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Early View

Original research article

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Prime-boost, double-dose influenza vaccine immunity in COPD: a pilot observational study

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Author contributions

All authors met the ICMJE critieria for authorship. Specifically they all:

- Made substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work; AND
- Drafted the work or revising it critically for important intellectual content; AND
- Gave their final approval of the version to be published; AND
- Agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Take home message: Prime-Boost Double-Dose influenza immunization does not further improve vaccine response in COPD patients, underscoring the need to design more effective vaccine strategies to overcome immune hypo-responsiveness.

Abstract

Rationale

COPD patients are more susceptible to viral respiratory infections and their sequalae and have intrinsically weaker immune responses to vaccinations against influenza and other pathogens. Prime-Boost Double-Dose immunization has been suggested as a general strategy to overcome weak humoral response to vaccines, such as seasonal influenza vaccination, in susceptible populations with weak immunity. However this strategy, which may also provide fundamental insights into the nature of weakened immunity, has not been formally studied in COPD.

Methods

We conducted an open label study of seasonal influenza vaccination in 33 vaccine-experienced COPD patients recruited from established cohorts (mean age 70 yrs (66.9-73.2); mean FEV_1/FVC ratio 53.4% (48.0-58.8)). Patients received two sequential standard doses of the 2018 quadrivalent influenza vaccine (15 μg haemagglutinin per strain) in a prime-boost schedule 28 days apart. We measured strain-specific antibody titres, an accepted surrogate of likely efficacy, and induction of strain specific B-cell responses following the prime and boost immunizations.

Measurements and Main Results

Whereas priming immunization induced the expected increase in strain-specific antibody titres, a second booster dose was strikingly ineffective at further increasing antibody titres. Similarly, priming immunization induced strain-specific B cells, but a second booster dose did not further enhance the B cell response. Poor antibody responses were associated with male gender and cumulative cigarette exposure.

Conclusions

Prime-Boost Double-Dose immunization does not further improve influenza vaccine immunogenicity in previously vaccinated COPD patient. These finding underscore the need to design more effective vaccine strategies for COPD patients for influenza.

Introduction

Double-dose, prime-boost vaccination is generally considered an effective strategy to provide protective immunity in susceptible populations, particularly the elderly and those with predisposing co-morbidities such as chronic lung disease, where vaccines are less effective.[1] However, it remains widely underappreciated that COPD patients are more susceptible to respiratory viruses *per se*, and have intrinsically weaker immune responses to vaccinations against influenza and other pathogens[2] than their peers; accelerated "immuno-ageing/senescence" may contribute to this phenomenon.[3] This may particularly predispose COPD patients to acute influenza infections, subsequent bacterial pneumonia and life-threatening thromboembolic events in the year after recovery.[4,5]

In the elderly the immunosenescence that attenuates immunity to influenza vaccination can be overcome, in part, by higher-dose and adjuvanted vaccines.[6,7] Similarly, homologous Prime-Boost vaccination protocols, where second doses of the same vaccine are separated by 3-4 weeks, can strengthen humoral immune responses.[1,8,9] Much less is understood about vaccine responses in COPD.[10,11] Antibody titre is conventionally used as a surrogate endpoint in inactivated flu vaccine trials[12] particularly because humoral rather than cellular immunity is mostly elicited by this technology[13,14]. Of these antibodies those against haemaglutanin are most often used, by convention, to measure response although those against neuaminidase can also confer protection[15,16]. We have previously reported that humoral immunity and seroconversion rates were markedly attenuated in COPD patients to the 2010 trivalent inactivated influenza vaccine against the pandemic (H1N1) 2009 strain (A/California/7/2009), a H3N2 strain (A/Perth/16/2009), and a B strain (B/Brisbane/60/2008).[11] Such inactivated vaccines effectively elicit humoral antibody responses but typically fail to elicit effective CD8+ T cell immunity. A recent Cochrane Review identified only one double-blinded randomized controlled trial on doubling vaccine dose in COPD.[17] In that study, which was performed in a predominantly vaccine naïve population, a trivalent vaccine used at double the standard dose was found to be 76% effective in preventing influenza infections.[18] However, the effectiveness of higher dose, prime-boost immunizations in

vaccine-experienced COPD patients receiving annual recommended vaccinations has not been reported.

In 2018 Australian COPD Guidelines ((COPDX Guidelines – Version 2.55 (August 2018)) were changed to recommend doubling influenza vaccination dose in COPD patients . This change in guidelines enabled us to study the effectiveness of doubling the standard dose of influenza vaccine using a prime-boost immunisation schedule in COPD patients in Melbourne and Brisbane who were already screened and enrolled in ongoing NHMRC-funded cohort studies on vaccine immunity. We assessed antibody titres and strain-specific B cell responses. In contrast to previous reports we observed a striking lack of effectiveness of this immunization protocol to further increase antibody titres in the majority of COPD patients. Our study suggests that the Prime-Boost strategy is unlikely to be effective, mitigating against undertaking larger randomised studies on this strategy and underscores the need to specifically study vaccine effectiveness in COPD in order to develop more effective strategies.

METHODS

Study Population

Participants were recruited across two sites as a sub-study of ongoing cohort studies on vaccine immunity at the Princess Alexandra Hospital (Brisbane, Australia) and the Royal Melbourne Hospital from the Melbourne Longitudinal COPD Cohort. Eligible participants were over 55 years of age with a current clinical diagnosis of mild-to-very-severe COPD, and post-bronchodilator FEV_1 <80% predicted, an FEV_1/FVC ratio <0.7, with no COPD exacerbations in the 28 days prior to the study. Subjects were enrolled if: co-morbidities (e.g. cardiac diseases including; ischaemic heart disease, cardiac arrhythmias, cardiac failure; diabetes mellitus, hypertension) were stable and well controlled; and, use of inhaled or low-dose oral corticosteroid was stable in the 28 days preceding and remained stable post-vaccination. Exclusions were: invasive malignancy within the

past two years, renal impairment (eGFR < 40 mL/min), acute febrile illness with fever >38.5 °C, hypersensitivity to egg proteins, use of oral prednisolone or equivalent ≥10 mg/day, or use of other immunosuppressive therapy. Patient characteristics are shown in Table 1 (where heart conditions included Ischaemic heart disease, cardiac arrhythmias and cardiac failure). Patients were also asked if they ever had doctor diagnosed asthma and a proportion of patients were likely, therefore, to have COPD-asthma overlap which is very common in the general community.

Study Design

Recruitment occurred preceding the southern-hemisphere influenza season and written informed consent was obtained from each subject prior to commencement. Detailed clinical assessment, blood collection and spirometry were performed at day 0, prior to intramuscular administration of a single dose of the standard 2018 inactivated, quadrivalent, split-virion influenza vaccine (FluQuadri™, Sanofi Pasteur, immunisation protocol and sample collection shown in Figure S1). The 2018 vaccine consisted of 15 μg haemagglutinin (HA) each of: A/Michigan/45/2015 (H1N1) pdm09-like virus; A/Singapore/INFIMH-16-0019/2016 (H3N2)-like virus; B/Phu/3073/2013-like virus and B/Brisbane/60/2008-like virus. Participants returned at day 28 post-vaccine for a second visit, where blood samples were taken prior to administration of a second vaccine dose. The first patient data was collected on 9th April, 2018 and the last sample was collected on 29 August 2018. Participants attended two further clinic visits on days 56 and 84 to provide blood samples. Blood samples were processed on the day of collection: PBMCs and sera were stored at -80°C.

Annual vaccination against influenza, which is subsidized and widely available, is recommended for all COPD patients in Australia but not mandated. As a matter of record the quadrivalent vaccines available in the 2016 and 2017 prior to our study comprised:

2016

- A (H1N1): an A/California/7/2009 (H1N1)-like virus

- A (H3N2): an A/Hong Kong/4801/2014 (H3N2)-like virus (H3N2 is generally the most severe form of influenza A)
- B: a B/Brisbane/60/2008-like virus.
- B/Phuket/3073/2013-like virus.

The 2017 vaccine comprised

- A (H1N1): an A/California/7/2009 (H1N1)-like virus
- B: a B/Brisbane/60/2008-like virus.
- B/Phuket/3073/2013-like virus.
- H1N1_A/MICHIGAN/45/2015 like

This study was conducted in accordance to the Declaration of Helsinki Principles and the Australian National Health and Medical Research Council (NHMRC) Code of Practice. Ethical approval was granted by the local ethics committee for each site: The University of Queensland Human Ethics Research Office (clearance number: 2011000502), Metro South Health Human Research Committee (HREC/09/QPAH/297) and Royal Melbourne Hospital/Melbourne Health (HREC MH 2019:086). The first patient data was collected on 9th April, 2018 and the last sample was collected on 29 August 2018.

Immunogenicity

Hemagglutination inhibition assay (HIA) was performed on receptor-destroying enzyme treated sera, against components of each vaccine strain using microtitre techniques at VIDRL (Victorian Infectious Disease Reference Laboratory, WHO Collaborating Centre for Reference and Research on Influenza).[19] Briefly, serial two-fold dilutions in PBS (1:10 to 1:1280) were incubated with 4 hemagglutinating units of the vaccine strain-specific influenza antigen, and 1% turkey erythrocytes

(H1N1 and B strains), or 1% guinea-pig erythrocytes in the presence of oseltamivir (H3N2). Strain specific HA antibody titres were calculated as the reciprocal of the highest dilution of sera that inhibited hemagglutination. Titres below the limits of detection (<10 or <20) were arbitrarily designated a value half the threshold of detection. The primary study endpoint was seroconversion (defined a four-fold or greater increase in hemagglutination inhibition antibody titre. Seroprotection (defined as a hemagglutination inhibition antibody titre ≥ 1:40) was a secondary endpoint.

Strain-specific B cell response

Peripheral blood mononuclear cells (PBMCs) were isolated using density gradient centrifugation and stored at -80°C until required. To determine the quantity and phenotype of B cells elicited, influenza-specific B cells were identified using recombinant HA (rHA) probes and assessed by flow cytometry as described (Koutsakos et al., 2018). Probes were developed for two 2018 southern-hemisphere influenza vaccine strains: A/Michigan/45/2015 (H1N1) pdm09-like virus and B/Phuket/3073/2013-like virus. Due to the lack of availability of probes for the A/Singapore/INFIMH-16-0019/2016 (H3N2)-like virus and B/Brisbane/60/2008-like virus strains, B-cell data for these strains was not assessed. To phenotype strain-specific B memory cells, CD19+IgD- B cells were identified with rHA probes, and CD27 and CD21 surface marker expression determined by flow cytometry to differentiate between classic memory B cells (CD27+CD21+), activated memory B cells (CD27+CD21-), naïve B cells (CD27-CD21+), and atypical memory B cell (CD27-CD21-, also referred to as “anergic” or “exhausted”) B cell populations. The gating strategy and antibodies used are described in Figure S3 and Table S1 respectively). We have previously observed in our COPD cohort that inactivated vaccines do not elicit CD8 T cell responses, so these were not measured in this study.

Statistical analysis

Forty-one participants were recruited. Seven participants did not supply blood samples for all four days and one did not provide full demographic information and were excluded from statistical analysis. For the remaining 33 study participants descriptive statistics were calculated separately for each vaccine strain. Antibody HIA titres were summarised as geometric mean titre (GMT) ± 95% confidence intervals (CI). Antibody titres were log transformed and significant differences for each time points compared to baseline determined by repeated measures ANOVA. Changes in mean percentages and phenotype of strain specific B cells were assessed with Wilcoxon matched-pairs signed-rank tests. Correlations between day 28 HIA titre and clinical characteristics, and day 28 HIA titre and strain specific B cells and titre, were assessed using Spearman's correlation coefficient (r_s) for non-Gaussian distributions. With ability to seroconvert as the dependent variable, generalised linear models (GLMs) were used to identify factors independently associated with antibody response, and any interactions between such factors. Descriptive statistical analyses were calculated with GraphPad Prism, version 8.4.2 (464), (GraphPad Software, San Diego, California USA, www.graphpad.com). GLMs and clinical correlations were calculated using R, version 3.5.2 (2018, The R Foundation for Statistical Computing Platform, Vienna, Austria). A P value of <0.05 was considered to indicate statistical significance.

Results

Study participants

Characteristics of the thirty-three study participants are described in Table 1. Most were aged in their 60s and 70s and had moderate to severe COPD (mean FEV1 % predicted (95%CI), 52.8(45.8-59.8)). More participants were male and just over 40% were current smokers. Most participants had been vaccinated within the preceding two years; only four participants were influenza vaccine naive (Table 1).

A second vaccination dose at day 28 does not improve strain-specific antibody titres

Vaccination with the 2018 seasonal quadrivalent influenza vaccine (15μg of each strain) resulted in the predictable increase in mean serum titres of A/H1N1 specific antibodies at D28 (Figure 1), though seroconversion rates were less than 40% across all vaccine strains (Table S2). However, a second vaccination with the same dose at D28 did not induce any additional increase in mean antibody titre at D56 or D84 (Figure 1), nor did it improve seroconversion rates (Table S2). In fact, mean antibody titre was lower at both these time points than at D28, demonstrating an inability of double dose (sequential prime-boost) vaccination to maintain or augment antibody titres (Figure 1, S2). Similar response patterns were observed with the other vaccine strains (A/H3N2, B/Phuket and B/Brisbane): the second vaccine dose at D28 did not induce any additional increase in mean antibody titres at D56 or D84, and did not prevent a decline in mean antibody titres at D84 (Figure 1). GLM analysis (Table 2) indicated that females were more likely to seroconvert than males, although this was only significant for A/H1N1 strain (P=0.0278). Stepwise GLM analysis using current smoking status, pack years and FEV_1/FVC ratio, indicated that pack years had more influence on ability to seroconvert than current smoking status or FEV_1/FVC ratio. The negative association between pack years and seroconversion was statistically significant for the A/H1N1 strain in univariate analysis (P=0.049), however, was not significant when adjusted for current

smoking status and FEV_1/FVC ratio. Further details on seroconversion rates for each strain are provided in the Supplementary Results (Table S2).

A second vaccination dose at day 28 does not improve the induction of strain-specific B cells and does not alter the phenotype of circulating B-cells.

We next asked whether a second sequential vaccination altered induction of vaccine strain-specific B cells. This involved the use of validated probes recognising type A (H1N1) and type B (B/Phu) antigens.[20-22] Vaccination led to an increase in both A/H1N1 and B/Phu specific B cells at D28 (Figure 2). However, a second vaccination at D28 did not induce any additional B/Phu specific B cells, and was unable to maintain the A/H1N1 specific B cells induced by the initial vaccination (Figure 2). There were strong associations between specific antibody titres and the proportions of A/H1N1 specific B cells and B/Phu specific B cells are shown in the Supplementary Results (Figure S4).

Similarly, while initial vaccination at D0 induced an increase in activated memory B-cells, and a concomitant reduction in the percentage of A/H1N1-specific classic memory B-cells at D28, a second vaccination at D28 did not induce any additional effects on the percentage of either populations at D56 (Figure 3). Vaccination had minimal effects on the relatively low percentages of naïve and atypical memory B cell populations (see Supplementary Results S5).

Discussion

In the present study, we evaluated the outcome of delivering double the usual dose of seasonal influenza vaccine to COPD patients using a Prime-Boost immunisation schedule to determine whether this might enhance immunogenicity. Priming immunization induced a significant increase in strain-specific antibody titres across all four vaccine strains. However, our results reveal a striking lack of effectiveness of the second “boost” arm of this strategy which was unable to further increase antibody titres or B cell responses. These results contrast markedly with a previous report

in influenza vaccine-naive COPD patients, however, this study did not use as prime-boost vaccination schedule.[18]

Notwithstanding the failure of the boost to enhance immunity it is important to note that we, as have others, did observe a positive humoral response to the priming vaccination in many patients. Given the susceptibility of COPD patients to influenza, post-infection pneumonia and its sequalae, our results clearly support the continued use of influenza vaccination in this population. Our study was not designed to understand if a double-dose prime-boost strategy actually prevents influenza infection, as such studies require very large cohorts of thousands of patients over an extended period of time.

In our study we observed that the majority of patients had some increase in antibody titre and up to 36 percent reached the 4-fold increase conventionally regarded as sero-conversion. Of note several of the antigens present in the 2018 vaccine were also present the 2016 and 2017 vaccine as detailed above and this may have influences the 2018 response to those antigen, for example by yielding a higher base line and therefore an apparently smaller change in titre. These antibody responses were mirrored by increases in vaccine strain-specific B cells. Immunologically, it is known that inactivated influenza vaccines activate both B cells and T follicular helper (TFH) cells to induce antibody-secreting cells and increases in serum antibody titres.[20] This study in a general population of healthy adults demonstrated that inactivated influenza vaccinations induce three main B cell responses comprising a transient CXCR5−CXCR3+ antibody-secreting B cell population, CD21hiCD27+ memory B cells, and CD21loCD27+ B cells. Activation of circulating TFH cells was shown to correlate with the development of both CD21lo and CD21hi memory B cells in this study. In our study, while the trend was not marked or statistically significant, many patients actually showed a small decrease in antibody titre after boost. Previous studies have observed a negative correlation between prior antibody levels and subsequent B cell responses. Whether repeat immunisation to the same epitope leads to clinically important decreases in humoral immunity, and if so what mechanism(s) might cause this, are areas of current controversy.

In order to better understand correlates of poor humoral responses, we analysed multiple potential determinates using a GLM statistical approach. Males seroconverted less readily relative to females. Interestingly, current smoking was not independently associated with seroconversion, though overall pack-year history was negatively correlated. Active smoking, or even exposure to passive cigarette smoke, has repeatedly been shown to enhance susceptivity to influenza and other viruses and this is thought to reflect mucosal immune suppression. It is however likely that long exposure to smoke reflected in pack-year history damages the immune system to an extent that active smoking no longer appears as a risk factor.[23] Age, a well-established determinant of weak vaccine responses, was not significantly associated with seroconversion, most likely because all our patients were elderly and in a narrow age range. It is known that T follicular helper cell responses (T_{FH}) are required to consolidate high-affinity antibody production and that these wane with age[24] although it is not known if COPD specifically weakens this compartment.

Our study has several key limitations. Key amongst these are that our study is also, for ethical reasons, was constrained to an open label in design, and is that it is not possible to measure actual protection against influenza infection for studies of this size and duration. Protection studies require very large cohorts of thousands of subjects and a lengthy duration. We did not measure antibodies at the respiratory mucosal surfaces nor could we assess the anatomical distribution of memory cells which, in post-mortem studies, have shown a predilection to redistribute from blood to lung tissue. While it is conceivable that COPD patients might show favourable changes in antibody titres in these compartments, but not in the circulation, this seems unlikely. Even in healthy people inactivated flu vaccines produce relative weak and usually transient increases in mucosal antibodies.[25]

All of the patients in our study met GOLD criteria for diagnosis of COPD however a proportion of patient reported a past diagnosis of asthma, which has a high population prevalence in Australia, and some were likely to have at least some feature of asthma-COPD overlap (ACO), common in “real-world” cohorts. Our study was not sufficiently powered to determine if responses

to double dose influenza vaccine differed in patients with pure COPD, versus those with both COPD and asthma. This is a limitation of our study and warrants further investigation in larger studies. However, it is noteworthy that our recent much larger study of single dose influenza vaccination found that neither COPD nor asthma were associated with seroconversion and seroprotection [26].

We would also like to explicitly state that our results are not generalisable to adenoviral or RNA vaccine technologies being employed against SARS-CoV-2. For example inactivated influenza vaccines produce no, or negligible, anti-viral T cell responses.

Despite these limitations our study clearly demonstrates that a double-dose prime-boost immunisation schedule is not effective in increasing humoral immunity in COPD. Given that COPD patients are a large and highly vulnerable population, our studies highlight the need to specifically study this population and, more importantly, to develop much more effective influenza vaccination strategies in this group.

Table 1: Demographics and baseline characteristics of the study population

Baseline Demographics and Clinical Characteristics	Total
N (%)	33
Mean age (95%CI)	70 (66.9- 73.2)
Female - n (%)	11 (33.3)
Ever Smoked	33 (100)
Current Smokers - n (%)	11 (33.3)
Mean Pack Years (95%CI)	54.8 (42.9 - 66.7)
Mean BMI (95%CI)	28.3 (25.7 - 30.9)
Diabetes - n (%)	5 (15.1)
Heart condition - n (%)	14 (42.4)
Asthma - n (%)	15 (45.5)
High blood pressure - n (%)	12 (36.3)
High cholesterol - n (%)	11 (33.3)
Bronchiectasis - n (%)	4 (12.1)
Mean FEV1 predicted% (95%CI)	52.8 (45.8 - 59.8)
Mean FVC predicted% (95%CI)	77.5 (69.5 -85.6)
Mean FEV1/FVC % (95%CI)	53.4 (48.0 - 58.8)
Flu vaccine 2017	26 (78.8)
Flu vaccine 2016	28 (84.8)
Flu vaccine naïve	4 (12.1)

Table 2: **Binomial GLMs determining factors influencing Ab response (seroconversion) for each vaccine strain**

glm(formula = foldsite ~ bmi + gender + age, family = "binomial", data = data)				
	Estimate	**Std. Error**	**z value**	**Pr(>|z|)**
H1N1 A/Michigan				
Intercept	1.757163	4.618047	0.38	0.7036
BMI	0.003058	0.060796	0.05	0.9599
Gender(Male)	-1.786016	0.811875	-2.2	0.0278 *
Age	-0.01836	0.050812	-0.361	0.7179
H3N2 A/Singapore				
Intercept	10.60108	6.04039	1.755	0.0793
BMI	-0.05018	0.07228	-0.694	0.4875
Gender(Male)	-1.91511	0.98814	-1.938	0.0526
Age	-0.13534	0.06981	-1.939	0.0525
B/Phuket				
Intercept	5.4915	5.6524	0.9720	0.3310
BMI	0.0030	0.0677	0.0450	0.9640
Gender(Male)	-0.8661	0.9737	-0.8890	0.3740
Age	-0.0967	0.0671	-1.4400	0.1500
B/Brisbane				
Intercept	-4.7166	8.2767	-0.5700	0.5690
BMI	0.1061	0.0975	1.0880	0.2770
Gender(Male)	-1.7665	1.4190	-1.2450	0.2130
Age	0.0010	0.0913	0.0110	0.9910

glm(foldsite ~ currentsmoke + pkyrs + FEV1.FVCratio., data = data, family = "binomial")					
	Estimate	**Std. Error**	**z value**	**Pr(>|z|)**	**Univariate Pr(>|z|)**
H1N1 A/Michigan					
Intercept	-1.4239	2.9707	-0.4790	0.6320	
CurrentsmokeTRUE	-1.0267	0.9946	-1.0320	0.3020	0.446
Pack years	-0.0173	0.0203	-0.8510	0.3950	0.049*
FEV1/FVC ratio	3.7675	4.2039	0.8960	0.3700	0.0752
H3N2 A/Singapore					
Intercept	-1.2816	3.1968	-0.401	0.688	
CurrentsmokeTRUE	0.94844	0.9662	0.982	0.326	0.258
Pack years	-0.0150	0.0225	-0.665	0.506	0.275
FEV1/FVC ratio	0.94756	4.3672	0.217	0.828	0.2014
B/Phuket					
Intercept	-1.7461	3.9157	-0.4460	0.6560	
CurrentsmokeTRUE	0.8875	1.1078	0.8010	0.4230	0.3465
Pack years	-0.0217	0.0290	-0.7490	0.4540	0.188
FEV1/FVC ratio	1.5926	5.2619	0.3030	0.7620	0.1417
B/Brisbane					
Intercept	-7.7828	5.30766	-1.466	0.143	
CurrentsmokeTRUE	-0.6934	1.41095	-0.491	0.623	1.00
Pack years	0.01336	0.02633	0.508	0.612	0.542
FEV1/FVC ratio	8.62764	7.17196	1.203	0.229	0.2319

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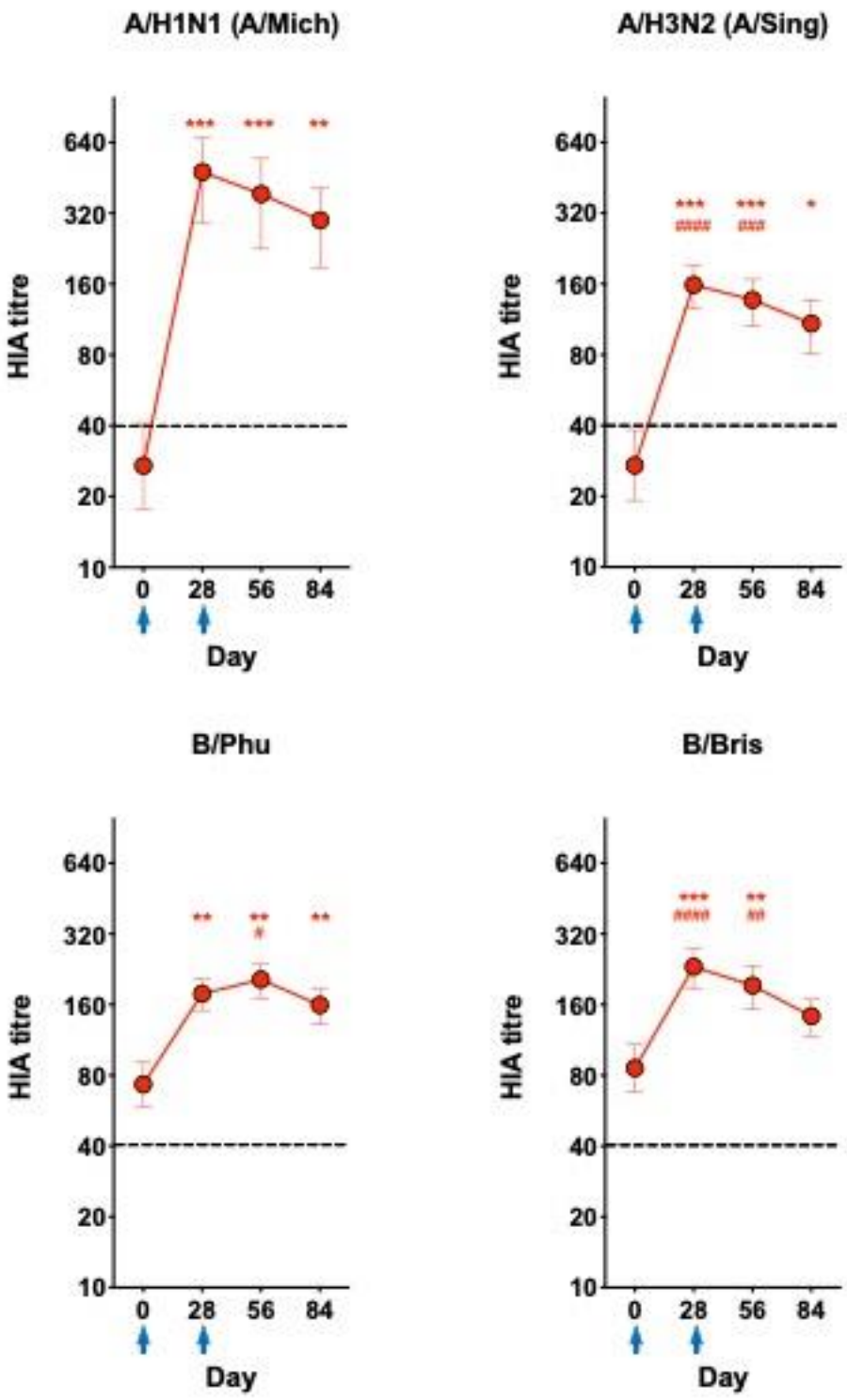

Figure 1: A second vaccination dose does not increase or sustain strain-specific antibody titres. Serum antibody titres were determined by HIA. Blue arrows indicate days of vaccinations. Data represented by geometric mean titre ? 95% confidence interval, and statistical significance determined by repeated interval ANOVA. n=33 (**P < 0.01, ***P < 0.001 relative to D0; #P<0.05, ##P<0.01 relative to D84). Dotted horizontal black line indicates seroprotection titre (1:40).

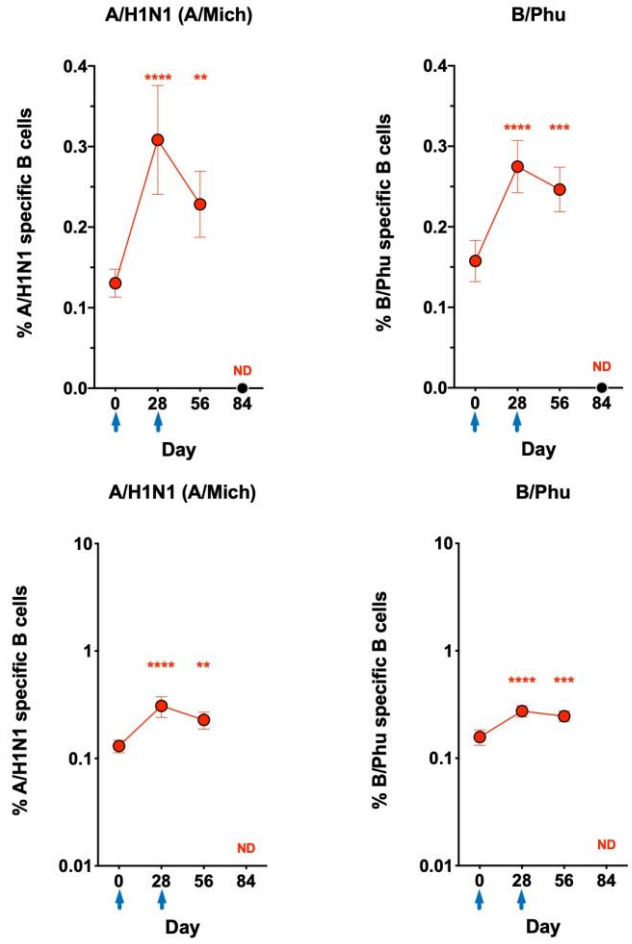

Figure 2: A second vaccination dose does not improve the induction of strain-specific B cells. PBMCs were stained with A/H1N1 and B/Phu specific probes to determine the percentage of cells within the blood CD19+IgDneg B cell population by FACS. Data shown as mean ? SEM. n=33. Statistical significance was determined using Wilcoxon matched-pairs signed-rank test. (**P < 0.01, ***P < 0.001, ****P<0.0001 relative to D0).

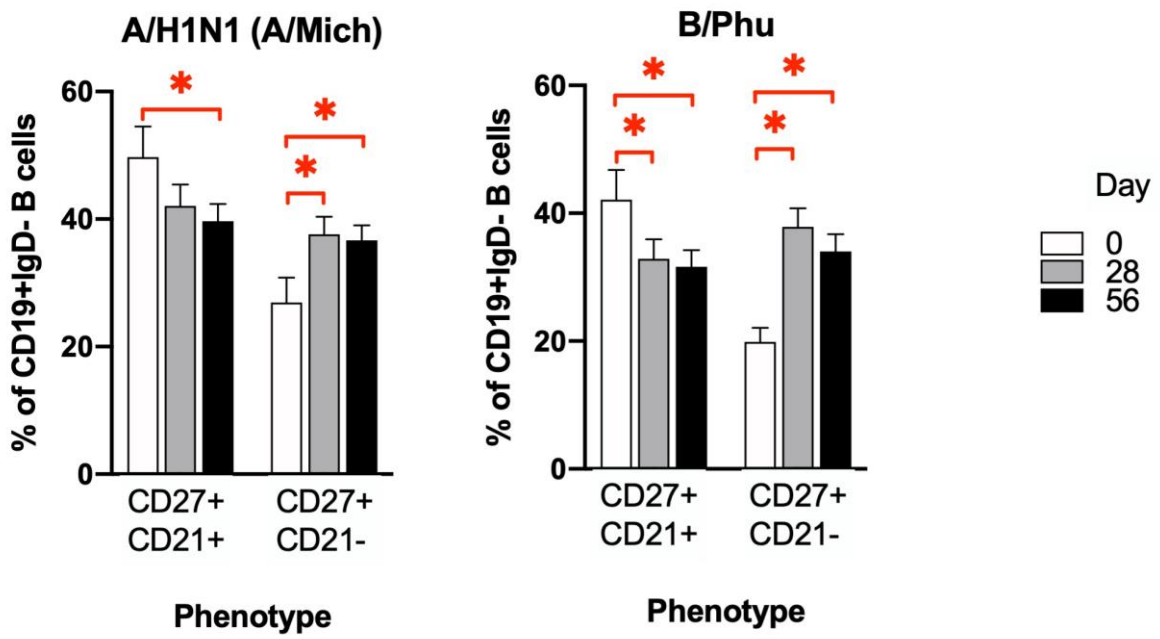

Figure 3: A second vaccination dose does not affect vaccine-induced changes in B cell memory. CD19+IgDPBMCs were stained with A/H1N1 and B/Phu probes, and cells differentiated on the basis of CD27 and CD21 expression by FACS. n=33 Statistical significance was determined using Wilcoxon matched-pairs signed-rank test. (*P < 0.05).

Prime-boost, double-dose influenza vaccine immunity in COPD

Supplementary material

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Figure S1: Vaccination strategy. COPD patients were given two doses of the 2018 influenza vaccine 28 days apart. Blood was collected at four timepoints for determining antibody titres. PBMCs were collected at 3 timepoints.

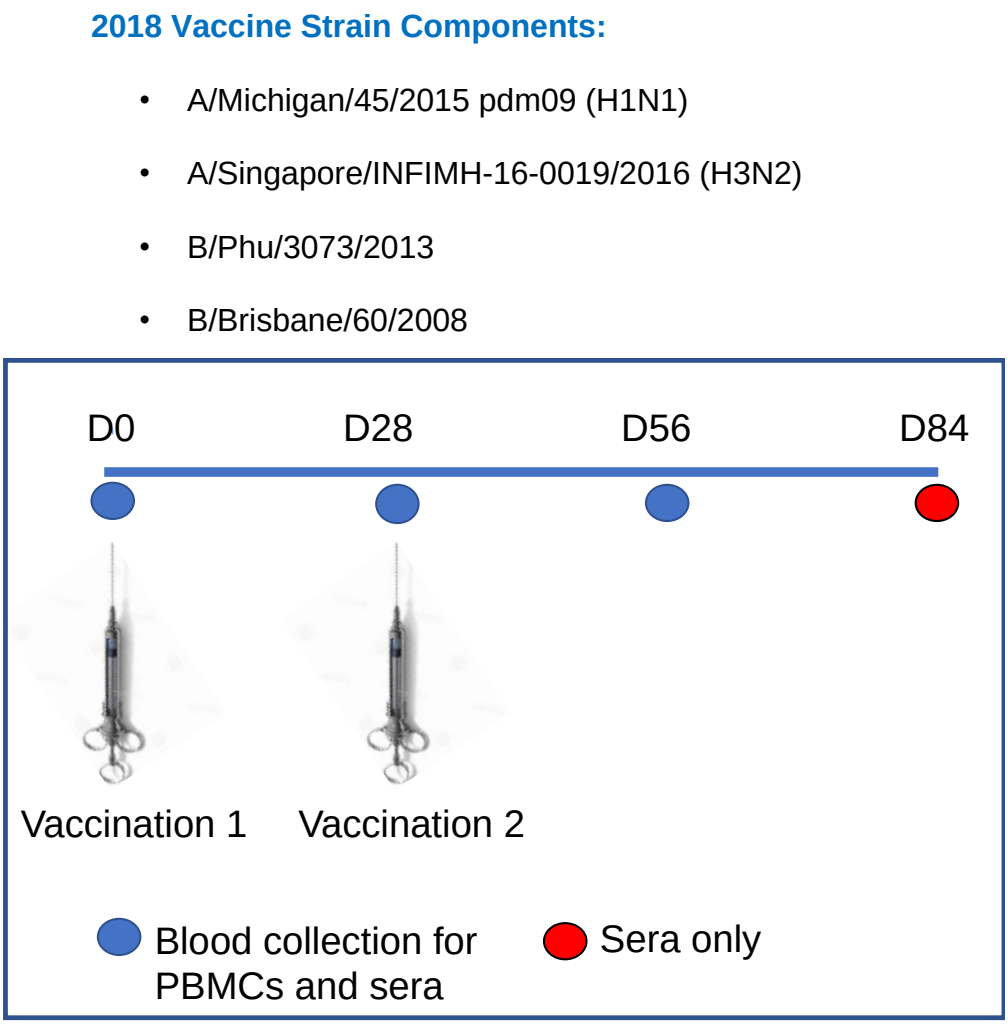


Figure S2. HIA antibody titres for all patients. Data represents titres with geometric mean and 95% CI. n= 33.

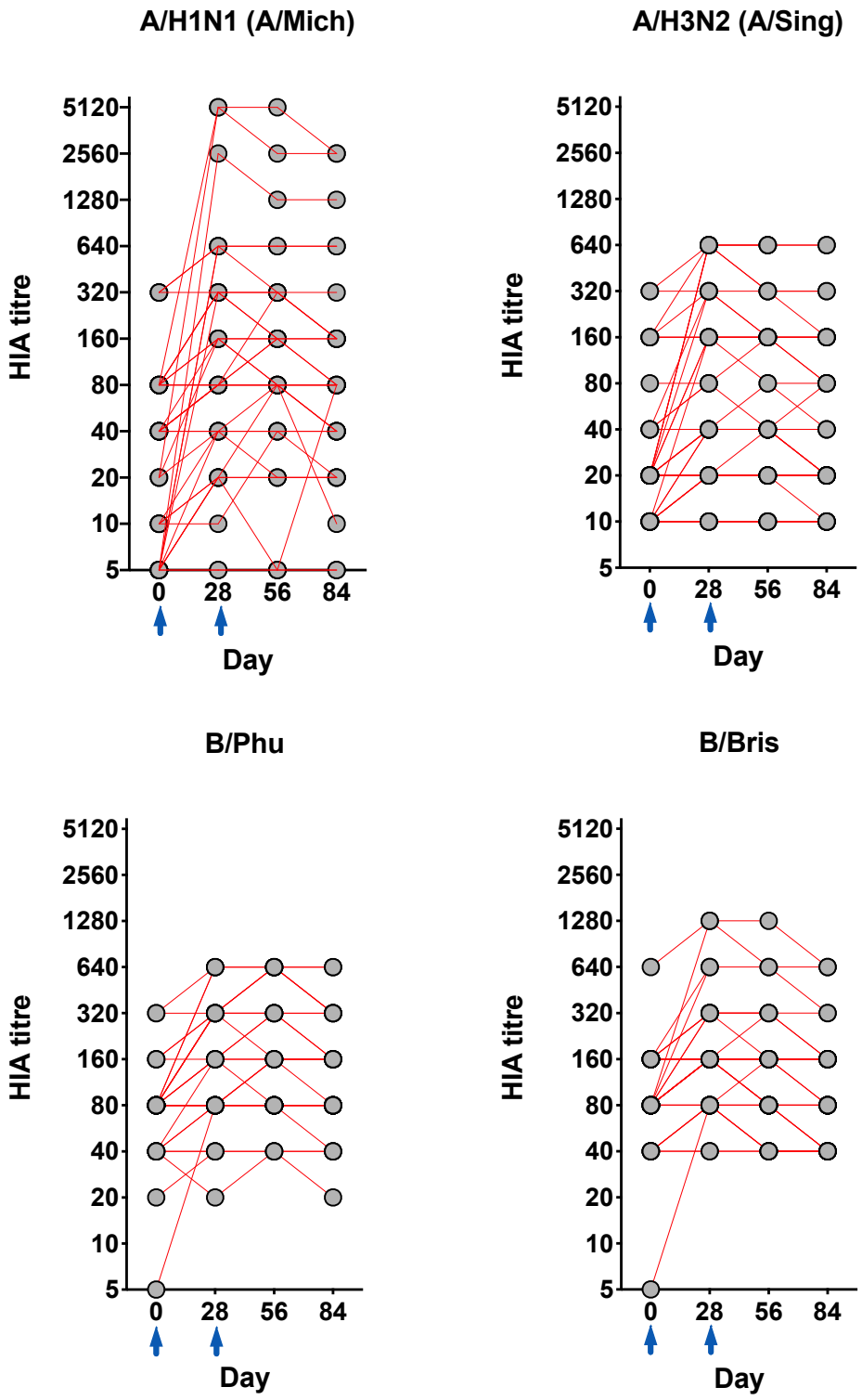


Figure S3. FACS gating strategy. PBMCs were collected and analysed using the outlined gating strategy as per Koutsakos et al.,2018.[1]

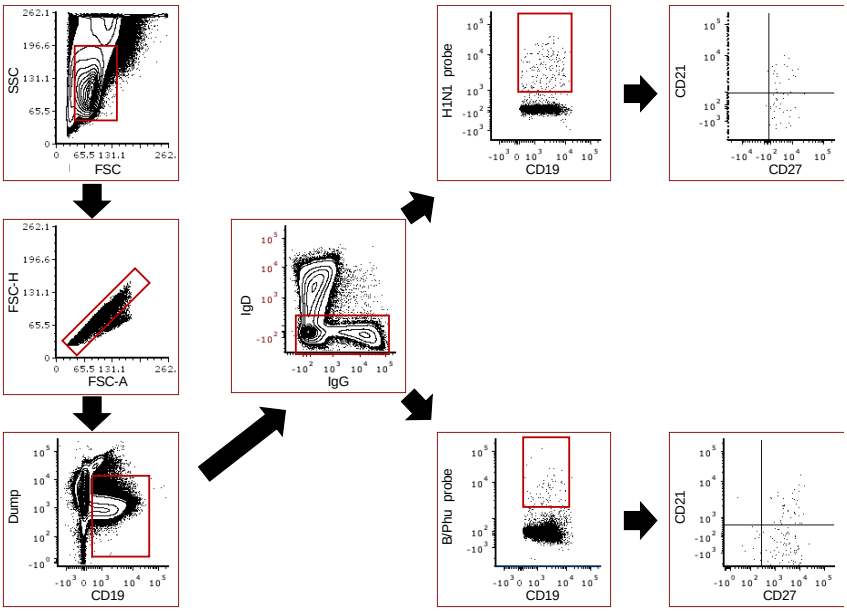


Figure S4. Correlation between strain-specific B cells and antibody titres (D28).

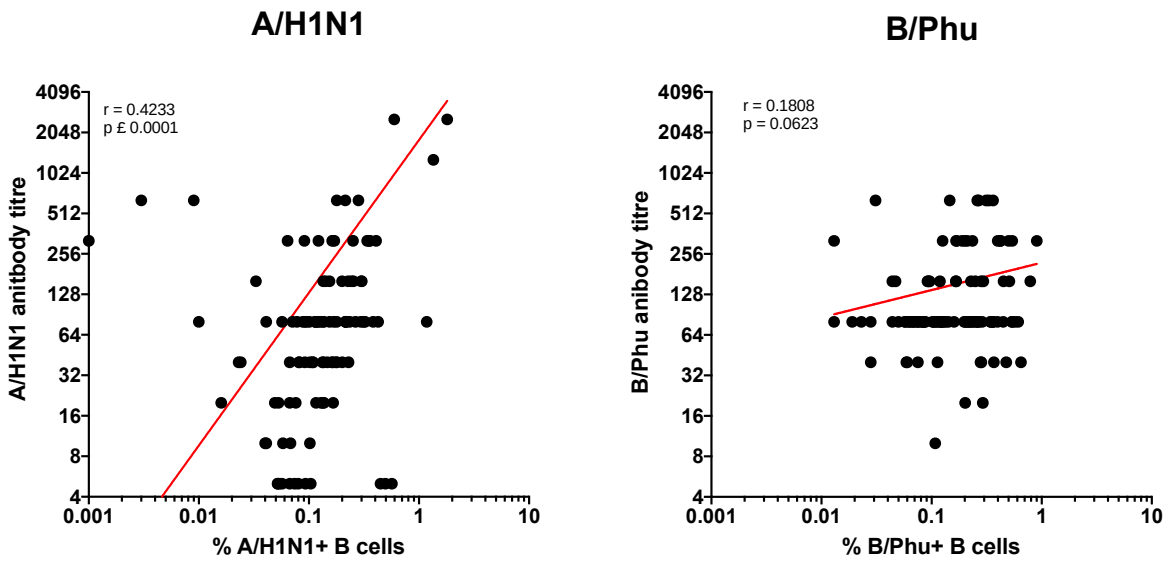
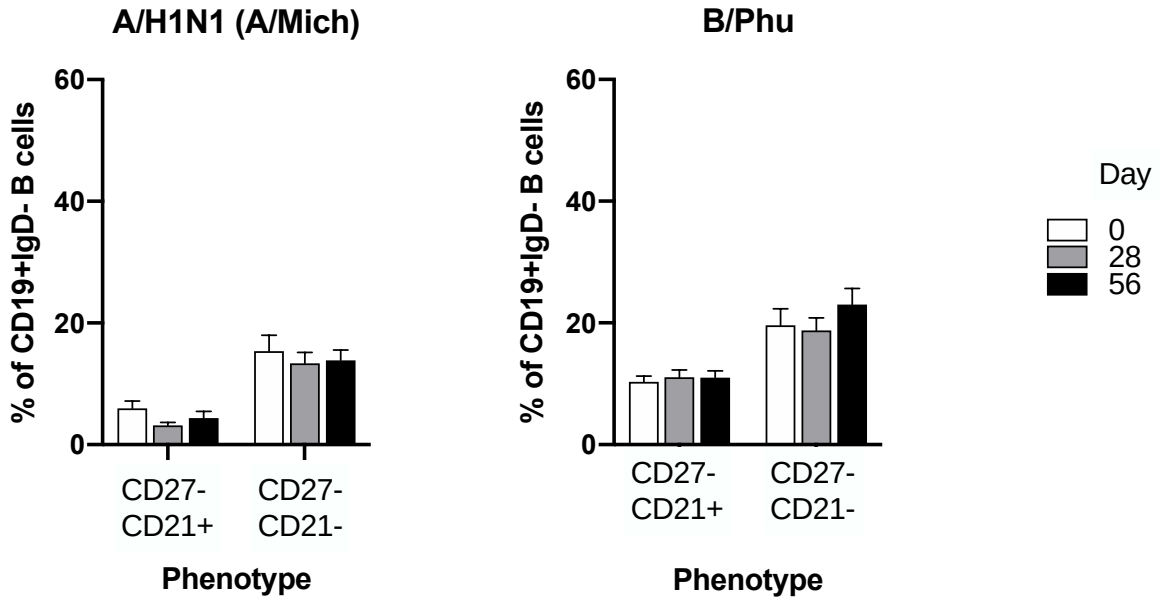


Figure S5. Percentage of naïve ($CD27^-/CD21^+$ and $CD27^-/CD21^-$) cells within the CD19+IgD- strain-specific B cell population.



Supplementary Table S1: Antibodies used in FACS analysis

Antibody	Cat #	Fluorochrome	Dilution	Vendor
Free SA	563261	BV510	1:600	BD
CD20	560631	AF700	1:150	BD
BPHU rHA	X	BV421		
Live Dead	L34957	AQUA	1:500	ThermoFisher
CD3	317331	BV510	1:600	Biolegend
CD8	301047	BV510	1:1500	Biolegend
CD10	312219	BV510	1:750	Biolegend
CD14	301841	BV510	1:300	Biolegend
CD16	302047	BV510	1:500	Biolegend
CD27	302829	BV605	1:150	Biolegend
IgG	564230	BV786	1:75	BD
H1 rHA	X	PE		
CD19	IM2708U	ECD	1:150	Beckman
IgD	561314	PE-Cy7	1:500	BD
IgM	563903	BUV395	1:150	BD
CD21	564595	BUV737	1:300	BD

Table S2: Percentage of patients seroconverting (seroconversion = four-fold change to baseline)

	Strain			
Day	**A/H1N1**	**A/H3N2**	**B/Phu**	**B/Bris**
28	36.4	24.2	18.2	9.1
56	39.4	30.3	18.2	12.2
84	27.3	21.2	15.2	6.0