NATURAL T CELL MEDIATED PROTECTION AGAINST SEASONAL AND PANDEMIC INFLUENZA

RESULTS OF THE FLU WATCH COHORT STUDY

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AUTHOR'S CONTRIBUTIONS

In recognition of the complementary roles of epidemiology and immunology in the paper ACH and LW are joint first authors with AMJ and AM as joint senior authors. ACH developed the epidemiological design for Flu Watch as a framework for assessing the hypothesis that T cell responses protect against detectable nasal shedding in those infected with influenza with input from AMJ and AM. LW developed the approach to conducting large scale ELISPOT assays and led the T cell laboratory analysis with support from AM and NG.

ACH is the PI of the Flu Watch study. He conceived the idea for and designed the original seasonal influenza study and the pandemic extension in discussion with AMJ, JMW and MZ. AB, AM, JVT, IN, JMW, AMJ and MZ were co-applicants on the seasonal and pandemic grants, members of the steering group and contributors to study design. AC was a co-applicant for the pandemic extension and joined the steering group in 2009 contributing to design of the pandemic phase. Authors LW, NG, EBF and ERCM also contributed to study design. Data collection was done by LW, EBF, ERCM and AB. EBF led the data management with contributions from ERCM and OD. ACH led the development of the analytical strategy with major contributions AM, AMJ, LW and NG as well as contributions from EF, AB, AC, ERCM, IN, JVT, JMW and MZ. LW, AM and NG led the T cell laboratory analyses. MZ and AB led the serological and PCR laboratory analyses with contributions from LW. Alison Bermingham tragically and unexpectedly died shortly prior to publication, her tireless work was central to the success of this study. AC was the statistical advisor for Flu Watch, with input from OD. ACH analysed the data with contributions from LW. All authors contributed to interpretation of findings. ACH and AM wrote the manuscript with major contributions from LW, NG, AMJ, EBF and OD and input on

drafts from all authors. Literature search was conducted by ACH, LW, NG and AM. Tables and figures were created by ACH with input from LW, NG, AM, EBF and OD.

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AT A GLANCE COMMENTARY:

Scientific Knowledge on the Subject

Evidence from previous studies has supported the hypothesis that T cells protect against influenza disease and virus shedding. However, the extent of naturally pre-existing T cell responses across the community and their protective effect against nasal viral shedding (which is the best measure of influenza infectiousness) in naturally acquired pandemic and seasonal infection has not been determined previously.

What This Study Adds to the Field

In those infected with seasonal or pandemic influenza A, commonly occurring cross protective T cells targeting internal proteins have a major effect on population immunity by protecting against symptomatic PCR-confirmed disease. Vaccines stimulating T cells may provide important cross-protective immunity.

This article has an online data supplement, which is accessible from this issue's table of content online at www.atsjournals.org

NATURAL T CELL MEDIATED PROTECTION AGAINST SEASONAL AND PANDEMIC INFLUENZA

ABSTRACT

Rationale: A high proportion of influenza infections are asymptomatic. Animal and human challenge studies and observational studies suggest T cells protect against disease among those infected, but the impact of T cell immunity at the population level is unknown.

Objectives: To investigate whether naturally pre-existing T cell responses targeting highly conserved internal influenza proteins could provide cross-protective immunity against pandemic and seasonal influenza.

Methods: We quantified influenza A(H3N2) virus specific T cells in a population cohort during seasonal and pandemic periods between 2006-2010. Follow-up included paired serology, symptom reporting and PCR investigation of symptomatic cases.

Measurements and Main Results: 1414 unvaccinated individuals had baseline T cell measurements: (1703 participant observation sets). T cell responses to A(H3N2) virus nucleoprotein (NP) dominated and strongly cross-reacted with A(H1N1)pdm09 NP ($p<0.001$) in participants lacking antibody to A(H1N1)pdm09. Comparison of paired pre- and post-season sera (1431 sets) showed 205 (14%) had evidence of infection based on four-fold influenza antibody titre rises. The presence of NP specific T cells before exposure to virus correlated with less symptomatic, PCR-positive influenza A (overall adjusted odds ratio 0.27 (95% Confidence Interval, 0.11-0.68), $p=0.005$, during pandemic ($p=0.047$) and seasonal periods ($p=0.049$)).

Protection was independent of baseline antibodies. Influenza specific T cell responses were detected in 43% indicating a substantial population impact.

Conclusions: Naturally occurring cross-protective T cell immunity protects against symptomatic PCR-confirmed disease in those with evidence of infection and helps to explain why many infections do not cause symptoms. Vaccines stimulating T cells may provide important cross-protective immunity.

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INTRODUCTION

Our recent research shows influenza infects around 18% of unvaccinated individuals each year, but up to 75% of those infections are asymptomatic.[1-3] The underlying immunological correlates of asymptomatic infections are not well understood. Antibodies, specific for hemagglutinin (H) and neuraminidase (N), protect against repeat infection[4], but these proteins are highly variable enabling the virus to cause annual winter epidemics.[5] Sporadically, a new pandemic virus emerges, with introduction of genes from animal reservoirs.[6] Absence of pre-existing antibody immunity during pandemics can cause more severe illness and high mortality rates.[7] In 2009, a new pandemic A(H1N1) virus appeared with gene segments from avian, porcine and human viruses.[8] H1 and N1 proteins were sufficiently distant from previously circulating viruses to evade antibodies prevalent in the human population.[8] Most infections were asymptomatic or minimally symptomatic[1] and mortality rates were considerably lower than feared.[8,9] However, the emergence of pathogenic avian influenza viruses, such as the A(H7N9) subtype which has caused recent human deaths in China,[10] remains a constant threat.

Whilst antibodies prevent infection, T cell responses which target the highly conserved internal proteins of influenza A virus such as nucleoprotein (NP) and matrix (M) protein may prevent or modulate symptoms following influenza infection. CD8+ T cells cannot prevent initial infection, but they, and to some extent CD4+ T cells, mediate viral clearance after infection in mice.[11-15] Experimental influenza A virus infection induces murine CD8+ T cells responses that reduce the severity of subsequent infection with viruses of different H and N subtypes.[11-13,16,17] Human challenge studies provided the first evidence that pre-existing T cells protect against seasonal influenza disease; but generalizing findings from artificial infections has been difficult.[18,19]

A recent study reported pre-existing T cell protection against natural pandemic influenza disease in healthy adults amongst 25 participants infected with influenza.[20] However, it is not clear whether such protection is important in the wider community, whether T cells provide protection against both pandemic and seasonal strains, and what the extent of protection is against PCR-positive disease.

There is substantial interest in alternative vaccine strategies since cross-protection with conventional vaccines is minimal. Stimulation of T cell responses targeting highly conserved internal proteins of influenza A virus offers the promise of both reducing morbidity and spread of virus shed from the upper respiratory tract.[21,22] Vaccines targeting the T cell response are in development.[23-25] A further, suitably powered demonstration of the population importance of T cell mediated protection against seasonal and pandemic infection would help to justify future investments in trials of such vaccines. This study aimed to define the role of T cell based immunity in protection against seasonal and pandemic influenza in the general population.

METHODS

Cohort Design

The Flu Watch Study has been described previously.[1] In brief; each autumn (2006-2009) general practices (GP) across England randomly selected individuals from GP lists and invited their households to participate over the coming winter. Participants were asked weekly for symptoms of "cough, cold, sore throat or 'flu-like illness" and completed prospective daily symptom diaries throughout any illness. The Oxford MultiCentre Research Ethics Committee approved this study and participants gave written-informed consent.

PCR detection of virus shedding

Participants submitted, by mail, nasal swabs on day 2 of any illness for Reverse transcription polymerase chain reaction (RT-PCR) identification of influenza and other respiratory viruses.[26,27] Since qualitative real-time assays were used, we felt it was inappropriate to use C_t values to make quantitative inferences about viral shedding. Meaningful quantitative measurement would require samples to be taken in a highly standardised way, with close attention to time from symptom onset and rapid transfer to the laboratory. This was not possible due to the geographically dispersed community nature of this research which relied on self-sampling and postal submission by participants.

Serology

Pre- and post-season sera were batch-tested for anti-influenza virus antibody using haemagglutination inhibition assays (see the online data supplement). Seroconversion was defined as a four-fold titre rise and considered indicative of infection occurring

between baseline and follow up samples. We excluded vaccinated participants because of the difficulty in interpreting four-fold titre rises in the context of vaccination.

T cells

Ex vivo T cell responses to overlapping peptides representing the whole proteome of influenza A(H3N2) virus, prevalent in 2006/7 and 2008/9, were measured by interferon-γ ELISpot assay[28] before each influenza season (see the online data supplement).

The ELISpot assay did not distinguish CD4 from CD8 T cell responses. Therefore in stored samples from a random subset of unvaccinated individuals taken during the winter wave of the pandemic (n=174), T cells were further cultured and CD4+ and CD8+ T cell responses were then quantified by measuring intracellular interferon-by flow cytometry (see the online data supplement).[29] Staff conducting serological and virological assays were blind to T cell results and vice versa.

Statistical Analysis

We focused on the immunoprevalent, highly cross-reactive NP specific T cell responses and the dominant strain of influenza circulating in any given year. Linear regression models were used to investigate the relationship between log-transformed T cell responses and serological/symptom variables. We used poisson regression models to explore the effect of NP response and other variables on rates of infection. Logistic regression models were built to test the primary hypothesis that pre-existing T cell responses to NP would protect against detectable viral shedding in individuals serologically infected with influenza. Robust standard errors accounted for correlation between repeated measurements in the same individual, and potential confounders

were adjusted for. Fisher's exact test was used to assess the protective effect of response separately for pandemic and seasonal influenza. Sensitivity analyses using the less specific respiratory illness outcome are reported in the online supplement. Estimates are presented with a 95% confidence interval and a p-value ($p<0.05$ was considered statistically significant). Analyses were performed in STATA 12.

There is no accepted threshold for quantifiable ex-vivo influenza specific T cell responses. Therefore values of ≥20 spot forming units per million (SFU/M) peripheral blood mononuclear cells (PBMC) (above the 99th percentile of the negative control well distribution) were considered detectable if the original pooled test well result was also significantly higher than the pooled negative control well results ($p<0.05$, negative binomial distribution).

RESULTS

1414 participants met inclusion criteria, some contributing to more than one season: 2006/7 (n=321); 2007/8 (n=404); 2008/9 (n=322); 2009/10 (n=656) giving 1703 observation sets. Follow-up serology was available for 84% (1431/1703). Of these, 14% (205/1431) were infected with the season's dominant circulating influenza A strain (four-fold rise in specific antibody titre). Infected participants contributed 2289 person weeks follow-up with illness status reports returned for 1877 (82%) weeks. Amongst those infected there were 143 illnesses reported with 112 (78%) submitting nasal swabs (see Table E1 in the online data supplement).

Baseline influenza-specific T cell responses to peptide pools spanning individual proteins in the majority of participants were low (Figure 1A and B) but comparable to previous studies.[18,30] The median total A(H3N2) specific T cell response after subtraction of background no-peptide control responses was 83 SFU/M PBMC. NP was the immunodominant antigen (median ex vivo NP T cell response 15 SFU/M PBMC). 25% of the total T cell response was specific for NP and 19% for M (Figure 1C summarises variation in the proportion of the total response due to each protein by individual). 43% (730/1703) of observations had a T cell response to NP (≥20 SFU/M PBMC) compared to 35% for M (Figure 1D). NP T cell responses were detectable in 45% (65/146), 53% (302/570), 40% (319/804) and 24% (44/183) of baseline ex vivo assays in children aged 5-15, young adults aged 16-44, older adults aged 45-64 and those aged 65 and over respectively (chi-squared p<0.001). We found strong evidence (p<0.001) of cross-reactivity between T cell responses to H3N2 and A(H1N1)pdm09 (Figure 2A and B). Using a culture assay, to expand antigen specific T cells in the presence of peptide and IL-2, followed by peptide stimulated cytokine expression and flow cytometry, we found that the contribution of CD8+ T cells was higher for the internal influenza proteins compared to HA and N, and highest for NP (Figure 3). NP specific ELISpot responses *ex vivo* and cultured NP responses *in vitro* were strongly correlated (log transformed regression coefficient 0.86 (95% Confidence Interval (CI) 0.36-1.36) p<0.001). This correlation held for influenza specific CD8+ cells (1.65 (95% CI 1.02-2.28) p <0.001) but not for CD4+ cells (0.45 (95% CI -0.16-1.06) p=0.146) (See Figures E1A-C in the online data supplement). There was no evidence that NP-specific T cell responses differed at baseline between those vaccinated and unvaccinated in the last two years (age and baseline antibody adjusted odds ratio 0.96, 95% CI 0.71 to 1.28, p=0.77).

The primary outcome was the nasal shedding of influenza virus as detected by PCR. The average incubation period (time between infection and symptom onset) is 48 hours and viral shedding generally peaks another 24 hours later.[18] Viral shedding is associated with both disease severity and infectiousness.[2,31] The rate of serologically confirmed infections per 100 person seasons was strongly inversely associated with baseline homotypic antibody titre but as expected was not affected by baseline NP specific T cell response (Table 1). Amongst those infected 35/205 (17.1%) shed detectable influenza virus from nasal swabs taken during illness (cough, cold, sore throat or influenza like illness) (Table 1). In univariate analysis the primary outcome was only associated with an NP specific T cell response and gender, with viral shedding lower in those with a response and in females. In the final adjusted model only a pre-exposure NP specific T cell response ≥20 SFU/M PBMC was significantly associated with reduced odds of nasal viral shedding (adjusted odds ratio 0.27 (95% CI 0.11-0.68), p=0.005). The protective effect was significant for seasonal influenza (9.9% (6/66) of those with a detectable NP response shed influenza virus vs 21.7% (20/92) of those without, Fisher's exact test p=0.049). The protective effect was equally significant for pandemic influenza (0% (0/13) of those with NP response shed virus vs 26.5% (9/34) of those without, p=0.047). Although the study is underpowered for further breakdown by seasonal influenza strain, the direction of effect was the same for both seasonal H1N1 and H3N2. For those infected with seasonal H1N1 8/32 (25.0%) of those without a baseline T cell response shed seasonal H1N1 virus compared to 1/24 (4.2%) of those with a baseline T cell response (Fisher's exact p=0.063). For those infected with H3N2 12/78 (15.4%) of those without a detectable NP shed H3N2 virus vs 6/57 (10.5%) of those without a detectable NP response (Fisher's exact p=0.454). Amongst those who shed virus we found no evidence of a

positive or negative correlation between pre-exposure NP specific T cell responses (log transformed SFU/million PBMC) and log transformed symptom severity scores (correlation coefficient 0.30, 95% CI -0.19 to 0.79, p=0.225).

In sensitivity analyses, using the less specific outcome of self-reported respiratory illnesses (cough, cold, sore throat or influenza like illness) in those with serological evidence of infection (excluding illnesses known to be due to other viruses and those occurring outside the period of influenza circulation), we found no significant protection associated with the baseline NP specific T cell response in the seasonal epidemics. However, in 34 participants infected with the pandemic strain who did not have a positive baseline NP specific T cell response, there were 33 illnesses. This contrasts with 4 illnesses amongst 13 participants infected with the pandemic strain who had a positive baseline ex vivo NP T cell response (age and baseline antibody adjusted Incidence Rate Ratio 0.49, 95% CI 0.25-0.96, p=0.037).

DISCUSSION

We show that pre-existing cross-reactive T cell responses to NP were independently associated with decreased odds of nasal viral shedding in those infected with seasonal or pandemic influenza. Whilst homotypic antibodies provide strong protection against infection, our work supports our primary hypothesis that, in those who become infected, baseline T cells provide heterotypic protection against the highly specific outcome of detectable viral shedding. T cell responses at protective levels were

present in 43% of participants and correlated with a reduced risk of shedding virus by around two-thirds, indicating an important population level effect.

This is the first large community cohort study of human T cell immunity against influenza. The large study size allowed assessment of both confounders and interactions. It covered a cross section of the population and spanned both seasonal and pandemic periods. Weekly follow-up minimised recall bias but was less intense than during human challenge studies likely leading to some under-ascertainment of influenza illnesses and viral shedding. PCR on self-submitted nasal swabs have been shown to have comparable sensitivity to samples taken by healthcare workers.[32] Sensitivity analyses using the more sensitive but less specific outcome of respiratory illness show similar cross-protective immunity against illness in those infected with the 2009 pandemic strain, perhaps unsurprising given the virological dominance of A(H1N1)pdm09 during 2009-10.[1] Because nasal swabbing was only requested from symptomatic individuals we are unable to determine whether T cells affected viral shedding in asymptomatic infection.[33] We did not measure baseline innate immune responses, which by definition are not antigen-specific. Severe influenza involves hyper activation of innate immune responses in the lungs, particularly of monocytes and macrophages populations.[34] These responses are also involved in mild disease involving only the upper respiratory tract, particularly in children, but investigating this was beyond the scope of our study.[35-37]

Participants had baseline influenza specific memory T cell responses at low levels which is compatible with the known expansion and contraction of T cells following acute viral infection.[18] Stringent criteria were applied to define a robust threshold (≥20 SFU/M PBMC) for detectable ELISpot responses *ex vivo*. This cut-off is lower than that used in vaccine testing, but is statistically sound given the very low backgrounds

in the assays and is appropriate because of the low level of baseline T cell responses in the general population. The few very strong responses may have identified actively infected participants sampled during the early stages of the epidemic or pandemic, which was possible because the pre-season bleeds were not completed before the influenza season had started. However exclusion or inclusion of these donors in the sensitivity analyses did not affect conclusions.

The main previous evidence of T cell based protection in naturally acquired influenza comes from a London based cohort study over the 2009/10 and 2001/11 seasons of the pandemic. Healthy adults (n=342, median age=28) recruited at a University/Medical School were asked to report symptoms every three weeks and submit self-taken nasal swabs during illness. By contrast we report 1703 participant observation sets from 1414 general population participants across all age groups and including those with chronic illness followed up over periods of both pandemic and seasonal influenza. We used weekly symptom reporting and self-submission of nasal swabs to maximise ascertainment of influenza. In the previous study the infected group (n=43) was identified on the basis of 4 fold antibody titre rises or positive PCR. The authors do not make it clear whether those who were vaccinated between baseline and follow up bleeds were excluded (which would be expected to cause a 4 fold titre rise). We exclude those in whom vaccination could have caused 4 fold titre rises from our definition of infection. The previous study analyses T cell responses as a continuous variable whereas we categorised our T cell results into positive and negative responses as described above. This was both because the highly skewed distribution of the responses meant it could not be readily transformed to meet assumptions of appropriate statistical tests and because we saw no evidence of a quantitative effect above this binary cut off. Neither study found evidence of T cell

protection against infection. In the previous study infected patients who reported multiple illnesses during follow up were excluded as it was not considered possible to identify which illness was caused by influenza, leaving 25 adults for study of the protective effect of T cells. We did not exclude such patients as our primary outcome was PCR-confirmed disease which did not occur more than once during follow up and our secondary outcome was whether or not participants reported illness during follow up. Thus our main analysis focusses on 205 infected patients. In the previous study, when examining total pre-existing cross-reactive total cytokine-secreting T cells to live pH1N1 virus in the 25 infected patients, they report higher baseline T cell results amongst those who reported no fever (n=12, p=0.03), amongst those with completely asymptomatic infection (n=3, p=0.02) and amongst those with lower symptom scores (p=0.05), but no difference in those who were PCR positive (n=11). By contrast we found no protective effect of total cell response to H3N2 influenza proteins against PCR positive disease, symptoms or symptom scores. In the previous study, when examining cellular responses to highly conserved CD8 epitopes from the immunodominant internal PB1, NP and M1 proteins in these 25 patients, they reported higher baseline levels amongst those who reported no fever (n=12, p=0.02), no ILI (n=15, p=0.04) and those with lower symptom scores (p=0.01). Finally, when examining IFN-γ+IL-2− T cells specific for conserved CD8 epitopes they found negative correlations with symptom scores (p=0.004) and higher baseline levels in those who were PCR negative (p=0.05). By contrast we focussed analyses on the response to the immunodominant NP protein (where the response was shown to be dominated by CD8 cells). We found those with a positive response at baseline were less likely to shed virus (adjusted odds ratio 0.27 (95% CI: 0.11-0.68, p=0.005), during pandemic (p=0.047) and seasonal periods (p=0.049)), and during the pandemic were

less likely to report symptoms. We found no association with total symptom scores (although we used lower weights for severe symptoms compared to the previous report). Given the different population groups and methods utilised we consider both studies to provide consistent evidence that baseline cross reactive CD8 T cell responses to conserved internal proteins do not protect against infection with influenza but do protect against symptoms and PCR positive disease in those infected with the pandemic strain. In addition we show protection against PCR positive seasonal influenza. We also show that around 43% of the population have baseline T cell responses associated with protection indicating a substantial role of T cells for protection at a population level (see Table E2 in the online data supplement for a summary comparison of these two studies).

Influenza symptoms with fever appear around 48 hours after infection[18] and nasal swabs to detect virus shedding in the nose by PCR, were taken from the second day of illness. Therefore the T cells had at least 72 hours to act before the swab was taken. This time would allow accumulation of T cells at the site of infection in the upper respiratory tract and some expansion. Memory T cells can respond by releasing interferon-γ and other cytokines within 6 hours of antigen contact[38] and can expand up to 10,000-fold in ten days, approximately one division every 20 hours.[39] Therefore even low levels of functional memory T cells in the blood of 20/million, which is equivalent to 10^5 in the whole blood volume, could provide enough T cells to migrate to the site of infection to clear virus quickly. The lower frequency of detectable nasal virus shedding in infected participants (proven by an antibody rise) with positive baseline NP specific T cell responses compared to those without this response suggests that T cells promote more effective control of infecting virus in the upper respiratory tract. There was a strong correlation between ex vivo NP specific T cell

responses and cultured CD8+ T cell responses to NP, but no such correlation with cultured CD4+ T cell responses. Thus the NP specific T cell response, which was the most prevalent influenza protein-specific response and which showed an independent protective effect comprised proportionately more CD8+ T cells than CD4+ T cells. Therefore, as also reported by others NP specific CD8+ T cells are likely to be important mediators of protection.[20] However, we cannot exclude some additional protective effect of CD4 T cells, as shown in a previous small challenge study[19] just as that study could not exclude a role for CD8 T cells. Most likely both are involved.

We excluded vaccinated participants from the analysis, but found no evidence that T cell responses at baseline were associated with recent vaccination. This is consistent with previous research that has shown that subunit vaccines fail to, or only weakly, stimulate CD8 T cell responses.[12,40] At the time of the study the live attenuated influenza virus vaccine (LAIV) was not licensed in the UK and no volunteer had been given that vaccine. The relatively poor protection offered by that vaccine in adults could be related to pre-existing T cell immunity preventing virus take; this needs to be explored further in future studies.

Previous work, including animal models[11-13, 16, 41-44] and some studies in humans[18-20], suggests a potentially important role for T cell based immunity to provide cross-protective immunity to influenza. However this is the first study to show a significant strong effect for both seasonal and pandemic influenza viral shedding that is independent of baseline antibodies in a broadly based population sample.

Widespread adaptive heterotypic immunity provided by cross-reactive T cells along with innate immune responses could help to explain why many seasonal infections are asymptomatic and why this phenomenon was also common in the 2009 pandemic,

despite antibody based immunity being very low in the population at the outset.[45] These results suggest that vaccine induction of CD8+ and/or CD4+ T cells specific for NP could offer partial protection by reducing virus shedding resulting in clearance of infection, reduced respiratory symptoms and possibly reduced influenza transmission. This could be useful during the first six months of a pandemic before a conventional vaccine becomes available. This could also be of benefit in years when vaccine is poorly matched to circulating influenza strains, as happened in the 2014/15 influenza season.[46] There have been concerns that overstimulation of T cell responses could increase risk of post infection immunopathology.[47,48] The absence of a positive correlation between NP concentration and symptom severity in those who do become ill is therefore reassuring. Also, the experimental virus vectored vaccines currently being tested are unlikely to give very high levels of T cell responses in humans once the initial post vaccination peak is over reducing any risk.[49] Therefore this demonstration that quite modest levels of NP specific T cells in the blood correlate with protection, at least for the relatively weakly virulent viruses of recent years, is encouraging.

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FIGURE LEGENDS

Figure 1. Pre-existing *ex vivo* influenza specific T cell response

The pre-season *ex vivo* frequency of influenza (H3N2) specific T cell responses from 1703 baseline measurements from 1414 Flu Watch participants were quantified by IFN-γ ELISpot assay. Some participants contributed to more than one season, but no participant had more than one baseline measurement per season.

A: Each column represents a different baseline sample. The height of each column represents the *ex vivo* frequency (SFU/M PBMC) of H3N2 specific T cell response; each colour represents the T cell responses targeted at each of H3N2 viral antigens as indicated below the bar chart.

B: For the same samples as in Figure 1A the *ex vivo* NP specific T cell responses (y axis) are plotted on a $\log_{10}$ scale and ordered on the x axis by the strength of the response.

C: For the total *ex vivo* T cell responses in the whole study, the proportional contribution of each H3N2 viral antigen specificity is shown, indicating for instance that NP specific T cells constitute 25% of the whole response.

D: Percentage of Flu Watch baseline samples that had ex vivo detectable memory T cell responses (for each influenza protein response we subtracted the negative control well result from the test well result and accepted values of ≥20 SFU/M PBMC as reactors if the original result was also significantly higher than that of the negative control wells ($p<0.05$ based on the negative binomial distribution).

Figure 2. Pre-existing influenza NP and M specific T cell cross-react to pandemic H1N1 2009

The frequencies of T cell responses to pandemic H1N1 2009 NP and M peptides were quantified *ex vivo* from Flu Watch participants (N=222) who had no detectable antibody against pandemic H1N1 2009 HA at baseline. Each dot represents one Flu Watch participant. Panel A shows the $\log_{10}$ transformed *ex vivo* frequency of H3N2 NP specific T cell response shown on the X axis and the $\log_{10}$ transformed *ex vivo* frequency of the T cells targeted at pandemic H1N1 2009 NP showing on Y axis. The NP T cell responses were quantified by IFN-γ ELISpot assay and backgrounds were subtracted in the data presented in this figure. Panel B shows the equivalent for M responses.

Figure 3. CD4 and CD8 phenotype of pre-existing influenza specific T cells

The pre-season influenza (H3N2) specific T cell responses from 174 randomly selected Flu Watch participants were expanded by culture with peptide and IL-2 and the frequency of CD4+ and CD8+ influenza specific T cells were measured by intracellular interferon-γ staining (Y axis).The height of each column indicates the percentage of all influenza specific T cells (CD3+IFN-γ+) responding to H3N2 virus peptides representing each protein. The grey and red colours indicate the relative CD4+ and CD8+ T cell responses.

Table 1 – Cohort characteristics and analysis of risk factors for infection and PCR-confirmed symptomatic illness in those infected.

	Number with paired sera (person seasons)	**# infections (# per 100 person seasons)***	**Unadjusted Incidence rate ratio (IRR-95% CI), p**	**Adjusted IRR (95% CI), p**	**# (%) of infections with PCR-positive influenza A***	**Unadjusted OR (95% CI), p**	**Adjusted OR (95% CI), p**
NP SFU/M PBMC †							
NP < 20	869 (762)	126 (16)	1		29 (23%)	1	1
NP ≥20	630 (562)	79 (14)	0.94 (0.70-1.25), 0.659		6 (8%)	0.26 (0.10-0.64), 0.003	0.27 (0.11-0.68), 0.005
Year							
2006	294 (289)	51 (18)		1	6 (12%)	1	
2007	351 (339)	40 (12)	0.63 (0.43-0.94), 0.024	0.65 (0.44-0.95), 0.03	8 (20%)	1.5 (0.43-5.2), 0.52	
2008	285 (221)	67 (30)	1.35 (0.97-1,88), 0.073	1.45 (1.05-1.98), 0.023	12 (18%)	1.0 (0.31-3.3), 0.97	
2009	569 (475)	47 (9.9)	0.46 (0.34-0.67), <0.001	0.44 (0.30-0.64), <0.001	9 (19%)	1.3 (0.39-4.2), 0.69	
Gender							
Male	692 (612)	85 (14)	0.86 (0.65-1.14), 0.303		19 (22%)	2.3 (1.0-6.1), 0.04	1.9 (0.91-4.1), 0.09
Female	807 (712)	120 (17)	1		16 (13%)		
Age group							
5-15	105 (94)	25 (27)	1.76 (1.16-2.68), 0.008	2.56 (1.68-3.88), <0.001	2 (8%)	1	
16-45	499 (440)	77 (18)	1	1	14 (19%)	2.5 (0.58-11), 0.22	
45-64	734 (651)	89 (14)	0.77 (0.57-1.05), 0.107	0.74 (0.054-1.00), 0.05	19 (21%)	2.9 (0.65-13), 0.16	
65+	161 (139)	14 (10)	0.50 (0.28-0.91), 0.022	0.70 (0.37-1.29), 0.253	0 (0%))	n/a	
Baseline titre							
<16	1170 (1033)	186 (18)	1	1	34 (18%)	1	
16-32	206 (183)	18 (9.8)	0.58 (0.35-0.96), 0.034	0.42 (0.25-0.70), 0.001	1 (6%)	0.29 (0.04-2.0), 0.22	
64-128	89 (78)	1 (1.3)	0.06 (0.01-0.43), 0.005	0.04 (0.01-0.30), 0.002	0	n/a	
>128	34 (29)	0 (0.0)	n/a	n/a	n/a	n/a	

*Refers to dominant influenza strain: H3N2 in 2006/7 and 2008/9; seasonal H1N1 in 2007/8 and A(H1N1)pdm09 in 2009/10. Each year one strain of influenza A dominated: In 2006/7 there were 6 PCR-positive cases of H3N2; in 2007/8 there were 9 PCR-positive cases of seasonal H1N1 and one of H3N2; in 2008/9 there were 16 H3N2 and 2 seasonal H1N1 and in 2009/10 there were 12 PCR-positive cases of A(H1N1)pdm09. PCR and serology results refer to the dominant influenza strain circulating each year.

†Nucleoprotein spot forming units per million peripheral blood mononuclear cells.

FIGURE LEGENDS

Figure 1A

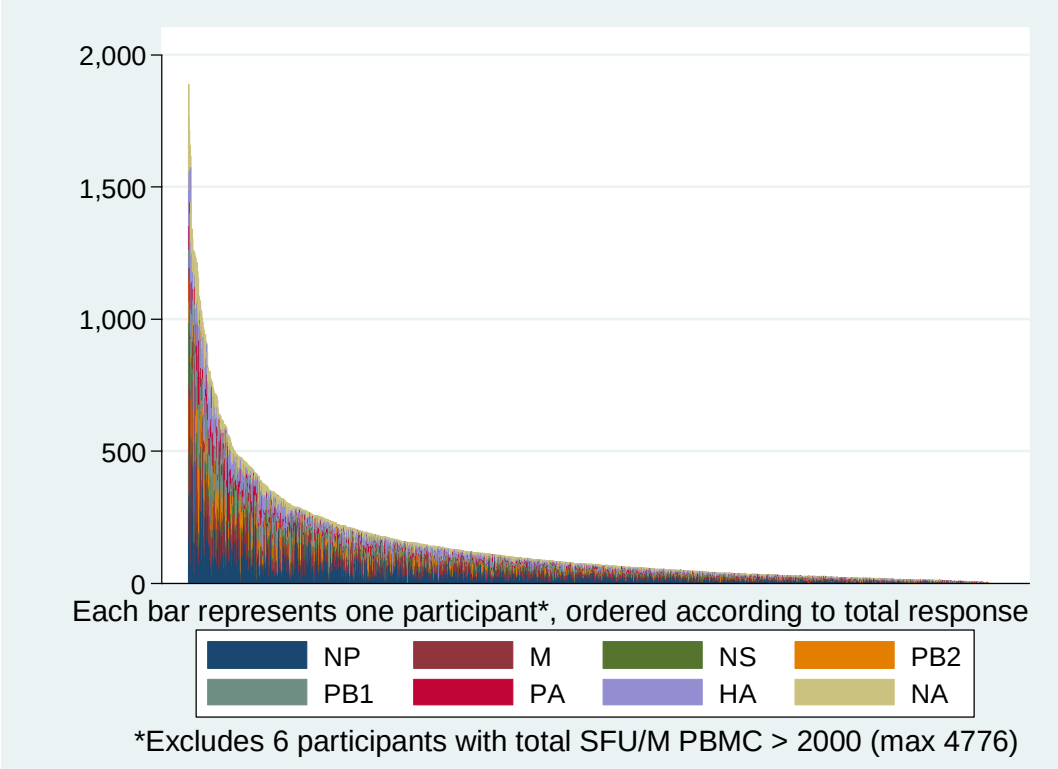

Figure 1B

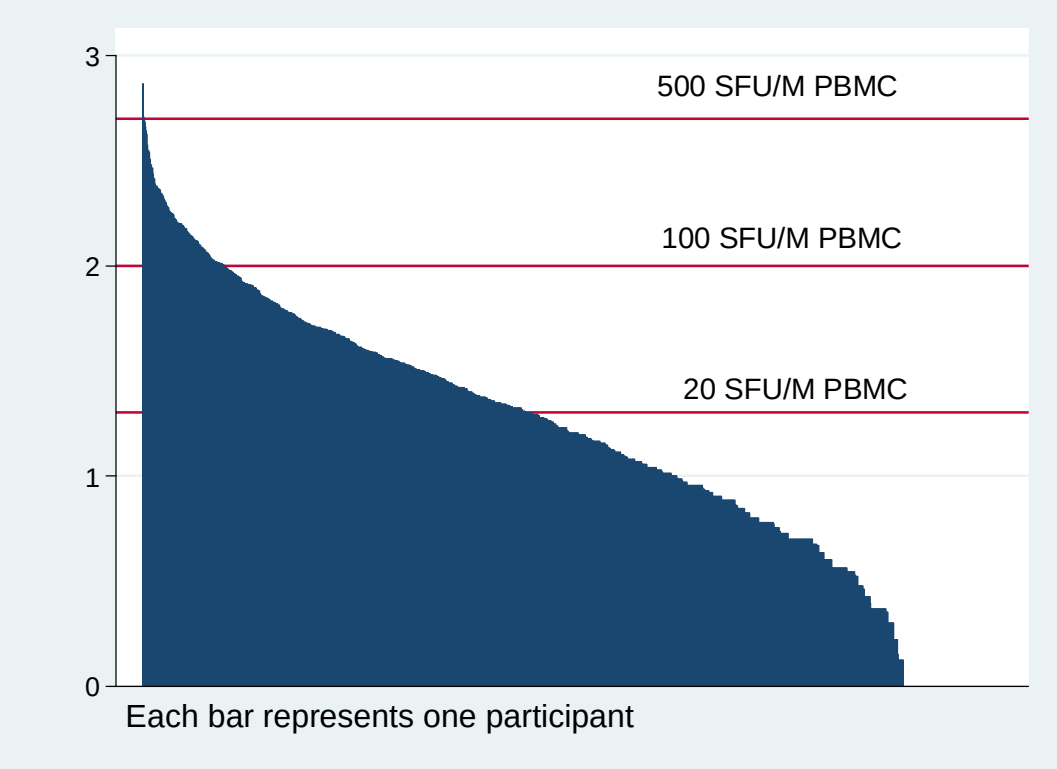

Figure 1C

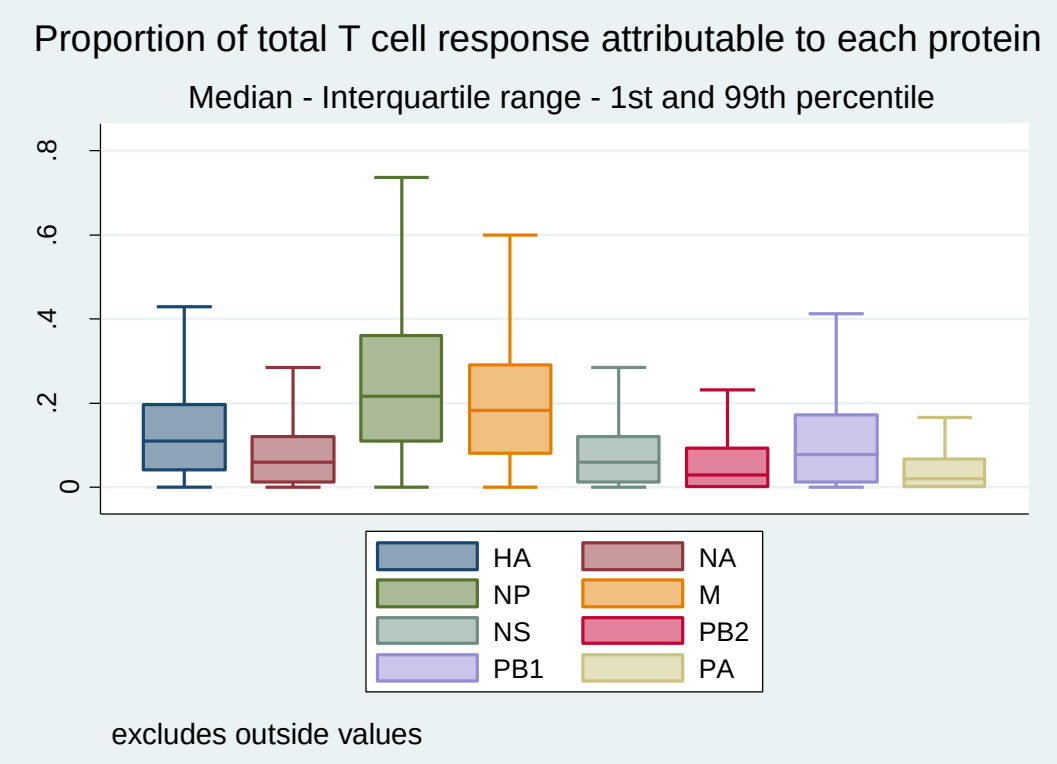

Figure 1D

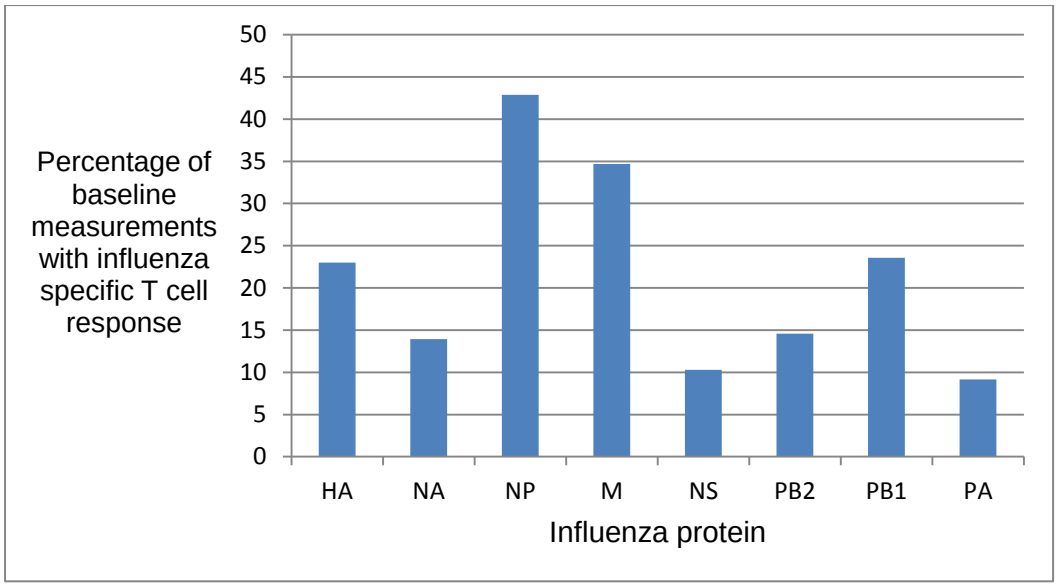

Figure 2A

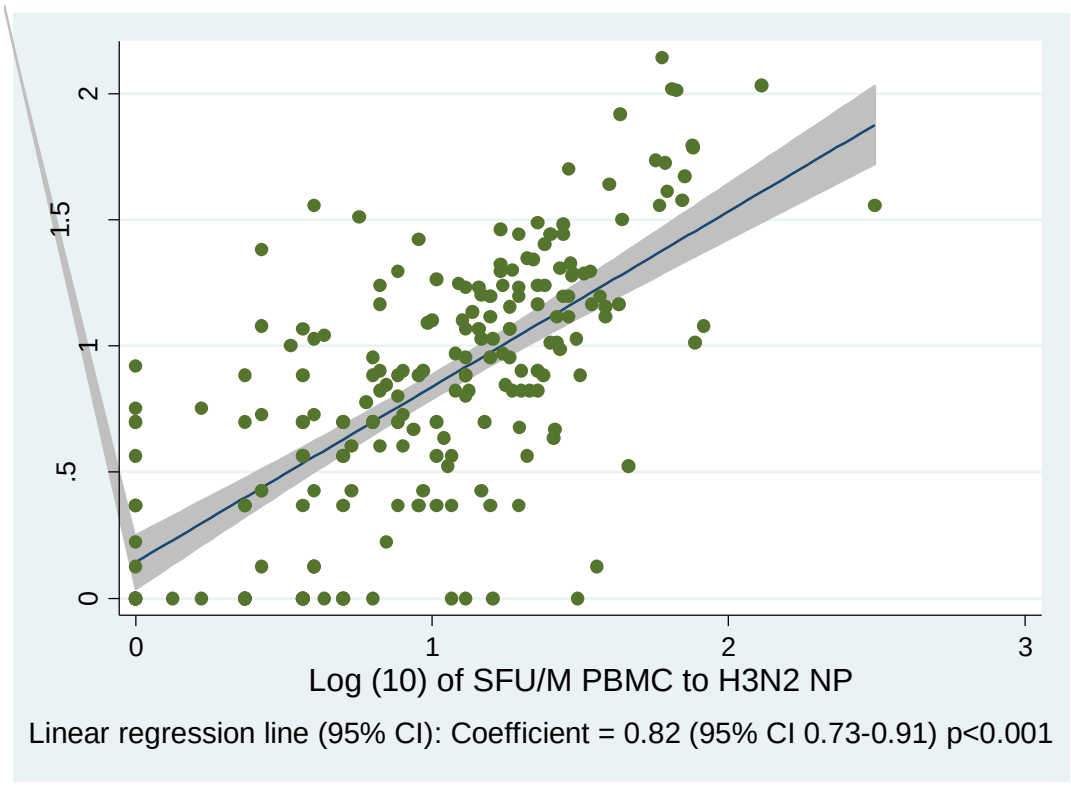

Figure 2B

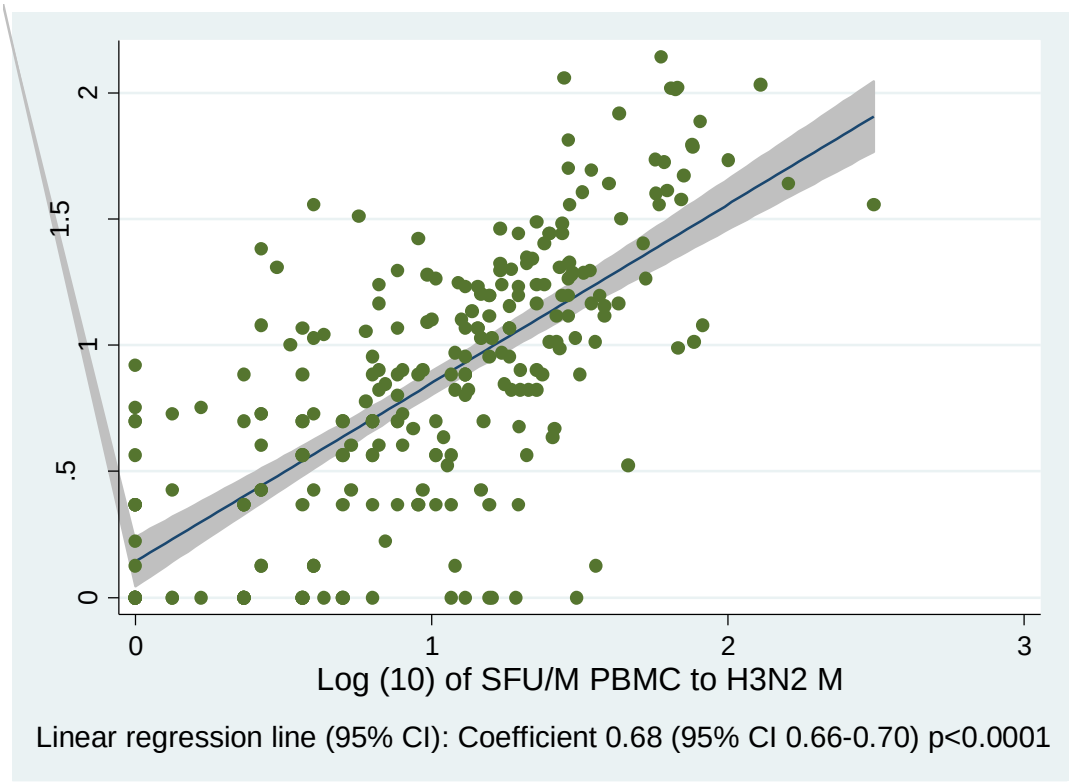

Figure 3