viral NP on the surface of influenza-infected cells.[11, 13, 22,
247 31] Our human anti-NP mAbs demonstrated a range of binding to influenza-infected A549
248 cells *in vitro*, indicating that some anti-NP mAbs may be able to promote Fc-mediated
249 cytotoxicity of infected cells.
250
251 Several of our human anti-NP mAbs had greater or equivalent binding to the surface of
252 influenza-infected cells compared to the anti-HA stem mAb CR9114. Previous studies have
253 demonstrated that CR9114 mediates Fc effector functions *in vitro* and confers protection in
254 an Fc-dependent manner in mice.[6, 7]. However, passively infused human anti-NP mAbs (N11-
255 C06 and N16-B03) were not protective following lethal influenza virus challenge, with all of
256 the treated mice ultimately succumbing to the infection. These results suggest that anti-NP
257 antibodies cannot protect mice from lethal influenza and may not contribute to protective
258 immunity during influenza virus infection. It is plausible that anti-NP human mAbs are not
259 protective due to relatively low levels of influenza NP (compared to HA and NA) on the
260 surface of infected cells[22] and/or extracellularly. We and others have shown that non-
261 neutralising anti-HA mAbs confer protection against both influenza A and B viruses in
262 mice.[6, 32] Low availability of NP *in vivo* may prevent anti-NP mAbs from performing certain
263 Fc-mediated effector functions that are required for protection. Differences in IgG subclass

binding and cellular expression patterns between human and murine FcγRs[33] may have also led to suboptimal FcγR engagement by human anti-NP mAbs *in vivo*. In contrast, Fujimoto *et al.* reported that transgenic mice expressing high concentrations of an anti-NP human mAb were partially resistant to lethal H5N1 and H1N1 influenza infections.[31] These conflicting results may be attributed to experimental differences including mAb administration (infusion vs expression) and distinct strains/subtypes of influenza virus used for lethal challenge. A recent study by Degan *et al.* showed that only 30% of human mAbs generated from single plasmablasts early after influenza virus infection were HA reactive, with the majority recognising other antigens including NP.[34] Cocktails of early infection-induced mAbs, containing high levels of anti-NP mAbs, had limited protective ability when passively infused into a murine influenza challenge model, with only 20% of mice surviving infection. This study supports our findings that human anti-NP mAbs are poorly protective against lethal influenza virus challenge in mice.[34]

Our results suggest that NP is a major target of the humoral immune response during influenza virus infection in humans and mice. Anti-NP human mAbs have the ability to engage FcγRs and bind to NP on the surface of infected cell lines; however, this did not confer protection from mortality in a murine infection model. Despite the wide prevalence of anti-NP antibodies in human adults, our findings suggest that NP may not be bioavailable as a target of ADCC in the influenza-infected lung. Influenza HA is more abundant on the surface of infected cells,[35] and non-neutralising anti-HA mAbs confer protection against both influenza A and B viruses *in vivo*.[6, 24] Since human anti-NP mAbs appear to have poor protective potential when infused into mice, future studies should explore whether anti-NP mAbs provide an additive or synergistic benefit when infused in combination with other influenza-specific mAbs. Overall, this study indicates that human populations remain susceptible to influenza because human antibodies targeting the conserved NP do not confer robust protection against influenza virus *in vivo*.

Methods

Murine infection

Animal procedures were approved by the University of Melbourne Animal Ethics Committee (#1714193). C57BL/6 mice aged 6-8 weeks were used. Mice were anesthetized by isoflurane

296 inhalation prior to infection. Mice were infected intranasally with 50 μL of 50 $TCID_{50}$ of
297 A/Puerto Rico/8/34 (PR8).
298
299 *Human peripheral blood mononuclear cells (PBMCs) and sera*
300 Blood from anonymous healthy adult donors was provided by the Australian Red Cross
301 (Melbourne, Victoria, Australia). PBMCs were isolated using Ficoll gradient (GE Healthcare,
302 Chicago) and stored in liquid nitrogen. Frozen PBMCs were thawed at 37 °C, washed twice
303 with RF10 medium (RPMI 1640, 10% FCS, 1× penicillin-streptomycin-glutamine; Life
304 Technologies, Thermo Fisher Scientific, Waltham) and once with PBS prior to staining for
305 flow cytometry. Following the Ficoll spin, sera was removed and stored at –20 °C for each
306 blood donor.
307
308 *HA and NP probes*
309 Recombinant HA proteins from H1N1 PR8 and A/California/07/2009 (H1N1pdm09) were
310 used as probes for flow cytometry as previously described.[36] Expression constructs were
311 synthesized (GeneArt, Regensburg) and cloned into mammalian expression vectors. Proteins
312 were expressed in Expi293 (Life Technologies, Thermo Fisher Scientific, Waltham)
313 suspension cell cultures. The proteins were then purified by poly-histidine-tag affinity
314 chromatography and gel filtration. Proteins were biotinylated using BirA (Avidity
315 Biosciences, La Jolla) and stored at −80°C. Prior to use, biotinylated HA proteins were
316 labelled by the sequential addition of streptavidin (SA) conjugated to phycoerythrin (PE) or
317 allophycocyanin (APC), and stored at 4°C. Recombinant influenza A H1N1 NP proteins,
318 PR8 (11675-V08B; Sino Biological, Shanghai) and A/California/07/2009 (40205-V08H;
319 Sino Biological, Shanghai) were labelled with PE or APC fluorochromes using commercial
320 conjugation kits, as per manufacturer's protocol (AB102918, AB 201807; Abcam, Cambrige).
321
322 *Flow cytometry*
323 NP and HA-specific B cells were identified within cryopreserved human PBMCs by co-
324 staining with NP or HA probes conjugated to SA-PE and SA-APC (from BD, Franklin
325 Lakes). Cells were stained with Aqua Viability Dye (Thermo Fisher Scientific, Waltham).
326 Antibodies used for surface staining include CD19-ECD (J3-119) (Beckman Coulter, Brea),
327 CD20 BV421 (2H7), IgM-BUV395 (G20-127), IgD-PE-Cy7 (IA6-2), IgG-BV786 (G18-145)
328 (all from BD), CD14-BV510 (M5E2), CD3-BV510 (OKT3), CD8a-BV510 (RPA-T8),

CD16-BV510 (3G8), and CD27-BV605 (O323) (all from BioLegend, San Diego). Background B cells interacting with SA were excluded by staining with SA-BV510 (BD, Franklin Lakes).

For murine samples, tissues [spleen or mediastinal lymph nodes (MLNs)] were mechanically homogenized into single-cell suspensions in RF10 media (RPMI 1640, 10% FCS, 1× penicillin-streptomycin-glutamine; Life Technologies, Thermo Fisher Scientific). RBC lysis was performed with Pharm Lyse (BD). Isolated cells were stained with Aqua Viability Dye (Thermo Fisher Scientific) and Fc blocked with an anti-CD16/32 antibody (clone 93; BioLegend). Cells were then surface stained with the relevant NP or HA probes and the following antibodies: B220 BUV737 (RA3-6B2; BD); IgD BUV395 (11-26c.2a; BD); CD45 Cy7APC (30-F11; BD); GL7 Alexa Fluor 488 (GL7; BioLegend); CD38 PE-Cy7 (clone 90; BioLegend); SA BV786 (BD); CD3 BV786 (145-2C11; BioLegend); and F4/80 BV786 (BM8; BioLegend). Cells were washed twice, fixed with 1% formaldehyde (Polysciences, Philadelphia), and acquired on a BD LSR Fortessa using BD FACSDiva.

IgG ELISA with murine and human samples

For murine samples, 96-well plates (MaxiSorp, Nunc, Rochester) were coated with 100 ng per well of recombinant NP or HA protein (Sinobiological) overnight at 4 °C. After blocking with 5% with bovine serum albumin (BSA, Sigma-Aldrich, St. Louis), serial 4-fold dilutions of sera were added to duplicate wells and incubated for 2 h at room temperature. Following washing, plates were incubated with a 1:10,000 dilution of HRP-conjugated goat anti-mouse IgG (KPL, SeraCare, Milford) for 1 h at room temperature. For human samples, 96-well plates were coated with 100 ng per well of recombinant NP or HA protein overnight at 4 °C. After blocking with 5% with BSA (Sigma-Aldrich), serial 4-fold dilutions of sera were added to duplicate wells and incubated for 2 h at room temperature. Following washing, plates were incubated with a 1:4,000 dilution of HRP-conjugated rabbit anti-human IgG (Aligent, Glostrup) for 1 h at room temperature. Plates containing mouse and human samples were washed and developed using 3’3’5’5’-tetramethylbenzidine (TMB; Sigma-Aldrich) and read at 450nm. Duplicate wells were averaged, and endpoint titres were calculated as the reciprocal serum dilution of 3× background (uncoated wells).

Sequencing, cloning and expression of B cell immunoglobulins (Ig)

B cell receptors (BCRs) from single IgG^+ B cells sorted from cryopreserved human PBMCs were sequenced, cloned and expressed as recombinant mAbs as previously described.[24] Recombined heavy chain (V-D-J) and light chain (V-J) Ig sequences were synthesised (Genscript, Piscataway) and cloned into expression plasmids encoding the constant regions of human IgG1. The expression plasmids were then transfected into Expi293F cells using ExpiFectamine (Thermo Fisher Scientific, Gibco, Amarillo). Recombinant human IgG1 mAbs were purified from culture supernatants using agarose Protein-A or G (Pierce, Thermo Fisher Scientific, Waltham) and were used for all subsequent assays including ELISAs, surface staining of influenza-infected cells and murine infusion experiments.

IgG and human FcγR dimer ELISA with anti-NP mAbs

Binding of the purified mAbs to NP was tested by ELISA as previously described.[37] Briefly, 96-well NUNC Immunolon plates (Thermo Fisher Scientific) were coated overnight at 4 °C with 2 μg mL^{-1} of commercially sourced rNP from the A/California/07/2009 H1N1 influenza virus (Sinobiological). Wells were blocked with 1% fetal calf serum (FCS) in PBS, duplicate wells of NP-specific mAbs at different dilutions (starting at 5 μg mL^{-1}, four-fold serial dilutions) were added and incubated for 1 h at room temperature. Plates were washed prior to incubation with 1:30,000 dilution of HRP-conjugated anti-human IgG (Sigma) for 1h at room temperature. Plates were washed and developed using TMB substrate (Sigma-Aldrich) and read at 450 nm. NP-binding intensity was calculated as the antibody concentration giving half-maximal signal (EC_{50}) using a fitted curve (4 parameter log regression). A negative control mAb (VRC01) was included.

The capacity of the anti-NP mAbs to bind human FcγRs was tested using the recombinant soluble FcγRIIIa or FcγRIIa dimer ELISA as previously described.[28] Briefly, 96-well NUNC Maxisorb plates were coated overnight at 4°C with 50 ng of commercially sourced rNP from the A/California/07/2009 H1N1 influenza virus or HIV-1 gp140 (Sinobiological). Wells were blocked with 1% BSA (Sigma-Aldrich) 1 mM EDTA in PBS (BSA/PBSE) for 1h at 37°C. Duplicate wells of anti-NP mAbs at different dilutions (starting at 5 μg mL^{-1}, four-fold serial dilutions) were added and incubated for 1h at 37°C. After washing, 50 μL of 0.1 μg mL^{-1} biotinylated human FcγRIIIa or 0.2 μg mL^{-1} biotinylated human FcγRIIa dimers were added to the wells and incubated for 1 h at 37 °C. Plates were washed prior to incubation with a 1:10,0000 dilution of Pierce High Sensitivity HRP-Streptavidin (Thermo Fisher Scientific)

for 1 h at 37 °C. The plate was washed and developed using TMB substrate then read at an absorbance of 450 nm. A negative control mAb (VRC01) was included.

Surface staining of influenza-infected cells with anti-NP mAbs

Binding of anti-NP mAbs to NP on the surface of live influenza infected cells was tested by flow cytometry. A549 cells were infected at a MOI of 3 with the A/California/07/2009(H1N1)-like virus A/Auckland/01/2009 for 4 h, 8 h and 16 h at 37°C, 5% CO_2 as previously described. The A549 cells were then washed twice with PBS to remove residual media and trypsinised. After washing, the A549 cells were resuspended at $1x10^6$ cells per mL and 200 µL (2×10^5 cells) was added to the wells of a round bottom 96-well plate. The A549 cells were spun down, the supernatant was flicked off and the infected A549 cells were incubated with the anti-NP mAbs (5 µg mL^{-1}, 0.5 µg mL^{-1}, 0.05 µg mL^{-1} and 0.005 µg mL^{-1}) for 30 min at 4°C. The A549 cells were washed to remove unbound anti-NP mAbs then surface stained with mouse anti-human IgG BV421 (G18-145; BD) and near-infrared (IR) live/dead staining (Thermo Fisher Scientific) for 30 min at 4°C. Cells were washed twice, fixed with 1% formaldehyde (Polysciences), and acquired on a BD LSR Fortessa using BD FACSDiva.

Passive infusion of NP-specific mAbs

Groups of five BALB/c female mice aged 8-12 weeks were used to assess prophylactic protection by two anti-NP mAbs generated, as well as a hemagglutination inhibition positive (HAI+) control mAb (HV-B10) and HIV-specific negative control mAb (PGT121). The HAI+ control mAb, HV-B10, is on the same vector backbone as the anti-NP mAbs and was generated following the same protocol described above (with the exception that HA probes were used for isolation instead of NP probes). The mice were administered the mAbs intraperitoneally at a dose of 10 mg kg^{-1} of body weight. Twenty-four hours after passive infusion with mAbs, the mice were anaesthetised by isoflurane inhalation and challenged intranasally with 50 µL of A/Auckland/01/2009(H1N1) at a dose of 10^4 $TCID_{50}$. Mice were monitored for weight loss and signs of infection for 14 days and were euthanised if they lost more than 20% of their pre-infection weight.

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Conflicts of Interest

All authors report no potential conflicts of interest.

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i.e. Should it be "Antibodies against conserved antigens provide opportunities for reform in influenza vaccine design."
And add e76.

Figure Captions

Figure 1. HA- and NP-specific IgG$^+$ memory B cells and IgG titres in healthy human donors. IgG titre **(a)** and percentage (%) of A/California/07/2009 (H1N1pdm09) HA- and NP-specific IgG$^+$ memory B cells **(b)** measured in 20 healthy human blood donors. Donors used for sorting of single B cells and BCR sequencing were marked with an asterisk in panel B. The data are shown as mean ± standard deviation. Wilcoxon matched-pairs signed rank tests were used to compare the magnitude of HA- and NP-specific responses. *** $P < 0.001$, ns = not significant.

Figure 2. HA- and NP-specific IgG titres in PR8 influenza-infected mice. HA- and NP-specific IgG titres in mock- and influenza-infected C57BL/6 mice (n = 6 per group) at 14 days **(a)** and 28 days **(b)** post-infection intranasally with PBS or 50 $TCID_{50}$ of A/Puerto Rico/8/34 (PR8). This experiment was conducted once, with n = 6 mice per group. The data are represented as mean ± standard deviation. Mann-Whitney *U*-tests were used to compare the magnitude of HA- and NP-specific IgG responses. ** $P < 0.01$.

Figure 3. HA- and NP-specific germinal centre and memory phenotype B cells in spleen and mediastinal lymph nodes of PR8 influenza-infected mice. Percentage (%) of germinal centre (GC) phenotype B cells binding to HA or NP probes in the spleen (SPL) **(a)** and pooled mediastinal lymph nodes (MLN) **(b)** of mock- and influenza-infected C57BL/6 mice (n = 6 per group) at 14 days and 28 days post-infection intranasally (IN) with PBS or 50 $TCID_{50}$ of A/Puerto Rico/8/34 (PR8). Percentage (%) of memory phenotype B cells binding to HA or NP probes in SPL **(c)** and pooled MLNs **(d)** of mock- and influenza-infected C57BL/6 mice (n = 6 per group) at 14 days and 28 days post-infection IN with PBS or 50 $TCID_{50}$ of PR8. Data in panels **a** and **c** are represented as mean ± standard deviation, and a one-way ANOVA with Holm-Sidak's multiple comparison test is used to compare the percentage of HA- and NP-specific B-cells. Panels **b** and **d** represent data from pooled MLNs from six mice (n = 6) per group. ** $P < 0.01$, **** $P < 0.0001$.

Figure 4. Human anti-NP monoclonal antibodies bind to H1N1pdm09 NP, dimeric human FcγRIIIa and FcγRIIa. The 11 human anti-NP monoclonal antibodies (mAbs) expressed were tested for binding to recombinant A/California/07/2009 (H1N1pdm09) NP in an IgG ELISA **(a)**, an FcγRIIIa dimer ELISA **(b)** and an FcγRIIa dimer ELISA **(c)**. Sigmoidal non-linear regression curves are shown for each ELISA and these were used to calculate half maximal effective concentrations (EC50s) for the 11 human anti-NP mAbs **(d)**. Grey tiles indicate that EC50 could not be calculated. An HIV-specific mAb, VRC01, was included as a negative control.

Figure 5. Human anti-NP monoclonal antibodies bind to NP on the surface of influenza-infected cells. The 11 human anti-NP monoclonal antibodies (mAbs) expressed were tested for binding to NP on the surface of influenza-infected A549 cells at 4 h **(a)**, 8 h **(b)** and 16 h **(c)** post-infection with the A/California/07/2009(H1N1)-like virus A/Auckland/01/2009 at an MOI of 3. An anti-HA stem mAb, CR9114, was included as a positive control and an HIV-specific mAb, VRC01, was included as a negative control.

Figure 6. Passive infusion of human anti-NP monoclonal antibodies in mice experimentally challenged with influenza. Mice (n = 5 per group) were infused with 10 mg kg^{-1} of the human anti-NP mAbs, N11-C06 (red) and N16-B03 (orange), or controls, anti-HA head mAb HV B10 (purple) or PGT121 (grey) 24 h before intranasal challenge with 10^4

591 $TCID_{50}$ of the A/California/07/2009(H1N1)-like virus A/Auckland/01/2009. Mice were
592 monitored for 14 days and euthanised if weight loss exceeded 20% of starting weight. Weight
593 loss **(a)** and survival curves **(b)** are shown for the mice experimentally challenged with
594 influenza virus. This experiment was conducted once, with n = 5 mice per group. Data shown
595 are mean ± SEM.

Figure 1

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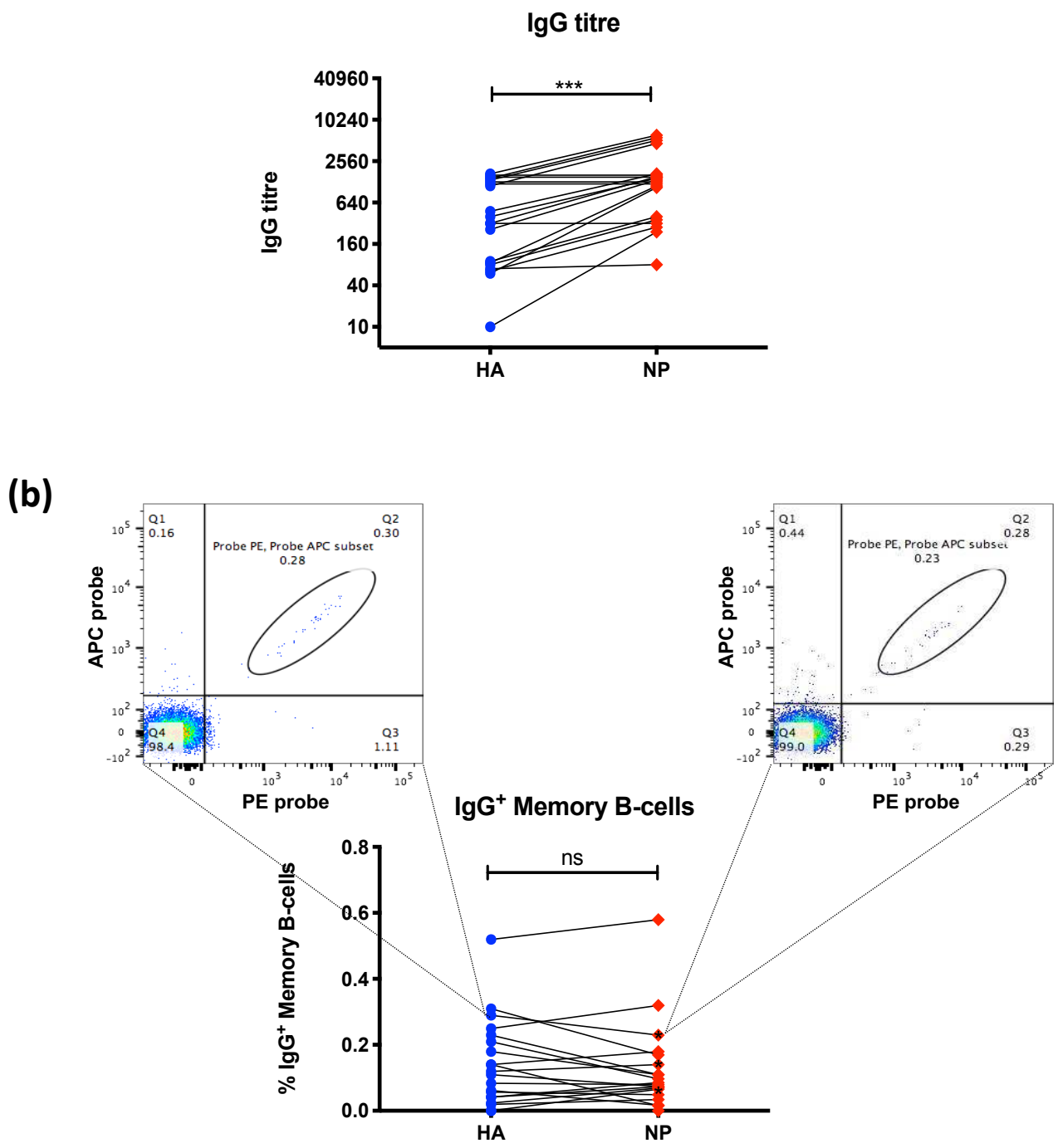

Figure 2

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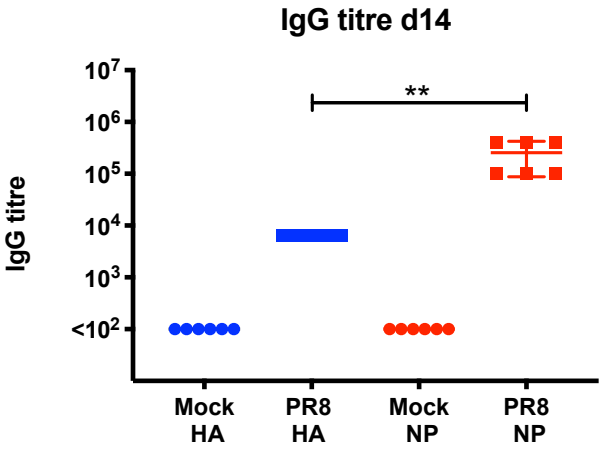

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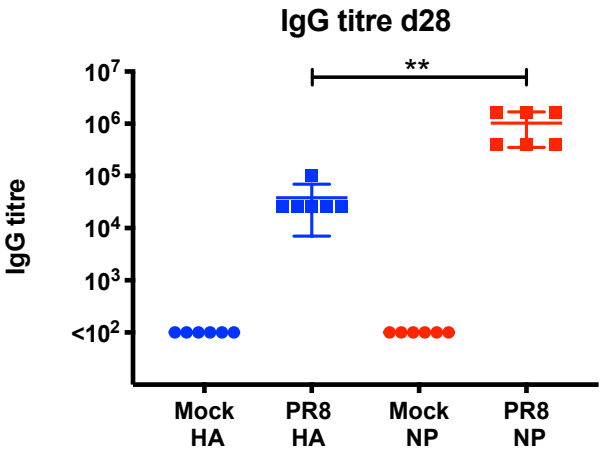

Figure 3

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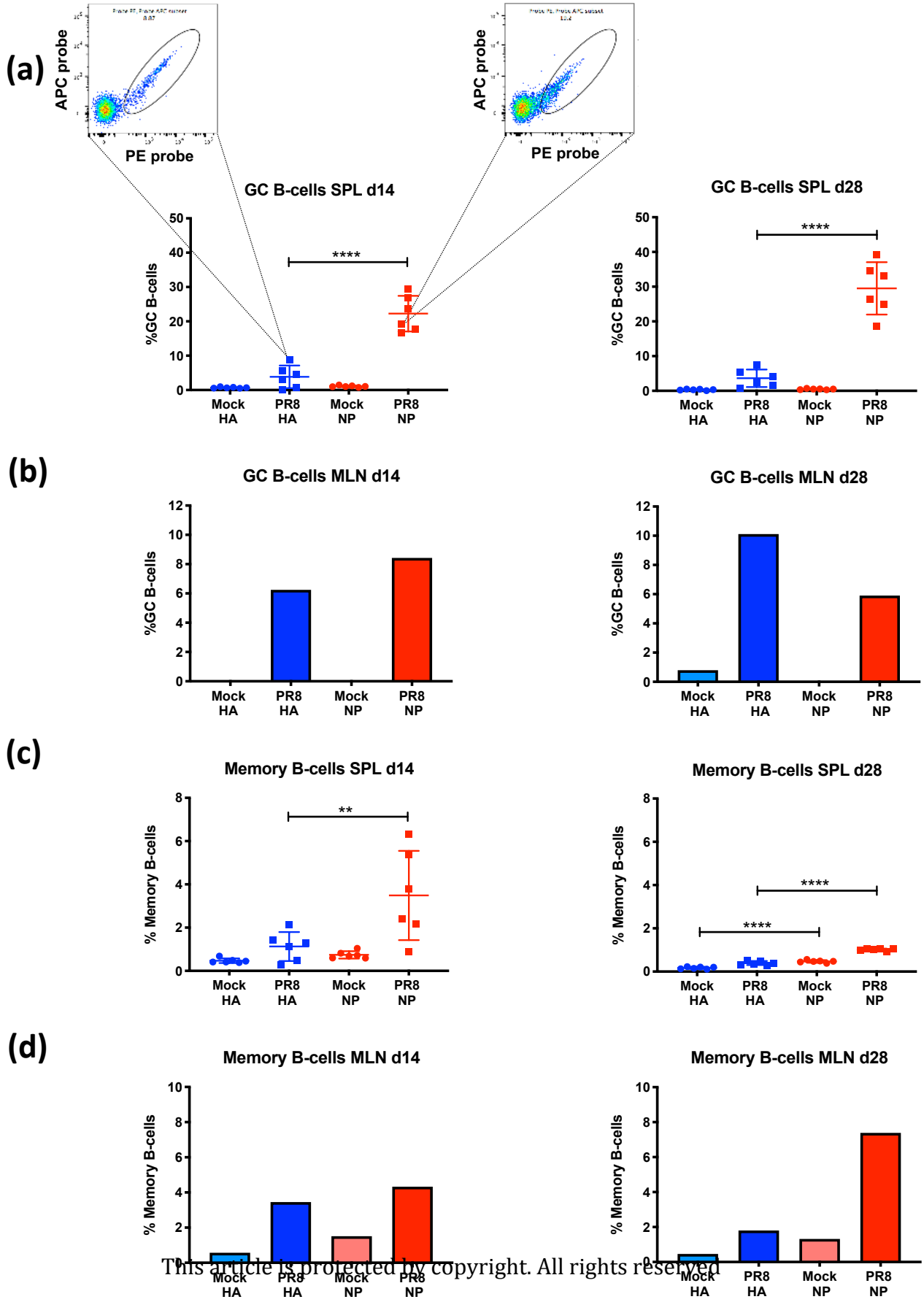

Figure 4

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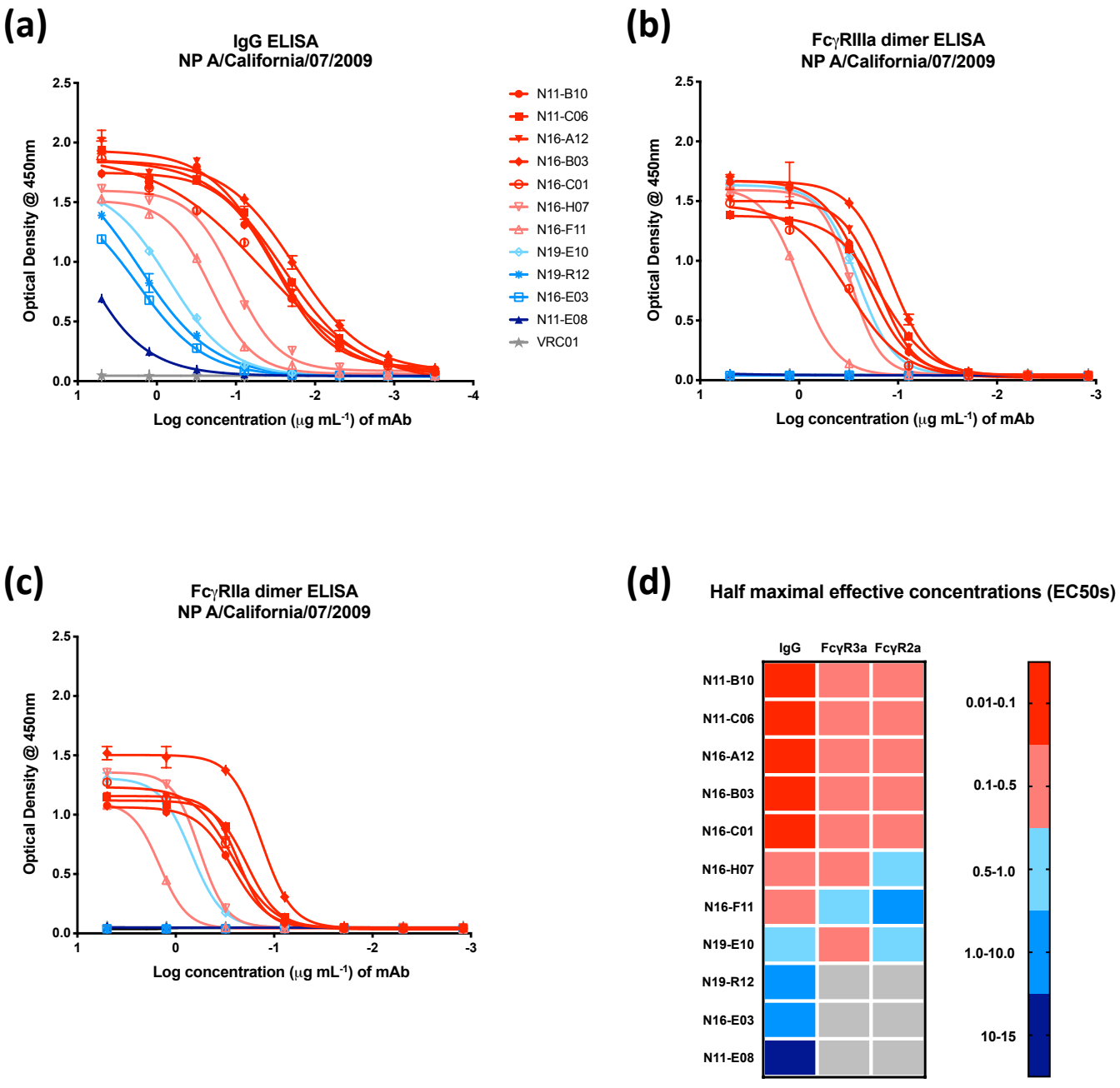

Figure 5

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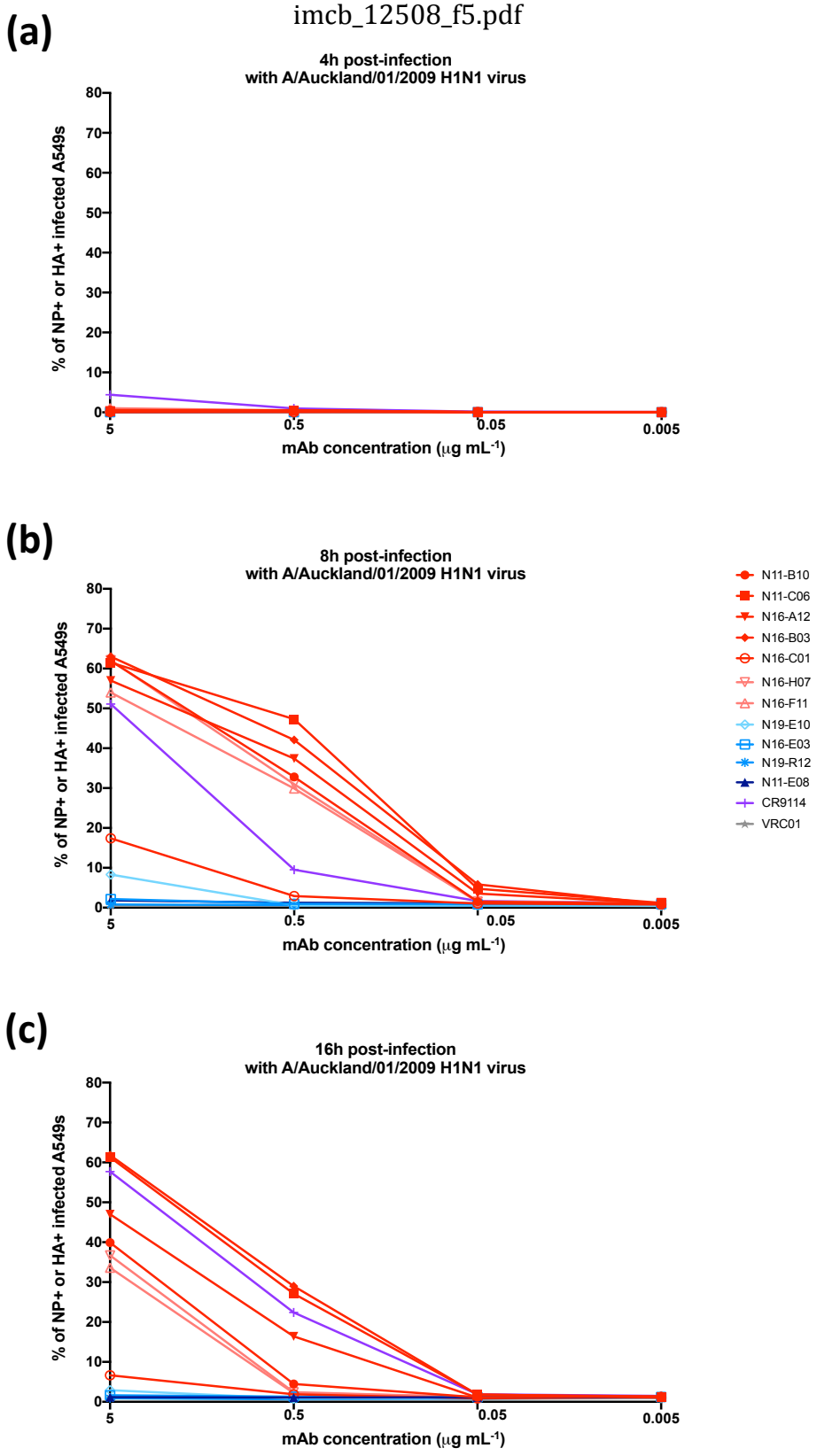

Figure 6

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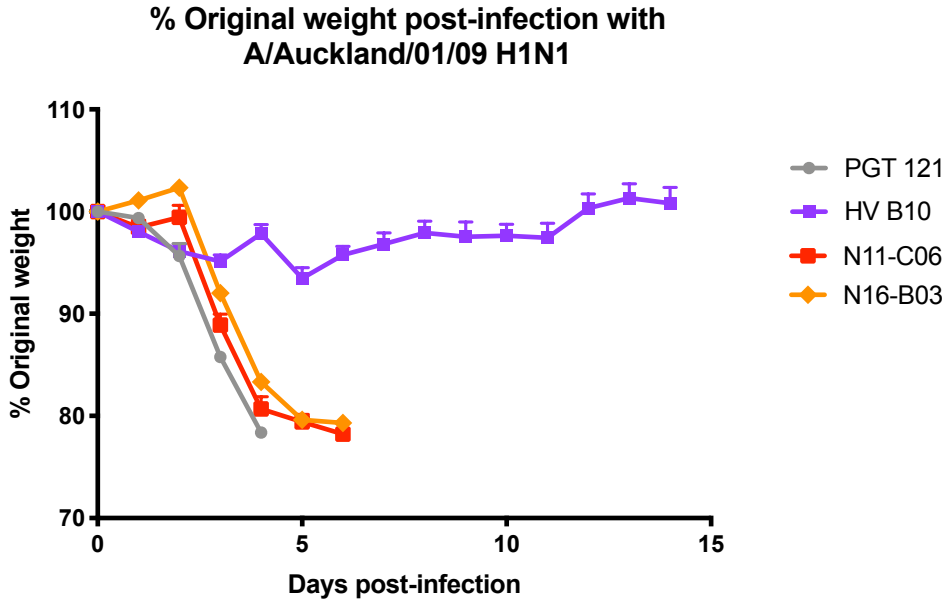
(a)



(b)