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ORIGINAL ARTICLE

Cross-reactive antibody-dependent cellular cytotoxicity antibodies are increased by recent infection in a household study of influenza transmission

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Abstract

Objectives. Influenza causes a spectrum of disease from asymptomatic infection to fatal outcome, and pre-existing immunity can alter susceptibility and disease severity. In a household transmission study, we recruited outpatients with confirmed influenza virus infection and prospectively identified secondary infections in their household contacts, therefore identifying infection cases with baseline samples for determining immune-mediated protection from influenza infection. **Methods.** We examined baseline broadly reactive immune correlates of relevance to universal vaccine development, specifically antibody-dependent cytotoxic (ADCC) antibodies and T-cell responses in functional assays. Antibodies were assessed in a cell-based NK cell degranulation assay by flow cytometry, and T-cell responses were assessed by IFN-γ intracellular cytokine staining flow cytometry assay. **Results.** The magnitude of antibody responses and ADCC function for multiple influenza-specific proteins was lower in participants who became infected, consolidating the role of pre-existing antibodies in protection from seasonal influenza virus infection. Among H1N1-infected contacts, we found that higher levels of pre-existing H1-haemagglutinin ADCC responses correlated with reduced symptom severity. Recent infection boosted the titre and magnitude of haemagglutinin-, neuraminidase- and nucleoprotein-specific ADCC antibodies. Limited T-cell samples precluded conclusions on the role of pre-existing T-cell responses. **Conclusions.** Overall, ADCC responses are a protective correlate against influenza virus infection that should be considered in future vaccine development and evaluation.Influenza-specific ADCC responses are elevated in uninfected subjects, associated with reduced symptoms and boosted by recent infection, whilst HA stem and NA IgG are also elevated in uninfected participants irrespective of ADCC function.

Keywords: antibody-dependent cellular cytotoxicity, baseline immunity, HA stem antibodies, influenza virus infection, T cells, universal immune correlate

INTRODUCTION

Current inactivated influenza vaccines have limited efficacy against antigenically drifted viruses and novel subtypes, and they only have a short duration of protection.[1] Therefore, there has been considerable interest to develop universal influenza vaccines, including studies of immune correlates of protection against influenza infection and disease.[2] The severity of influenza virus infections varies from asymptomatic infection with minimal viral shedding to debilitating illness and fatal disease, which can be attributed to a number of host factors and prior exposures of influenza.[3]

Cross-reactive memory responses against different influenza viruses have been found in both humoral and cellular compartments. T cells are capable of heterosubtypic cross-reactivity due to peptide epitope conservation,[4,5] and their breadth is evident by the presence of T cells specific for avian influenza A(H7N9) and A(H5N1) viruses in unexposed individuals.[4] In addition, HA-specific antibodies capable of antibody-dependent cellular cytotoxicity (ADCC) are also evident in the absence of haemagglutinin inhibition (HAI) for H7N9 and H5N1 viruses.[6,7] ADCC antibodies cross-link Fc receptors on B cells, macrophages and NK cells, to bridge the adaptive and innate immune responses and kill virus-infected target cells. ADCC antibodies are also proposed to be enriched for epitopes in the functionally conserved haemagglutinin (HA) stem and conserved nucleoprotein (NP) protein.[6,8]

For both these immune correlates, higher magnitude of pre-existing ADCC antibodies and memory T-cell responses has been found to reduce the risk of infection in human challenge models.[6,9] The mechanisms of these anti-viral responses are relatively well studied by experimental models, whilst their roles in preventing influenza infection and modulating influenza disease severity at the community level still need to be deciphered. We examined influenza-specific ADCC antibodies and T-cell responses as potential immune correlates of protection against influenza virus infection and illness in a household study of community-acquired infection, by identifying secondary influenza virus infections among household contacts exposed to influenza and evaluated their immune status at baseline before infection.

RESULTS

Household sampling for alternate correlates of protection studies

To enable the study of baseline immune correlates of protection against influenza virus infection, specifically ADCC antibodies from serum and T cells from peripheral blood mononuclear cells (PBMCs), blood samples were collected from household contacts of infected index cases (Figure 1a). Heparinised blood samples were collected from July 2013 to April 2017, for a total of 288 PBMC samples. To increase sample numbers for ADCC serum assays, archived serum samples were included (n = 124) from earlier years (2010–2013) of the same study and that were collected at the same exposure timepoints. RT-PCR with virus-specific subtype primers identified infections from 51 contacts ('infected contacts') for H1N1 (n = 13) and H3N2 viruses (n = 29) (Table 1). In addition, samples from 45 uninfected household members were selected and used as controls, based on being asymptomatic, from the same household if available, and similar age and sex as infected contact participants. Limited volumes of some subjects from archived samples limited paired analysis of pre- and post-exposure samples. Therefore, where possible results included both pre- and post-infection samples were paired, the data were stratified for influenza subtype.

Baseline antibody quantity, quality and function in infected and uninfected influenza-exposed household contacts

Sampling from infected index cases represents immune responses during acute influenza infection rather than baseline immunity; therefore, the primary comparisons of this study are immune responses at baseline, day 0, for infected and uninfected contacts (Table 1). Infected and uninfected contact participants were of similar age, gender and recent vaccination history (Table 1, non-significant by Fisher's exact

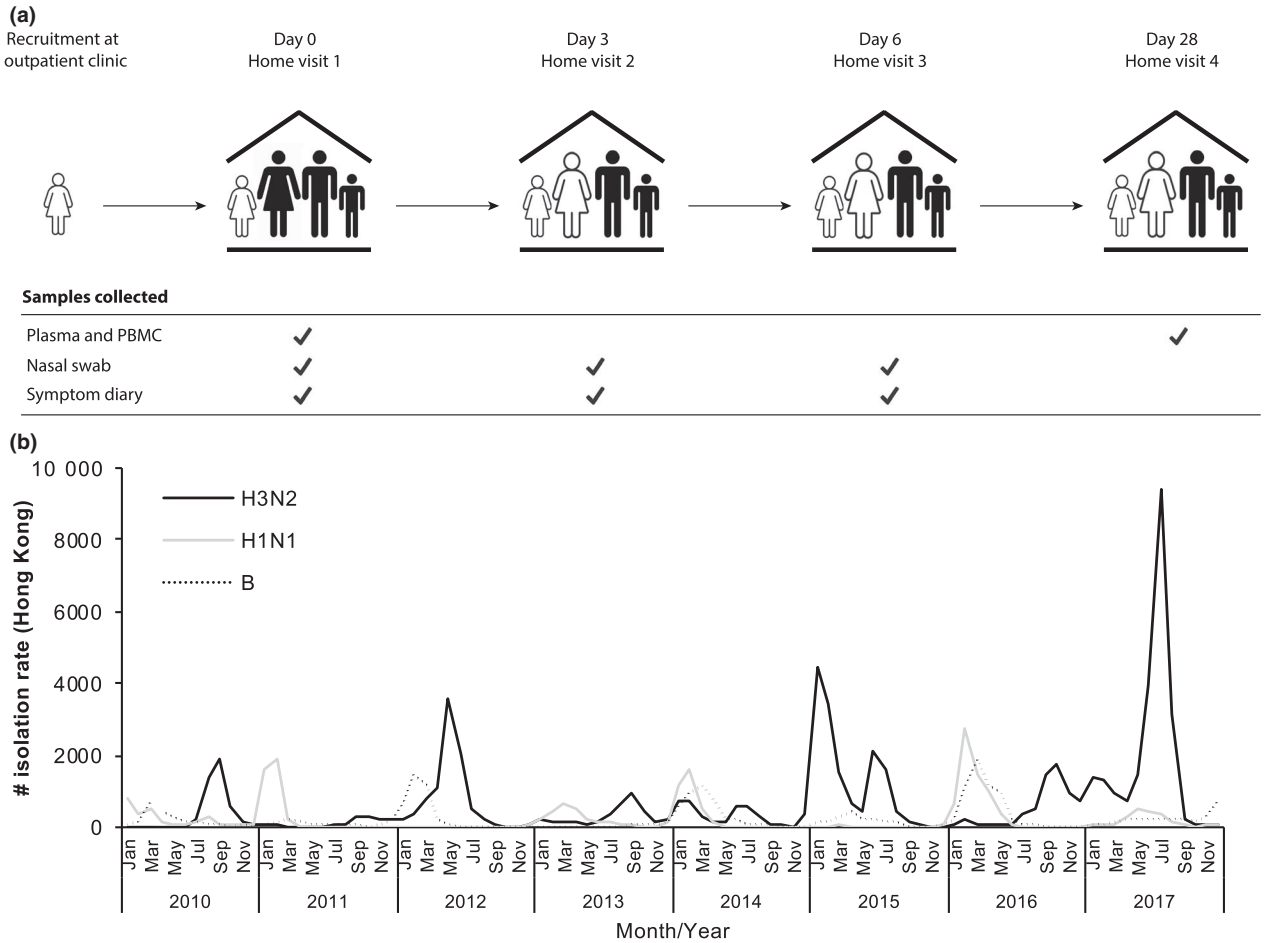

Figure 1. Household recruitment and sampling for the identification of influenza infection and baseline sample collection. **(a)** Influenza infected index patients were from outpatient clinics across Hong Kong. Home visits by nurses on the same day (visit 1, V1) recruited contact participants as volunteer household members for sample collection of blood for plasma and PBMC isolation, nasal swabs for viral RT-PCR using subtype-specific primers and self-reported symptom diary including body temperature. **(b)** Influenza virus isolation from hospital and outpatient clinics in Hong Kong during the study period (data extracted from the website of the Centre Health Protection, Hong Kong).[53]

Table 1. Baseline characteristics of household contacts

Characteristics	PCR-confirmed A(H1N1) infection (*N* = 13) *n* (%)	PCR-confirmed A(H3N2) infection (*N* = 29) *n* (%)	Negative (*N* = 45) *n* (%)
Age group (years)			
<18	0 (0)	2 (7)	1 (2)
18-50	13 (100)	22 (76)	31 (69)
50+	0 (0)	5 (17)	13 (29)
Male	3 (23)	10 (34)	25 (56)
Received vaccine in past 12 months	0 (0)	4 (14)	5 (11)
Current/ever smoker	3 (23)	5 (17)	11 (24)
Any chronic disease	1 (8)	10 (34)	14 (31)

test). ADCC responses of infected contacts and uninfected contacts at pre-exposure times were assessed for a protein panel representing recent viruses and conserved targets (Supplementary figure 1) and stratified for subtype-specific infections for H1N1 (Figure 2a) and H3N2 (Figure 2b) viruses. We found that there were significantly lower H1-HA and N2-NA ADCC

responses at the pre-exposure timepoint for H3N2-infected versus H3N2-uninfected contacts after adjusting for age. Whilst total H1-HA ADCC responses were not different at baseline for H1N1 infection (Figure 2a), the endpoint titre of H1-HA ADCC response was significantly lower at baseline pre-exposure timepoint for H1N1-infected contacts than H1N1-uninfected contacts (Supplementary figure 2).

We tested for H3N2 antigenic mismatch using the H3-HA proteins represented by vaccine isolates from 2009 (A/Perth/16/2009, clade 1, 96.1% homology versus H3-2013), 2011 (A/Victoria/361/2011, clade 3c1, 97.5% homology versus H3-2013) and 2013 (A/Switzerland/9715293/2013, clade 3c3a), spanning the WHO vaccine recommendations from 2009 to 2016, as the study was conducted from 2010 to 2017, with comparable total IgG responses for all H3N2-infected participants at pre-exposure (Supplementary figure 3a). For H3N2-infected subjects, pre-exposure baseline H3-HA ADCC responses increased with time from 2010 to 2015 (correlation $R^2 = 0.41$, $P = 0.03$, Supplementary figure 3b), which were not otherwise significant from 2010 to 2017 (correlation $R^2 = 0.04$), or from 2015 to 2017 (correlation $R^2 = 0.05$). Results may indicate H3-ADCC responses are sensitive to H3-HA antigenic changes with time, whilst ELISA assays that measure total antibody binding are less specific than functional assays and reflect cross-reactivity for H3-HA responses.

To determine whether the quantities of antibody were different between infected and uninfected household contacts, the total influenza virus-specific IgG in the serum was measured by HAI and ELISA. The baseline HAI responses (Supplementary figure 4a and b), H1-HA- and H3-HA-specific IgG (Figure 2c and d), were comparable between infected and uninfected contacts, reflecting similar results between ELISA and HAI assays. Importantly, IgG specific for NP, group 1 (G1) stem and N1-NA proteins was lower in H3N2-infected contacts, and G1 stem for H1N1-infected contacts at baseline than in H1N1-uninfected contacts (Figure 2c and d).

Effect of influenza infection on subtype-specific and cross-reactive antibody quantity, quality and function

To determine the relationship between infection and boosting ADCC responses, the fold-change of post-exposure serum samples was assessed (Figure 3). For H1N1-infected contacts (Figure 3a), infection resulted in a fold rise of ADCC responses towards the spectrum of proteins tested, including H1-HA, H3-HA, H7-HA, N1-NA, N2-N2 and NP proteins, but did not impact the ADCC activity of HA stem-specific responses. Whilst for H3N2-infected contacts, only the NA-specific (both N1-NA and N2-NA) ADCC responses were significantly boosted by infection (Figure 3b). These significant differences persisted after adjustment for age. In addition, the endpoint titres of H1-HA and H3-HA-specific ADCC responses were significantly boosted by recent H1N1 and H3N2 infection (Supplementary figure 4c).

The magnitude of the cross-reactive H7-HA-specific ADCC response was lower at baseline than other HA subtypes and limited in most participants[10]; however, low-level H7-HA ADCC responses were boosted post-infection (Figure 3a, $P = 0.04$). Pre-infection HA stem-specific ADCC responses were more common than H7-HA-specific ADCC responses and found in every participant tested (Figure 2c and d); however, HA stem ADCC response magnitude was not different at baseline for infected and uninfected contacts nor was HA stem-specific ADCC activity increased by infection (Figure 3a and b).

H1N1 infection and H3N2 infection resulted in significant fold rises for cross-reactive H7-HA- and NP-specific IgG (Figure 2c and d), and G1 stem-specific IgG was also increased by H1N1 infection. The IgG avidity (Supplementary figure 4d), and IgG1:IgG2 balance (Supplementary figure 4e) of HA-specific antibodies was comparable at baseline and after exposure between infected and uninfected contacts, whilst recent infection skewered the NP-specific IgG to a greater Th1 response by increased IgG1:IgG2 ratio (Supplementary figure 4d).

Association of baseline influenza-specific ADCC responses with influenza symptom severity

To assess whether ADCC responses were associated with reduced viral shedding or symptom severity once infection was established, we determined the correlation between the magnitude of baseline pre-exposure ADCC responses against symptom severity and peak viral loads and for H1N1- and H3N2-infected contacts.

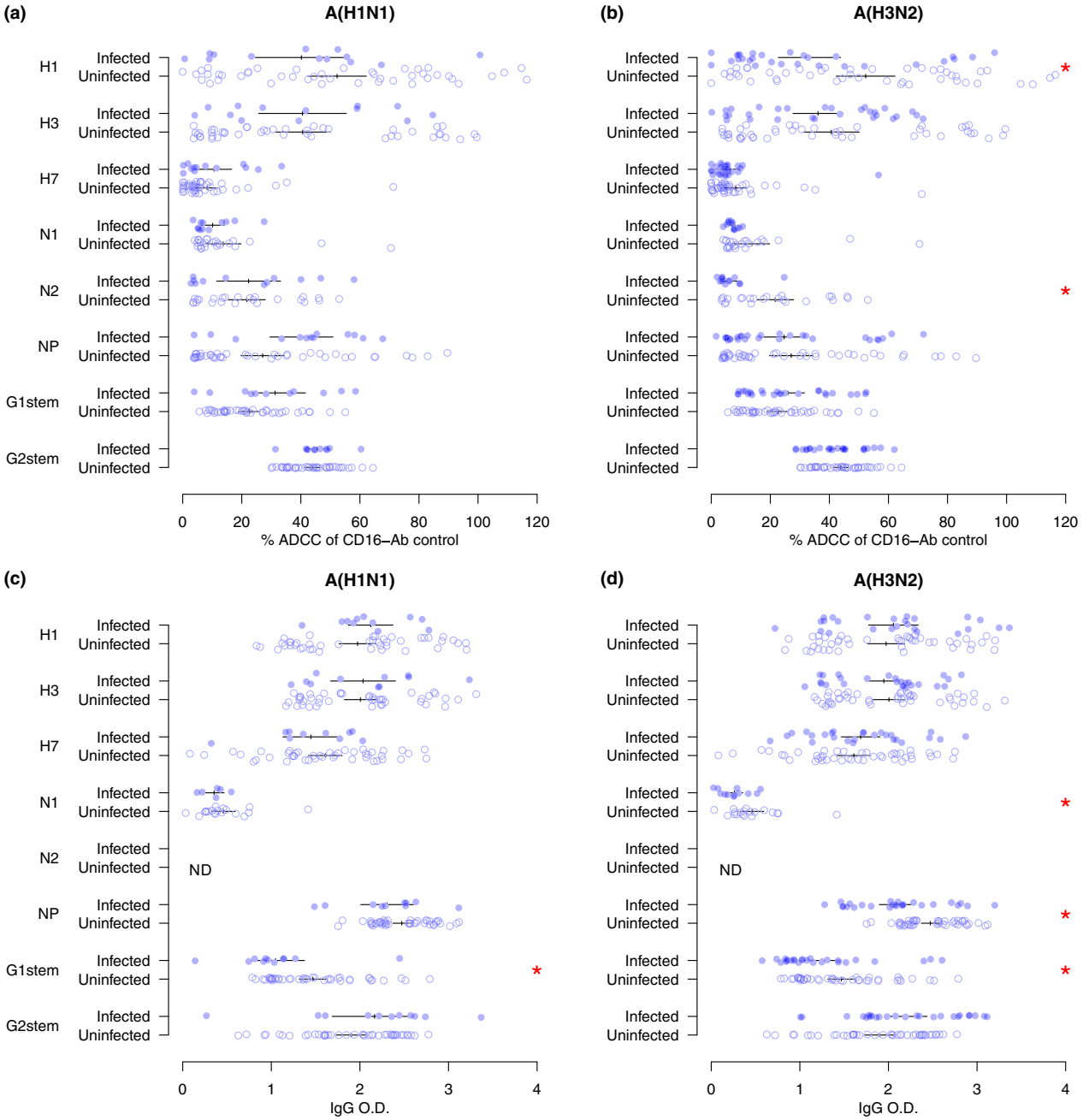

Figure 2. Pre-exposure influenza-specific ADCC responses for household contacts, for infected and uninfected contact participants. A panel of representative influenza viral proteins, including H1-HA, H3-HA, H7-HA, N1-NA, N2-NA, NP and HA group 1 (G1 stem) and group 2 stem (G2 stem) were screened with pre-exposure plasma for ADCC activation of NK cells from contacts infected with H1N1 **(a)** (n = 13) or H3N2 **(b)** (n = 29) and uninfected participants (n = 45). Protein-specific IgG for the same protein panel and participants was assessed by standard ELISA, from contacts infected H1N1 **(c)** (n = 13) or H3N2 **(d)** (n = 29) and uninfected participants (n = 45). Data represent the response of individual participant, group mean and 95% confidence interval. *P > 0.01 by Wilcoxon rank-sum test, and regression model adjusting for age to check the significance. FACS gating strategy is shown in Supplementary figure 1. Experiments were performed twice.

We observed that higher magnitude baseline ADCC H1-HA responses moderately but significantly correlated with reduced peak symptom severity score for H1N1 infection (Figure 4a) (correlation $R^2 = -0.30$, $P = 0.05$) but not for H3N2 infection (Figure 4b and d) or viral

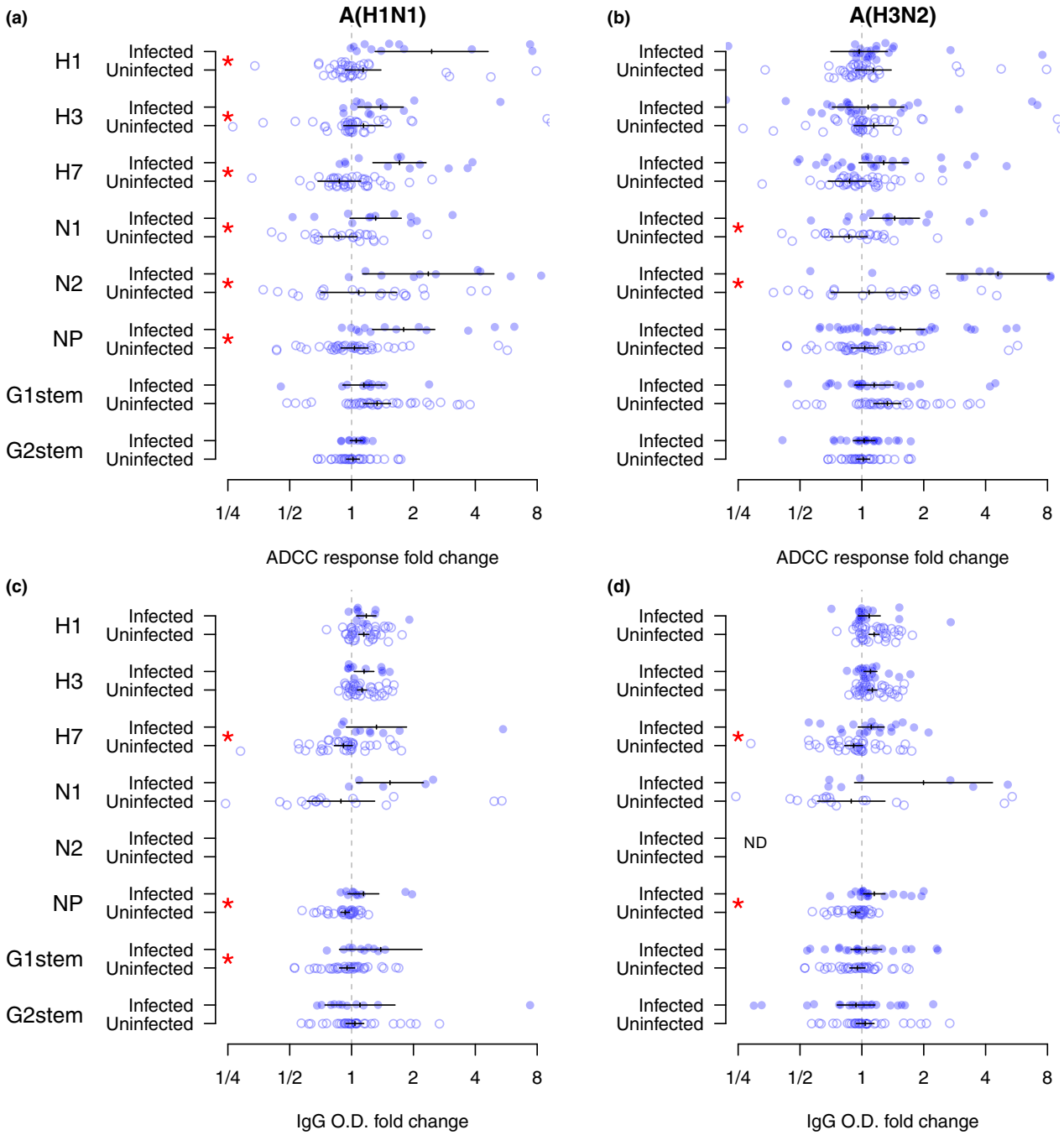

Figure 3. Infection boosts ADCC responses and total IgG for influenza proteins. The fold-change of ADCC responses to the protein panel from pre- versus post-exposure serum samples for **(a)** H1N1- and **(b)** H3N2-infected and **(a)** H1N1- and **(b)** H3N2-uninfected contacts. The fold-change of influenza-specific IgG by ELISA using the same protein panel for **(c)** H1N1- and **(d)** H3N2-infected contacts and **(c)** H1N1- and **(d)** H3N2-uninfected participants. Data represent the response of individual participant, group mean and 95% confidence interval. *$P > 0.01$ by Wilcoxon rank-sum test, and regression model adjusting for age to check the significance. ND, Not determined for ELISA for N2-NA because of limited serum. Experiments were performed twice.

shedding (Figure 4c). Furthermore, there was no significant correlation between fold-change in ADCC response magnitude or endpoint titre with symptom scores or viral loads. Combined regression analysis of symptom severity scores versus baseline ADCC responses H1-HA from

H1N1-infected and H3-HA from H3N2-infected participants for increased sample size did not increase the strength of the correlation.

Influenza-specific T-cell responses at baseline and post-infection

To measure $CD4^+$ and $CD8^+$ influenza virus-specific T-cell responses, PBMC samples were stimulated with influenza viruses to identify total influenza-specific IFN-γ^+ T cells by flow cytometry (Supplementary methods, Supplementary figure 5a),[11] including characterisation of phenotypic (CCR7/CD45RA, CCR5) and functional markers (CD107a, IFN-γ, TNF-α, IL-2). Influenza-specific IFN-γ^+ $CD8^+$ T cells (Supplementary figure 5b) and $CD4^+$ T cells (Supplementary figure 5c) were not significantly different between infected and uninfected contacts at pre- or post-exposure timepoints, nor were they increased at day 30 post-infection. Furthermore, no significant correlation was found for baseline IFN-γ^+ $CD4^+$ or $CD8^+$ T-cell responses and peak symptom scores or viral loads in H1N1- or H3N2-infected participants (data not shown). However, low response magnitude[12] and low viability in samples of some donors (<20% excluded from analysis) have limited our analysis of T-cell responses precluding further phenotypic or functional characterisation.

DISCUSSION

Our 8-year study of household transmission of influenza in Hong Kong enrolled influenza-exposed household contacts before infection was established enabling our present analyses on the role of baseline immunity, principally ADCC antibodies and T-cell responses, as broadly reactive protective immune correlates against influenza virus infection. Symptomatic index cases in our household study were identified after seeking medical attention, and we collected baseline blood samples from the household contacts before infection occurred, importantly providing a representative picture of infections acquired from natural influenza transmission.

Human influenza challenge studies are vital and provide the opportunity for baseline samples; however, screening for HAI low/negative participants and infection with a laboratory-grown virus at high dose in liquid instillation for intranasal delivery[6,9,13] of influenza virus may not represent community-acquired infection. Studies of acute influenza virus infection have considerable value,[3,14,15] but infection is established and immune activation ongoing. Longitudinal community-based cohort studies are also invaluable; however, in contrast to experimental challenge studies where pre-existing immunity can be correlated to viral loads and symptoms in a cohort of volunteers who are infected with the same viral strain, many additional factors other than pre-existing immunity can influence community-acquired influenza acquisition among household contacts, such as hand hygiene, and the extent of direct exposure to the index case. Previous community cohort studies of acquired pandemic H1N1 infection in 2009 with sample collection from prior to and early during the pandemic illustrated the foresight of established bio-banks to identify influenza virus infections.[16,17] The study by Sridhar *et al*. provided important data on the importance of influenza-specific memory $CD8^+$ T cells to reduce fever and symptoms,[16] whilst more recently Ng *et al*. showed the importance of HA group 1 (G1) stem binding antibodies in reducing the risk of infection.[17] The size of cohort required to identify sufficient number of influenza virus infections for the evaluation of the association of baseline immunity against infection is challenging and resource-intensive. Therefore, we performed active identification of symptomatic influenza cases and follow-up of their exposed household contacts to identify secondary natural influenza infections[18] and study of baseline immunity before infection.

Our present study found that cross-reactive influenza-specific antibodies, especially to non-neutralising targets such as NP, G1 stem and N1-NA, and cross-reactive H7-HA, to be a protective correlate of infection, as these antibodies were higher in uninfected contacts and boosted by recent infection. These results are consistent with a recent Nicaraguan household transmission study of pandemic H1N1 infection, which found G1 stem-specific IgG as an independent correlate of protection from infection at baseline.[17] Another recent study from our group has also shown a correlation of increased baseline NAI activity correlating with reduced symptoms scores during H1N1 infection.[19] Infected contacts at baseline timepoints compared to uninfected participants had lower NP and G1 stem-specific IgG, irrespective of ADCC function, suggesting antibodies to these conserved epitopes also serve

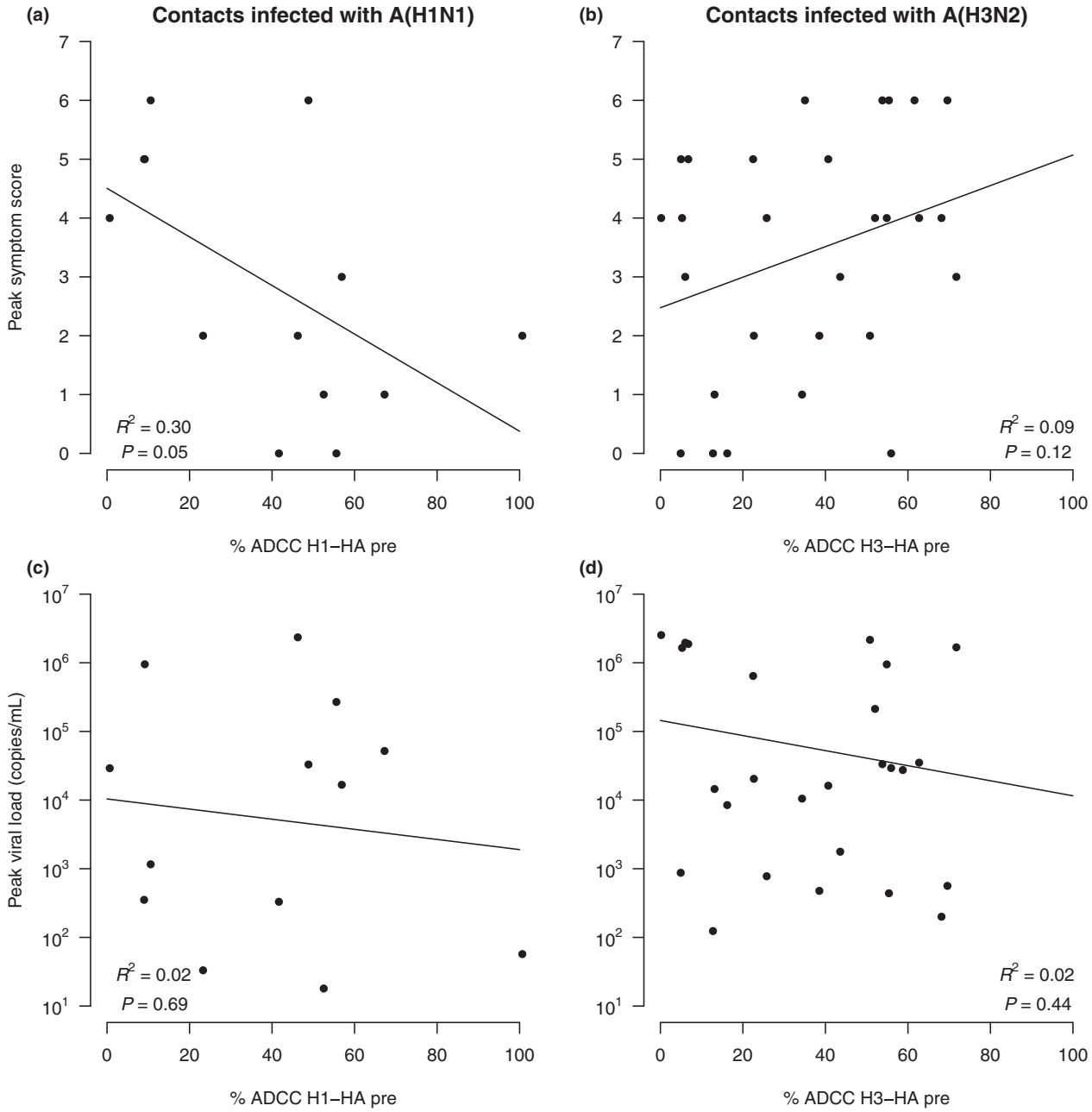

Figure 4. Higher baseline ADCC responses correlate with reduced symptom severity for H1N1 infection. The correlation of **(a, c)** H1N1- and **(b, d)** H3N2-infected contact participants baseline pre-exposure HA ADCC responses and peak symptom score, and peak viral loads for H1N1 infection **(c)** and H3N2 infection **(d)**. R^2 and slope *P*-value by the least squared method. Data represent the individual subject response.

a function in protection from influenza virus infection.[20,21]

Uninfected contacts had higher baseline total IgG for the G1 stem than H1N1- and H3N2-infected participants, and G1 stem-specific IgG levels were boosted by recent H1N1 infection. However, we observed no significant difference between infected and uninfected participants for the G2 stem antibodies by ADCC responses at baseline, fold-change after infection or ELISA assays despite clear and specific detection of G2 stem antibody responses.[22] The generation of G2 stem antibodies is notoriously more difficult because of an additional N-glycosylation

at HA1-Asn38 restricting access,[23,24] resulting in the isolation of far fewer G2 stem monoclonal antibodies,[25] and additional modifications required to stabilise the G2 stem headless structure for vaccination models.[26]

Antibody-dependent cellular cytotoxicity responses are reportedly highly cross-reactive, and avian influenza-specific ADCC antibodies can be found in healthy unexposed individuals[7,27] and enriched for the HA stem.[8,28] In our study, ADCC activity towards conserved NP, cross-reactive H7-HA, seasonal HA and NA proteins was boosted by recent H1N1 infection. Previously, we have reported limited boosting of ADCC responses post-vaccination with seasonal inactivated vaccines in older adults.[11] Influenza infection exposures in children boost ADCC responses but are less pronounced in adults post-infection,[8] and similarly cross-reactive ADCC responses plateau with age by 17 years of age.[7] In addition, we have previously found that ADCC responses in samples from adults collected a few months before and after H1N1 infection are stable.[29] We propose that ADCC antibodies are boosted shortly after infection and wane, which is also evident in macaque models of influenza infection.[30] In this study, post-exposure serum samples were collected within 30 days of infection, whilst in our previous community cohort study, serum samples were collected at 3- to 6-month intervals with infection determined serologically by HAI assay.

A limitation of these studies and ours is the use of recombinant influenza proteins to decipher antigen-specific IgG and ADCC responses, as protein conformation and orientation may impact antigenicity and binding. Whilst commercial protein conformation was not assessed in this study, we have previously confirmed the HA stem conformation and binding of conformational antibodies.[31] Importantly, the NK92 ADCC assay with recombinant proteins that we used has previously shown a strong correlation with cytotoxicity experiments using virus infected for total influenza-specific antibodies or transfected target cells,[8,27] IFN-γ production by NK cells derived from PBMCs[8] and other binding measures of FcR interactions.[32] Alternate antigenic targets such as the M2e with known FcR-dependent protection[33] are poor immunogens during natural infection (reviewed by Lee *et al.* [34]) and were not included in our study. NP-specific antibodies have previously shown FcR-dependent protection in mice.[20] In our study, we found that total NP-specific IgG was lower in infected subjects, but their ADCC function was comparable at baseline and boosted by recent H1N1 infection.

Typically, IgG1 and IgG3 isotype antibodies are attributed to ADCC function, requiring maturation of IgG subclasses,[35] whilst IgG2 is a Th2-type response and does not have ADCC function. We did not observe a difference in HA-specific IgG1:IgG2 balance between infected and uninfected contacts or after recent influenza virus infection, whereas NP IgG1:IgG2 increased post-infection. In addition, we observed a significant correlation with H1N1-infected contacts with higher baseline ADCC responses reporting lower symptom scores, but there was no effect on H1N1 peak viral loads. This may reflect the timing of anti-viral activity of ADCC antibodies, which may mediate immune regulation rather than significantly directly reducing viral loads by NK cell activation, whilst elsewhere HA-stalk antibodies have been proposed to reduce viral shedding rather than symptoms severity.[36] Furthermore, the fold-change in the magnitude and endpoint titre of ADCC responses during infection did not impact symptom severity or viral loads, suggesting that ADCC responses are more important at baseline before infection than during infection for limiting influenza disease severity as ADCC antibodies act early to engage innate cells during infection. Increased ADCC functional antibodies in uninfected contacts, and reduced symptoms scores in infected contacts with higher levels of ADCC antibodies, further demonstrate that ADCC antibodies are associated with protection against influenza virus infection and may also be a surrogate for greater antibody function by additional measures.

Antibody-dependent cellular cytotoxicity antibodies highly cross-reactive between influenza subtypes[27] and are not as exclusive in specificity as HAI responses, hence their attraction as an immune correlate for universal vaccine development. In our study, we have observed unexpected trends, with boosting of heterologous HA and NA responses, and discrepancies between infection with different influenza subtypes. We found that H3N2-infected subjects had significantly lower H1-HA ADCC antibodies than uninfected participants, whilst H3-HA-specific antibodies remained comparable between H3N2-infected and H3N2-uninfected participants, and H1-HA ADCC responses for H1N1-infected participants. A limitation of our study is the H3N2-infected participants' sample size over the years of collection for the study period as H3N2

viruses drifted during this time. There was a higher number of antigenic changes for H3N2 viruses versus H1N1 viruses during the 7-year collection period, with five H3N2 antigenic vaccine changes versus only one H1N1 strain dominating during the same time period; therefore, differences may have occurred for H3-HA of other representative strains. Previously, age-related increases in ADCC function have been found for H7N9 viruses from 2 to 17 years[7] and H1N1 pandemic-specific antibodies in individuals over 45 years of age.[37] Therefore, the novelty of the 2009 pandemic H1-HA may demonstrate the role ADCC plays for newer strains than H3N2 strains, which have not experienced a pandemic shift but gradual drift during our study. The importance of ADCC function may be related to the antigenic distance of the infecting strain versus prior exposures, with the novel factor of the pandemic H1-HA resulting in H1-specific antibodies, which are cross-reactive retaining ADCC function, whilst this was not seen for H3-HA-specific antibodies despite a high level of existing ADCC function in H3N2-infected and H3N2-uninfected contacts. HA imprinting[38] may also play a role in the differences of ADCC function for H1N1 and H3N2 infections, with the majority of our contact participants being aged 42 ± 12 years old, and H3N2 viruses circulating from 1968 and co-circulating with H1N1 viruses from 1977, coinciding with their first exposures most likely being H3N2 viruses, whilst this is difficult to prove without longitudinal samples from baseline we can still speculate.

Influenza-specific T-cell immunity has been shown to reduce viral shedding and symptom severity in human experimental infection for $CD4^+$ T cells[9] and community cohort studies for $CD8^+$ T cells.[16] A total T-cell memory response of >20 SFU/10^6 PBMCs has been defined as a protective threshold response from symptomatic influenza virus infection[5]. However, the correlation of baseline influenza-specific $CD4^+$ and $CD8^+$ T cells with the risk of infection by including both infected and uninfected participants has not been reported previously. Results from animal models[39,40] and human studies have repeatedly shown the necessity of T-cell immunity in clearing influenza virus infection,[9,13,16,41] especially heterologous infection with novel influenza subtypes in the absence of neutralising antibodies.[42] However, peripheral sampling of T-cell responses may not reflect mucosal immunity, with large numbers of T-cell resident memory found in the lung.[43] Therefore, whilst our study did not find a correlation between T-cell immunity and reduced risk of infection, viral loads or severity in our study compared to previous studies, this could be due to several possible limitations within our study such as T-cell antigenic match for seasonal influenza viruses is closer to existing memory responses than avian or pandemic strains, peripheral sampling, sample study size, PBMC viability and experimental limits of detection for *ex vivo* influenza-specific T-cell responses by IFN-γ ICS. Due to limited sample sizes, the T-cell responses of H1N1- and H3N2-infected participants were not split by infection subtype because of high levels of T-cell cross-reactivity, unlike our serological characterisation. The role of T-cell immunity as a protective correlate of infection remains elusive, and further studies are imperative for their inclusion in a universal candidate vaccine design.

The breadth, specificity and function of influenza-specific antibodies are actively shaped by multiple factors,[35,44] such as the route of exposure (e.g. peripheral vaccination versus local infection at mucosal surfaces), T cell help for B cells and inflammation levels of infection.[3] Systems serology approaches, which use a combination of multiple measures of antibody specificity, function and avidity for Fc receptors, are advancing the field of HIV,[32,45] Ebola,[46] dengue virus[47] and influenza vaccination.[35] Further work using systems serology approaches will advance our current observations and Fc-mediated functions in uninfected participants in community cohort studies. Our study further supports ADCC as a protective correlate for reduced risk of infection, boosted by recent infection and reduced symptoms during infection.

METHODS

Study design

We enrolled household index cases from outpatient clinics in Hong Kong from 2010 to 2017[48–50] that (1) presented with acute respiratory illness (ARI), defined as having ≥ 2 of 7 signs/symptoms (fever ≥ 37.8°C, headache, myalgia, cough, sore throat, runny nose and sputum production), and (2) were the first and only member in their household with a recent ARI. Screening of index cases was by rapid test (QuickVue or Sofia Influenza A + B, Quidel, Santa Clara, CA, USA) and confirmed by RT-PCR of nose and throat swabs, as previously described using virus subtype-specific primers.[51] We then arranged further follow-up by

active surveillance of index cases and their household contacts (Figure 1a). At a baseline home visit (day 0, same or next day of index case recruitment), we collected nose and throat swabs from each member of the household, and 4mL heparinised blood from a volunteer subset (Table 1). Symptoms were self-reported in diaries from days 0 to 6, and at days 3 (home visit 2) and 6 (home visit 3), we collected nose and throat swabs from all household members for testing by RT-PCR, and at 28 days (home visit 4, range 21–45 days) for blood collection from a volunteer subset. The study protocol was approved by the Institutional Review Board of the University of Hong Kong (UW:08-008).

Protein-specific ADCC responses

A protein plate-bound NK ADCC assay was performed as previously described.[11] The assay measures degranulation (CD107a) of activated human NK cells (NK92 cell line transfected with FcRγ IIIA, CD16) due to cross-linking of protein-specific IgG in participant sera (Supplementary figure 1). A panel of influenza viral proteins of representative influenza strains and cross-reactive proteins was assessed for ADCC activity. Commercial proteins (Sino Biological, Beijing, China) included H1-HA and N1-NA (A/California/07/2009(H1N1)), H3-HA and NP (A/Switzerland/9715293/2013(H3N2)), H7-HA (A/Anhui/01/2013(H7N9)), N2-NA (A/Hong Kong/4801/2014(H3N2)) and, for negative controls, non-specific protein (HIV gp120 and FBS block, Invitrogen, Carlsbad, CA, USA) at 400ng/well in PBS. Group 1 (G1) stem (from H1N1 consensus) and group 2 (G2) stem (from H3N2 consensus) proteins were made as previously described.[22,31] Positive controls include purified CD16 antibody (BioLegend, San Diego, CA, USA) and pooled H1/H3-HAI$^+$ human sera (n = 5 donors). Following stimulation, NK92 cells were stained for anti-human CD16-PE and CD107a-APC (BioLegend, USA). Responses were normalised to % of maximum CD107a$^+$ of CD16-stimulated positive controls, and background subtracted. Serum was typically tested at 1:20 dilution, or in a threefold dilution series from 1:20 to 1:43, 740 over 8 wells to determine endpoint concentrations for the highest dilution of antibodies inducing 5% CD107a$^+$, which was comparable to twice above background.[8] Baseline pre-exposure (day 0) responses and fold-changes from pre- and post-exposure (day 28) serum samples were assessed for infected and uninfected participants.

Enzyme-Linked Immunosorbent Assay (ELISA)

Influenza viral proteins (as above) (80 ng mL^{-1}) were coated on flat-ELISA plates (NUNC, Denmark) overnight. Following FBS blocking and washing (PBS with 0.05% Tween-20), diluted heat-inactivated serum samples (1:100) were bound for 2 hours, washed and detected by secondary anti-human IgG-HRP (Invitrogen, USA)), substrate/stop reaction (R&D systems, Minneapolis, MN, USA) and read at 450 nm. Additional experiments to assess the avidity of NP, H1-HA and H3-HA antibodies binding by incubation of 15 min with 8M Urea (Sigma, Kawasaki, Kanagawa, Japan), after serum binding to remove low avidity antibodies, and the % remaining IgG response representing high avidity antibodies.[52] IgG subclasses were determined using anti-human IgG1-HRP or IgG2-HRP (Southern Biotech, USA).

Statistical analyses

Serum baseline influenza virus-specific ADCC and IgG responses and their fold-changes were compared between viral subtype infected and uninfected contacts by the Wilcoxon rank-sum test. We further explored the differences by fitting linear regression model adjusting for age group. The relationship between peak symptom score and peak viral load versus pre-exposure ADCC responses was assessed by linear regression using the least squared method.

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CONFLICT OF INTEREST

BJC has received honoraria from Sanofi and Roche. Other authors declare no other competing interest.

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