How do cross-reactive memory B cells affect influenza vaccine titers

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Summary

Influenza causes substantial morbidity and mortality, and while vaccines are available, frequent update is required to keep up with virus mutation, and vaccine effectiveness can be poor despite careful strain selection and update. Humans have varied influenza exposure histories, and serological responses are also highly variable. This makes it difficult to use human sera for vaccine strain selection, which instead relies heavily upon first-infection ferret sera to characterize circulating strains. Nevertheless, it remains important to understand the effects of prior influenza exposure and immunity so that strains can be selected to generate optimal vaccine responses in humans. It is generally accepted that repeated exposure to different influenza strains favors generation of cross-reactive memory B cells, but their impact on protective antibody titers is unknown. We, and others, have demonstrated that titers are highest to strains encountered in childhood, supporting suggestions that cross-reactive memory B cells impede naïve B cell responses. Conversely, memory B cells can adapt to variant strains via B cell receptor (BCR) mutation, but it is not known if "adapted titers" match specifically primed naïve cell titers. Our research seeks to determine the cellular and molecular basis for variability in antibody responses to influenza, and for the effects of past influenza infections on responses to prevailing strains. The Ha Nam community influenza cohort is unique in that participants have been actively monitored for influenza illness and infection, defined by seroconversion, for eleven seasons. This provides a rare opportunity to understand how influenza exposure history and immune memory influence responses to influenza vaccination and the protection that is generated. In addition, we have developed key resources to investigate exposure history and immune memory including; a computational tool (antibody landscapes) to analyse titers in the context of antigenic difference between strains; and high throughput BCR sequencing to determine if responding cells derive from memory or naïve cells. The influence of memory cells on titers will dictate whether vaccine strains should be selected to avoid cross-recognition and interference or promote cross-recognition and adaptation. This project will investigate vaccine responses in cohort participants, and will relate titers to exposure history and the contribution of memory-derived cells to the acute B cell response.

Aims and objectives

The overall aim is to determine the impact of prior influenza infection and cross-reactive memory B cells on neutralizing antibody titers to the prevailing strain, i.e. the current infecting or vaccine strain. ***We hypothesize*** *that responses dominated by cross-reactive memory B cells are inferior to responses with less memory cell involvement.*

We will investigate the following specific questions:

1) Does infection history affect prevailing strain titer?

2) Are prevailing strain titers related to the magnitude of memory-type humoral responses, defined as early, cross-reactive IgG responses?

3) What proportion of acutely responding cells are memory-derived, what proportion adapt to the prevailing strain, and how do these relate to titers to the prevailing strain?

Background

Influenza vaccination, strain variation and prior immunity

Most vaccines work by generating neutralizing antibodies and memory B cells that can respond rapidly to infection. Repeated boosting may be required to attain protective titers. Influenza neutralizing antibodies are directed towards the major virus envelope protein, namely hemagglutinin (HA). The globular head, or HA1 domain, of HA contains the conserved sialic acid receptor binding pocket. Neutralizing antibodies block attachment by binding epitopes (antigenic sites) surrounding this pocket (1). However, influenza virus mutation and immune-selection pressure drive virus evolution and the emergence of antigenically different influenza viruses every few years, a process called antigenic drift. Therefore, frequent vaccine strain updates are necessary to maintain vaccine effectiveness, and annual vaccination is recommended. Vaccine strain

selection is based on antigenic and genetic (HA1 sequence) analyses of circulating viruses, to identify the emergence and wide transmission of circulating strains that are antigenically distinct from existing vaccine strains (2). Antigenic characterization is largely based upon hemagglutination-inhibition (HI) assays with reference strain anti-sera. HI assays detect antibodies that block influenza virus agglutination of red blood cells via sialic acid receptors on the red blood cells. Strain-specific antisera are made by infecting influenza-naïve ferrets with a single virus strain. Two-way titrations are performed with sera raised against each virus, which are tested against all other viruses. Derek Smith and colleagues developed a computational tool that uses HI titers to quantitate the antigenic difference between multiple strains for visualization on a two dimensional antigenic map (3). Influenza A viruses of the H3N2 subtype (A/H3N2) emerged in humans in 1968. Analysis of A/H3N2 strains circulating since 1968 demonstrates that they fall into 14 discrete antigenic groups or clusters (3, 4), each differing by 1 to 13 amino acids, located in up to five antigenic sites (3, 5). However, independent introduction of each substitution indicates that antigenic change is mainly caused by a single amino acid substitution in a single antigenic site (5), consistent with monoclonal antibody driven virus selection. It has been suggested that all five antigenic sites may be relevant to escape from human immunity, and in turn that ferret sera may not be representative of human sera (6). While insights gained from the study of ferret antisera provide precise information regarding virus mutations that alter neutralizing antibody epitopes, it is unclear whether this infers escape from human immunity. The reactivity of sera from human vaccinees is considered during vaccine selection, but results are difficult to interpret due to cross-reactivity and high variability between individuals whose exposure histories are unknown. Cross-reactive antibodies and cells are induced by repeated exposure to variant strains (6), but it is unclear how they influence responses to new strains. Studies in the 1950's demonstrated that HI titers are highest against the first strain encountered in life (7), and led to the doctrine of original antigenic sin, which postulates that upon subsequent exposure to related but antigenically varied viruses, the antibody response to the primary strain is reinforced while the response to the prevailing virus is dampened (7). Numerous studies have reproduced these findings. Our collaborative group recently demonstrated that titers diminish progressively with each new strain encountered (4). Lessler et al observed a similar phenomenon and called it "antigenic seniority" (8). Studies directly comparing the outcome of single versus repeated or multiple vaccination are conflicting (9-12), indicating that effects of prior immunity may vary. Most recently, Ohmit, Monto and colleagues demonstrated lower vaccine effectiveness with repeated vaccination in both 2011-2012 and 2012-2013 seasons (12). This was particularly apparent for A/H3N2, and effectiveness is generally lower against A/H3N2 than against A/H1N1 and B. A mathematical model developed by Derek Smith's group indicates that antigenic difference may be a key factor contributing to variable effects of repeat vaccination (13). The model indicates increasing benefit of repeated vaccination with increasing antigenic distance between the first and second vaccine strains, and decreasing distance between the second vaccine strain and the outbreak strain. Despite careful and considered vaccine strain selection, vaccine effectiveness varies between subtypes and years, and can be poor (14). Strain mismatch contributes to poor vaccine effectiveness, but does not fully account for the observed variability. The need to identify determinants of vaccine effectiveness is well recognized (12).

The Ha Nam community influenza cohort was established by the Oxford University Clinical Research Unit (OUCRU), Viet Nam and colleagues the National Institute of Hygiene and Epidemiology (NIHE) in 2007, and has been ongoing since that time. 270 households with around 1000 occupants have participated in continued active surveillance for influenza-like-illness since 2007. Participants have never been vaccinated against influenza. To date, swabs have been collected from over 1060 influenza-like-illness cases and assessed for influenza virus by real time RT-PCR and culture. Cross-sectional sampling of the cohort has been performed between each peak in detection of confirmed influenza cases, yielding 7544 blood samples to date. More than

24000 HI assays have been performed on paired pre- and post-season sera to detect seroconversions to prevailing strains. Seroconverters each season included participants with little or no apparent illness.

The serological response to a panel of A/H3N2 viruses, covering 43 years of virus evolution, was studied extensively for 69 Ha Nam cohort participants, including all A/H3N2 illness cases and 38 seroconverters (4). Ian Barr and his team at The WHO Influenza Collaborating Center in Melbourne assembled the comprehensive collection of historical H3N2 viruses, produced ferret antisera, and developed robust quality assurance and control measures for HI assays to achieve highly stable results (4). Derek Smith's team developed a computational tool, called antibody landscapes, to assess titers as a function of antigenic distance between viruses (4). This method builds upon the two-dimensional antigenic maps, described earlier, and plots individual human serum HI titers in a third dimension, then fits a smooth surface. A summary path is taken through each antigenic cluster on the antigenic map, and HI titers along this path are extracted to generate a two-dimensional landscape of titer versus antigenic distance. Landscapes were remarkably stable over time in non-infected individuals but varied greatly between individuals (4). Infection with A/H3N2 resulted in an unexpectedly strong back-boost of the pre-exposure antibody landscape. Responses were far broader than a typical primary response in ferrets. To investigate the effect of antigenic distance on back-boosting, sera from two vaccine studies were retrospectively assessed: one involved a vaccine strain that was antigenically well-matched to circulating strains, and the other a vaccine strain that was considered relatively novel because there had been little prior circulation. The more novel vaccine induced higher titers against both advanced and previous antigenic clusters. We hypothesize that the response to the well-matched vaccine was relatively poor because of greater interference from cross-reactive immune responses induced by prior exposures, but exposure histories were unknown. Consequently, our **first aim** is to determine if infection history affects prevailing strain titers by comparing responses to Trivalent Influenza Vaccine in Ha Nam cohort participants with and without A/H3N2 infection since surveillance commenced in December 2007. The cohort provides a rare opportunity evaluate immune responses to vaccination in people with known infection histories.

Influenza antibody titers and immunological memory – boost or avoid?

Robust back boosting of influenza neutralizing antibody titers (4) indicates that cross-reactive memory B cells are induced, but their impact on prevailing strain titers is unknown. In the antibody landscape study with Ha Nam sera, absolute titers were highest against strains encountered in early childhood. However, titer rises following infection were highest for infecting strains and gradually declined with increasing antigenic distance from the infecting strain (4). A range of memory B cell effects could account for these observations including: -

1) During each infection memory cells are boosted and naïve cells are primed, without effect on each other. As such, titers against a given strain would reflect the number of subsequent infections or boosts.

2) Memory B cell boosting interferes with naïve cell responses to new epitopes, and this increases with successive infections as the size and epitope range of the memory pool grows. Memory B cells have undergone affinity maturation and could interfere with naïve cell responses by monopolizing antigen and T cell help.

3) Memory B cells monopolize responses to new variants, and their ability to adapt declines with increasing antigenic difference.

These effects may not be mutually exclusive, for example memory cell adaptation may influence the extent of interference with primary responses. The impact of memory responses will dictate whether vaccine strains should be selected to boost or avoid cross-reactive memory responses. In the Ha Nam landscape study titers were assessed several months after infection when it was too late to define memory cell involvement or peak titers, and how they are related. Our **second aim** is to determine the relationship between prevailing strain titers and the magnitude of memory-type humoral responses, which will be defined based on the specificity and isotype of acute responses.

Cellular responses underlying antibody generation and maintenance

Influenza strain-specific neutralizing antibodies can be detected decades after the strain has ceased circulating, although antibody half-life is ~ 20 days. Antibody titers are maintained by long-lived antibody secreting cells (ASC), which reside within survival niches in bone marrow and secondary lymphoid organs (17). ASC number, and the proportion that are clonal, peak around 7 days after vaccination, at least one week before memory B cell frequencies peak (18, 19). Most are short-lived ASC clones, that persist for 2-7 months, whereas up to 5% attain sufficient antibody affinity to compete for survival niches, and produce antibodies for decades (17, 20). High affinity B cell receptor (BCR) mutants develop, and are selected to differentiate into long-lived ASC and memory B cells, within germinal centers in secondary lymphoid organs (17). The process requires interaction with follicular T helper cells, and involves somatic hypermutation, switch from IgM to IgG isotype, and regulation of genes that modulate survival and stimulation requirements such as CD80 (17). Somatic mutation leads to affinity maturation, and increases the capacity of B cells to capture and present antigen to follicular T cells, which provide help for further rounds of maturation (17). Memory B cell differentiation is less dependent on affinity, and memory cells have a broader repertoire than LL-ASC (17). Therefore, memory B cells provide a means to rapidly diversify the antibody response to include epitopes that escape long-lived ASC recognition (20, 21). BCR sequencing and expression have been used to investigate memory B cell responses to new influenza strains. Wrammert and colleagues suggested that memory B cells were the source of a substantial fraction of the acute ASC response to new influenza vaccine strains because ASC were dominated by expanded clones, that used extensively mutated, isotype-switched BCR (19). Recombinant monoclonal antibodies (mAbs) were derived from these ASC, and influenza B reactive mAbs had higher affinity for the vaccine strain than for past strains. This indicates that memory cells rapidly adapt to new strains via somatic hyper mutation, and the authors suggest original antigenic sin does not limit reactivity to new influenza strains (19). However, it should be noted that the influenza B strain in the vaccine and those circulating previously were from distinct antigenic lineages (Victoria and Yamagata, respectively), as opposed to being drift variants. Tan and colleagues identified a subset of clones (3 of 20) induced by an A/H3N2 vaccine that had higher affinity for past strains, providing some evidence that original antigenic sin shapes the antibody response to influenza (22). However, a closer inspection of the data presented by Wrammert et al, demonstrates that, unlike influenza B, A/H3N2-reactive clones with equivalent affinity for past and vaccine strains predominated, less than 25% had higher affinity for the vaccine strain, and more than 25% had higher affinity for a past strain. Scott Boyd's team at Stanford utilized high throughput sequencing to compare the BCR repertoire before and after pandemic H1N1 2009 vaccination. They revealed striking convergence in BCR gene use by cells from different vaccinees, and vaccinees from different studies (18). These so called "Signature BCR" detected post-vaccination were also detected amongst pre-pandemic memory B cells, albeit rarely (18). Repeated vaccination with the same strain induced the same convergent signature, but new clones were recruited, suggesting that antibody induced during the first vaccination may prevent restimulation of memory B cells specific for identical epitopes, whereas B cells that recognize somewhat different epitopes can respond (18). A similar study of BCR repertoire changes during seasonal influenza vaccination did not identify convergent rearrangement signatures, and observed extreme variation between individuals (23). Only one of three vaccinees exhibited a highly clonal response at day 7, as described by Wrammert et al (23). It is suggested that factors such as age and prior exposure may underlie this variability. Substantial gaps in knowledge remain. Foremost, the relationship between molecular and cellular responses and antibody titers has not been quantified. It is apparent that memory B cells can adapt to new strains but it is not known if adaptation is constrained by antigenic distance, and hence if adapted memory cell titers are equivalent to titers made by specifically primed naïve cells. Similarly, it is unclear whether clones derived from memory cells can attain sufficient affinity to become LL-ASC and maintain titers. Further it is not clear whether memory cell boosting occurs at the expense of stimulating new clones, and thus whether titers are reliant on memory cells.

Our **third aim** is to define the proportion of acutely responding cells that are memory-derived, and that adapt to the prevailing strain, and relate these proportions to peak and sustained titers.

Methods

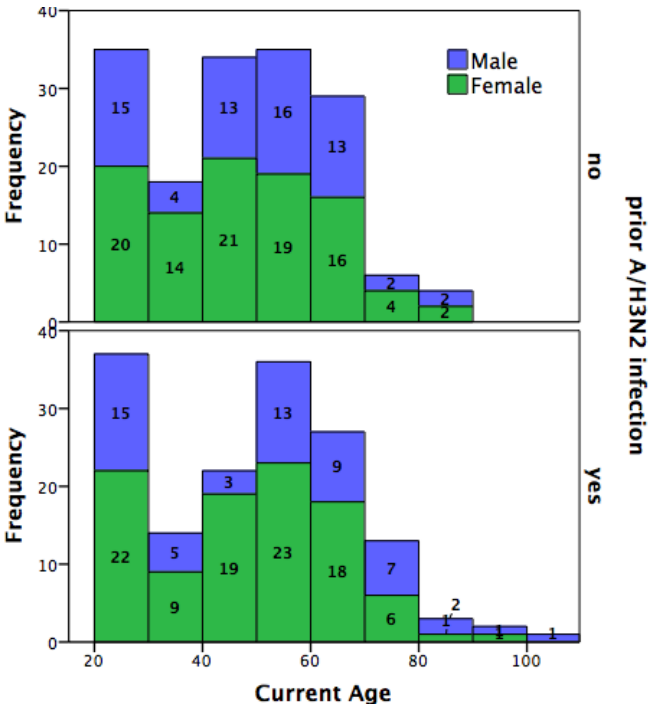
Study Design

Our primary approach is to examine influenza vaccine responses in participants with divergent histories of past infection, which we assume will affect memory cell involvement. We will investigate both peak titers and sustained titers, which are presumed to reflect LL-ASC. We will focus on responses to vaccination, in keeping with the goal of enhancing vaccine selection. However, infection generates broader responses than vaccination (24), such that effects of repeated infection and vaccination may differ. This will be explored by investigating natural infections detected through ongoing surveillance. We will study A/H3N2, rather than A/H1N1, strains because we have found that infection is more strongly associated with HI titer and more common in adults, and antigenic drift is greater (15). We aim to define the effect of past infection on prevailing strain titers, but infections prior to December 2007 are overlooked. Therefore, a more detailed characterization of the serological response will be used to detect memory more broadly and evaluate effects on prevailing strain titers. Finally, participants will be selected on the basis of divergent humoral responses to investigate the underlying cellular and molecular basis for variation. ASC, detected in peripheral blood during the acute response, will be defined as naïve- versus memory cell-derived based on time of appearance; use of IgM versus IgG; B cell receptor (BCR) clonality and extent of mutation, and clonal relationship to pre-infection memory and naïve pools. BCR adaptation will be quantified based on the extent of intraclonal diversity amongst large clones, and the proportion that express BCR with highest affinity for the prevailing strain.

Subject identification and selection

This study involves participants of the existing Ha Nam cohort, and has 2 components: (1) a study of vaccine immune responses in selected adult participants; and (2) a study of infection immune responses in all other adults who are not included the vaccine study. We will purposefully, rather than randomly, select adults for the vaccine immune response study in order to include 50 who have had confirmed A/H3N2 infection since 2007, and 50 who have not been infected. The two groups will be selected to obtain similar age and gender distributions. The age and gender distribution of those with and without documented A/H3N2 infection is similar.

As shown below, the number, and age and gender distribution of adult cohort participants with and without A/H3N2 infection since 2007 is similar. We will recruit around one third of each age band to maintain a representative distribution (Table below). We may not be able to obtain sufficient participants from some strata, depending upon people's willingness to participate, but age and gender matching is not essential for the study objectives and analyses.



Approximate numbers per group by age

Age	No prior H3N2	Prior H3N2
18-39	18	17
40-59	23	19
60-79	8	12
>=80	1	2

Sample size is based on the assumption that mean log 2 titer increase, i.e. (mean post - pre log 2 titer), will differ by at least 0.7 between the two groups, and that the standard deviation will be up to 1 for each group. The calculated sample size of 33 per group has been inflated to account for loss to follow-up and for additional comparisons of people infected with A/H3N2 in different years since 2007, and with viruses from different antigenic clusters (i.e A/Brisbane/2007 in 2008; A/Perth/16/2009 from ~ 2009-2011; A/Victoria/361/2011 from ~ 2011-2013). This sample size is similar to previous studies investigating repeat vaccination (11). Adult cohort participants who are not included in the vaccine study will be asked to participate in the study of immune responses to infection, and to provide a baseline blood sample for cellular immunology studies. The first 30 adults who develop influenza A/H3N2 illness within 18 months will be asked to provide post-infection blood samples.

Pre-selected candidates for influenza vaccination will be approached first, and will first be requested to participate in the vaccine component of the study. They will only be requested to participate in the infection component if they don't wish to participate in the vaccine component. This process will continue until 100 people have been recruited for vaccination. Thereafter, all remaining adults in the cohort, who are not in the vaccine study will be asked to join the infection component of the study.

Inclusion and exclusion criteria

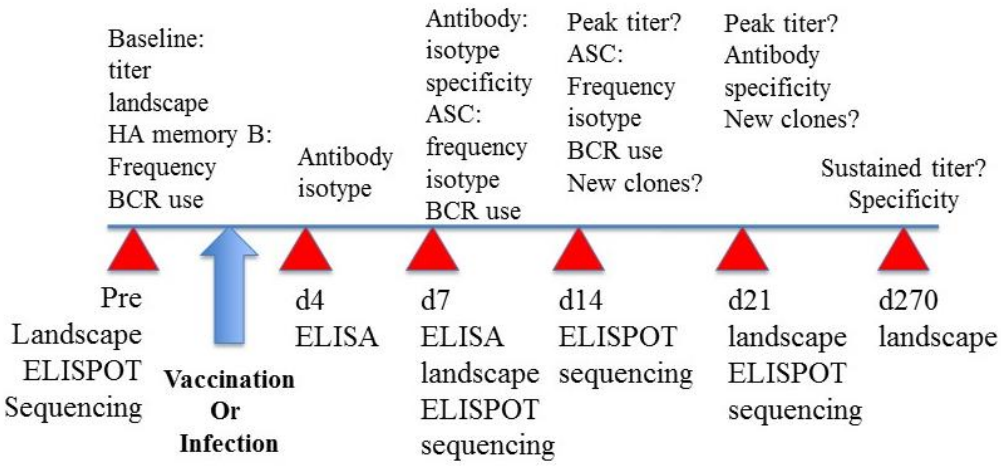
People included in this study will be consenting participants of the Ha Nam cohort, aged at least 18 years. Inclusion in the vaccine component of the study requires provision of blood samples at all prior cohort times points so that A/H3N2 infection history can be determined. Immunisation will be postponed if participants have a febrile illness or acute infection.

Adults will be excluded from the vaccine component if they have a history of reactions to vaccination, or have a condition that renders a person ineligible for influenza vaccine as listed in the summary of product characteristics. Children are excluded from the study due to the blood volumes required.

Participant Procedures

The procedures involved, and the investigation schedule are listed in the Table below.

Procedure	**Research**	**Timing**	**Undertaken By**
Influenza Immunization	100 adult cohort participants	One time, ~ July 2016	Immunization programme staff
20 ml venous blood	Adult cohort participants (up to 635)	Baseline, i.e. < 1 week before vaccination, or at the next cohort cross-sectional sampling time if not vaccinated	Provincial Hospital Nurses who routinely perform

20 ml venous blood sample	100 vaccine study participants, and up to 30 adults who develop influenza A/H3N2 illness within 18 months of baseline sample collection	Days-7 and -21 after vaccination or confirmed illness	phlebotomy
10 ml venous blood sample	"	Days-4, -14 and -270* after vaccination, or day-270* after confirmed illness.	
			

* Participants will not be asked to provide blood samples as part of usual cohort activities (i.e. one 7 ml blood sample is collected annually), if they have already provided a blood-sample as part of this study in that month.

Frequent blood sample collection is required to determine both peak and sustained titers and titer changes, and to characterize influenza virus-reactive effector and memory B cells, whose numbers peak in blood at around day-7 and 14-28 post-exposure, respectively. Larger blood volumes are required compared to the ongoing cohort study because B cell subsets will be isolated from peripheral blood mononuclear cells. Up to 30 participants with A/H3N2 illness, detected during up to two years of ongoing surveillance, will be assessed following the same schedule. As a part of ongoing cohort study activities, participants are asked to allow a health worker to visit them weekly to identify people who have influenza-like-illness (ILI), and those with are asked to provide nose/throat swabs and symptom diaries. Viruses are isolated from swabs for antigenic characterization by Ian Barr's group.

Risks and Ethical Issues

Inactivated influenza vaccines (GSK or Sanofi Pasteur) may cause mild side-effects (localised pain, redness, swelling; mild febrile response; mild myalgia, arthralgia, drowsiness or tiredness) lasting for 1–2 days. The vaccines contain traces of egg protein at doses (≤ 1.0 microgram) that can be safely given to people with an egg allergy; however, as a low risk of anaphylaxis is present, vaccination will be administered through a government Immunization Service. Vaccine will not be given to anyone who has previously experienced anaphylaxis after exposure to any vaccine component. Vaccinees

will be asked to stay at the vaccination clinic for 30 minutes post vaccination monitoring of severe side-effects.

Venipuncture can be unpleasant and can cause momentary discomfort and/or bruising. Infection, excess bleeding, clotting, or fainting are also possible, although unlikely. We have collected over 7500 blood samples to date and these events have not occurred. Vietnamese-speaking research team members will conduct face-to-face meetings with participants to explain the study risks as well as the benefits of influenza vaccination, and the voluntary nature of participation.

An adverse event protocol and adverse event report form has been developed. Staff who conduct vaccination or phlebotomy or who manage these activities will receive training to follow this protocol.

Statement of Insurance

The University of Melbourne will provide indemnity upon receipt and acceptance of a clinical trial declaration form.

Consent

Commune health workers will arrange an appointment with adult members of each Ha Nam cohort household during routine household visits. Experienced Vietnamese field staff from the National Institute of Hygiene and Epidemiology will be responsible for providing information and seeking consent. They will undertake training on specific procedures and information and consent forms for this study. They will meet in person with each study candidate, and use the information and consent sheets to explain study aims, methods, anticipated benefits and potential risks of the study. Field-workers will explain that participation is entirely voluntary, that all participants have a continuing right to withdraw at any time, and that the decision to participate in this additional study will not affect participation in the ongoing cohort study. Participants will be shown blood collection tubes to ensure they are aware of the volume of blood being requested.

Candidates will be able to consent or refuse at that time, or can retain the information and consent form and delay their decision until the following day. Candidates who wish to participate will be asked to sign the consent form, and indicate whether they agree to secondary use of specimens. The study member who obtains consent will also be asked to sign. If the person providing consent is unable to read, a witness will be required to verify that they were present throughout the entire informed consent process, that form was read accurately to the volunteer, that all questions from the volunteer were answered, and the volunteer has agreed to take part in the research.

Investigation of study samples

Aim 1- Does infection history affect prevailing strain titer?

Sera will be tested in HI assay with vaccine and infecting strain A/H3N2 viruses. Assays will be performed in laboratories in Melbourne (WHO) and Hanoi (OUCRU and NIHE), by personnel who have vast influenza serology and virology experience.

Log 2 transformed reciprocal titers for the two groups will be determined and compared at baseline and various times after vaccination with a focus on peak and sustained titers, and titer differences.

Absolute titers will be compared using Wald test (for non-zero difference between groups, a one-sided test). Titer differences (as per sample size) will be compared using t-test. A serological protection rate will be determined for each group assuming that titers ≥40 and ≥160 confer 50 and 100% protection, respectively, and will be compared using combined chi-squared tests.

Aim 2 - Are prevailing strain titers related to the magnitude of memory-type humoral responses?

Antibody landscapes and antibody isotype ELISA will be used to characterize the serological response in more detail and evaluate effects of memory-type humoral responses on prevailing strain titers. Antibody landscapes will be determined for serum collected pre- and 7, 21 and 270 days post-exposure. Others report that prevailing strain HI titers are undetectable at day 7 post-exposure, although a rise in HA-reactive antibodies can be detected by ELISA (26). At least 30 A/H3N2 strains, including representatives of each antigenic cluster, and infecting and vaccine strains, will be used. Assays will be performed by Ian Barr's lab (WHO center in Melbourne). Base map viruses and QA/QC reagents will be sent to the National Influenza Center laboratory in Ha Noi to develop capacity for landscape quality HI assays.

Serial dilutions of serum collected on days-4 and -7 after vaccination or infection will be tested in isotype ELISA with plates coated with recombinant HA1 subunit proteins representing prevailing and past strains (Sino Biologicals). Recombinant HA1 ELISA has been found to differentiate strain specific antibodies, and to have high concordance with HI assay (27). If necessary, serum will be pre-adsorbed against HA from a non-human influenza virus (H4 subtype, Sino Biologicals) to reduce broadly cross-reactive antibody detection. Coating concentration will be optimized to obtain a large linear range with serially diluted, post-vaccination sera. Separate plates will be coated with HA to determine levels of HA-reactive IgM and IgG. Reference sera will be used to control for variability, and to identify cut-offs that discriminate specific from non-specific sera.

Analysis and outcome

Landscape and isotype trends associated with infection history will be used to infer memory more broadly, i.e. inclusive of infections prior to 2007, and the impact on prevailing strain titers.

Landscape data will be analysed by Derek Smith's group (Cambridge University). For each individual, landscapes at each post-exposure time (d7, 21 & 270) will be compared to pre-exposure landscapes to obtain differentials. Mean differential landscapes will be compared to identify regions of difference over time, and between groups with and without prior A/H3N2 infection. This analysis of how landscapes change during the acute response to vaccination will indicate how specificity for prevailing versus past strains evolves and is affected by prior infections.

IgM:IgG isotype ratios for prevailing and past-strain specific antibodies produced within seven days of vaccination or illness onset will be determined and compared for between groups to determine whether recent prior infection induces early IgG biased/memory-type antibody responses.

Aim 3 - What proportion of acutely responding cells are memory-derived, what proportion have adapted to the prevailing strain, and how does these relate to peak and sustained titers to the prevailing strain?

In depth analyses of B cells and B cell receptors will mainly be performed on samples collected before, and 7 and 21 days after vaccination or infection (Study 2 and 3), when 20 ml blood samples are requested, with more limited analysis on day 14 when 10 ml of blood is requested. As explained

earlier, larger volumes of blood are required compared to previous protocols to obtain sufficient PBMCs for multiple in-depth analyses.

Flow cytometry will be used to quantify and sort $CD3^{-}CD19^{+}$ B cells into naïve (CD10-, $CD20^{+}$, $CD27^{-}$, $IgG/A/E^{-}$, CD38-/^{low}, $CD138^{-}$), memory ($CD20^{+}$, $CD27^{+}$, $CD38^{-/low}$, $CD138^{-}$ (switched IgM^{-} IgG^{-}), and ASC ($CD20^{-/low}$, $CD27^{++}$, $CD38^{++}$, $CD138^{+}$, surface $Ig^{low/-}$) subsets. Fluorescent-labeled influenza hemagglutinin (HA, Sino Biologicals) will be used to enrich for influenza-reactive naïve and memory cells. Our previous studies with Vietnamese patients indicate that absolute B cell counts are between 82000 and 533000/ml of which around 30% or at least 20000/ml are memory B cells. Influenza HA-binding cells account for around 0.2% of resting B cells (28), equivalent to around 150/ml. ASC counts on day 7 post-exposure are around 6000/ml, of which around 60% are influenza specific (19), including around 600 H3-HA-specific ASC/ml (29). Memory B cell counts peak at around 1000/ml 14-21 days post infection (19). To isolate influenza-reactive naïve and memory B cells, PBMCs will first be incubated with recombinant HA from a virus that does not infect humans (H4-HA from Sino Biologicals) to block non-specific binding via sialic acid on the surface of B cells (28). An optimized concentration of fluorescent labeled H3-HA will then be used to detect and sort specific cells. The frequency of HA-reactive cells in each subset, and the efficacy of H3-HA enrichment, will be further defined by ELISPOT with H3-HA coated plates. Different cell numbers will be used per well depending on sorting (e.g. 200000 unsorted PBMC, 10000 sorted naïve or memory, 2500 sorted ASC, and 100 HA-reactive naïve or memory). Cells will be incubated on two separate plates to enumerate cells making IgM and IgG. Reduced cell numbers will be used to enumerate total IgG and IgM producing cells in each sample. Naïve and memory subsets will be stimulated with CD-40 Ligand/TRAP and recombinant human IL-21, to induce antibody secretion, prior to ELISPOT (30). HA-reactive cells will be recovered from positive ELISPOT wells for EBV transformation in order to generate antibodies for testing in HI assay.

The **molecular basis for variation in antibody** titers will be investigated in a subset of participants. These will include up to 10 each classified as high titer, primary-type vaccine responders; low titer memory-type vaccine responders; and A/H3N2 illness cases. High throughput DNA sequencing using the Illumina MiSeq instrument will be performed on libraries of antibody heavy and light chains amplified from both bulk- and single-sorted cells, to permit deep repertoire analysis, and paired heavy and light chain analysis, respectively. High throughput antibody gene sequencing is technically challenging and requires careful validation to ensure reliable representation of repertoires. Sequencing will be performed in Scott Boyd's lab, where protocols for single and bulk cell antibody sequencing are established. We will single-cell sort up to 192 cells per subject and subset at each time-point, including HA-binding naïve and memory cells pre-exposure, ASC on days 7 and 14, and HA-binding memory cells on day 14 or 21. Others have been able to establish that tetanus toxoid-responsive ASC and memory cells are clonally related using only 126 and 20 antigen binding ASC and memory cells, respectively (31). RNA and DNA will be extracted from bulk-sorted cells, including at least 1000 HA-binding naïve and memory cells and 5000 naïve, memory or ASC of any specificity. Genomic DNA template represents individual cells equally, assuming no bias in amplification, whereas RNA yields greater repertoire depth. Katherine Jackson, from Scott Boyd's lab, will lead sequence analysis. The data analysis pipeline in the Boyd lab consists of the following elements: run quality control monitoring from instrument run parameters; merging of paired-end sequence reads; primer trimming and sample demultiplexing based on exact matches to sample-specific sequence

barcodes included in the primers; alignment of V, D and J gene segments to germline repertoires, and parsing of junctional nucleotides at the V(D)J junctions, using the iHMMune-align and IgBLAST programs and output parsing scripts; determination of positions of somatic hypermutation with reference to germline V and J gene segments; identification of clonally-related sequences based on shared V and J gene segment usage, common complementarity-determining region 3 (CDR3) length, and Hamming distance thresholds for the CDR3 nucleotide sequences; and a number of other secondary analyses, as previously published (18, 32). **Paired heavy and light chains** representing expanded clones will be selectively amplified and cloned into expression vectors for transfection of 293T cells. **Expressed recombinant antibodies** will be used to identify clones that make neutralizing antibodies and to **compare affinity for prevailing versus past strains.** In cases where high-throughput sequencing reveals additional members of clonal lineages with differing levels of somatic hypermutation, up to four representative sequences with higher and lower levels of mutation will be selected for expression and antigen affinities, to evaluate intraclonal variation and the effects of ongoing mutation on antigen specificity.

Analysis and Outcomes

- The proportion of H3-HA reactive ASC that make IgG versus IgM at day 4 and 7 will be used to infer the proportion that are memory derived, and will be related to infection history, pre-infection frequency of H3-HA reactive memory cells, and the isotype and titer of the humoral response.
- The comparison of BCR use by day 7 ASC from participants whose humoral responses diverge will indicate the contribution of memory B cells to acute plasmablast responses, and how this affects titers. Memory B cell contribution will be inferred from 1) the percentage that are clonal, that are isotype-switched and that have high mutation rates (~ 20 or more mutations); and 2) the percentage that are clonally related to pre-exposure memory B cells.
- We will identify clones that are generated from naïve cells by comparing BCR use by cells present from day 14 after exposure versus cells responding earlier and presumed to be memory-derived. In turn, we will determine the impact of memory responses on the generation of new clones.
- We will generate recombinant monoclonal antibodies from BCR representing clones that have expanded at day 7 and will compare affinity for prevailing and past strains to determine the proportion that have adapted and how this relates to titers.

Data management and storage arrangements

Paper study forms on are kept in locked cabinets in the commune health centre under the care of the local study coordinator until collection and transfer to OUCRU where they are kept in a locked cabinet. Data is entered into a secure online database that can be accessed by principle investigators and study staff who have been given a password with appropriate permission to enter, modify, view and/or download. Most field and lab-staff have permission to enter but not download data. A data manager form OUCRU has permission to modify data for the purpose of data cleaning but cannot download data. The principle investigator (Annette Fox) is responsible for overall data quality and has permission to review raw data and modify database entries. All modifications are tracked/audited with an explanation for changes made. Study team members with access to the database are required to complete the Good Clinical Practice course, as well as course on data protection where required. The data collected is under the joint custody of the Oxford University Clinical Research Unit (OUCRU); the National Institute of Hygiene and Epidemiology, Ha Noi, and The

University of Melbourne, Australia. Principle investigators from these institutions are provided with database permission that enables data extraction.

Access to the database is controlled by the data management unit of Oxford University Clinical Research Unit in Viet Nam, who developed the database under the direction of Annette Fox. Staff from this unit assign database access passwords with specific permissions that restrict whether a person can enter, view, modify or download data. Permissions are granted following instruction via email from Annette Fox.

The database will be retained indefinitely. Paper based forms will be retained until the data has been entered and validated, and results have been reported, or for at least three years. Thereafter, the paper files will be scanned and archived electronically and destroyed securely according to OUCRU standard procedures. Electronic data will be stored indefinitely on a secure OUCRU server.

Sample management and storage arrangements

Samples will be stored in secure OUCRU-VN freezers indefinitely after the completion of the study. Access to samples is controlled by OUCRU policy. Future studies to be performed on stored samples will be submitted to the appropriate research ethics committee as required. Cells and aliquots of sera will be sent to collaborating institutions at the Peter Doherty Institute in Australia. Access to the institute is controlled via key card with a separate key card access for the freezer room, as well as locked freezers. Samples will be kept until testing and reporting is completed. RNA and DNA samples sent to Stanford University will only be used solely for B cell receptor gene analysis. Amplified single cell B cell receptor genes and recombinant antibodies will be retained indefinitely but future use will require ethical approval.

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