Supplementary Information

Pre-existing influenza-specific CD4$^+$ T cells correlate with disease protection against influenza challenge in humans

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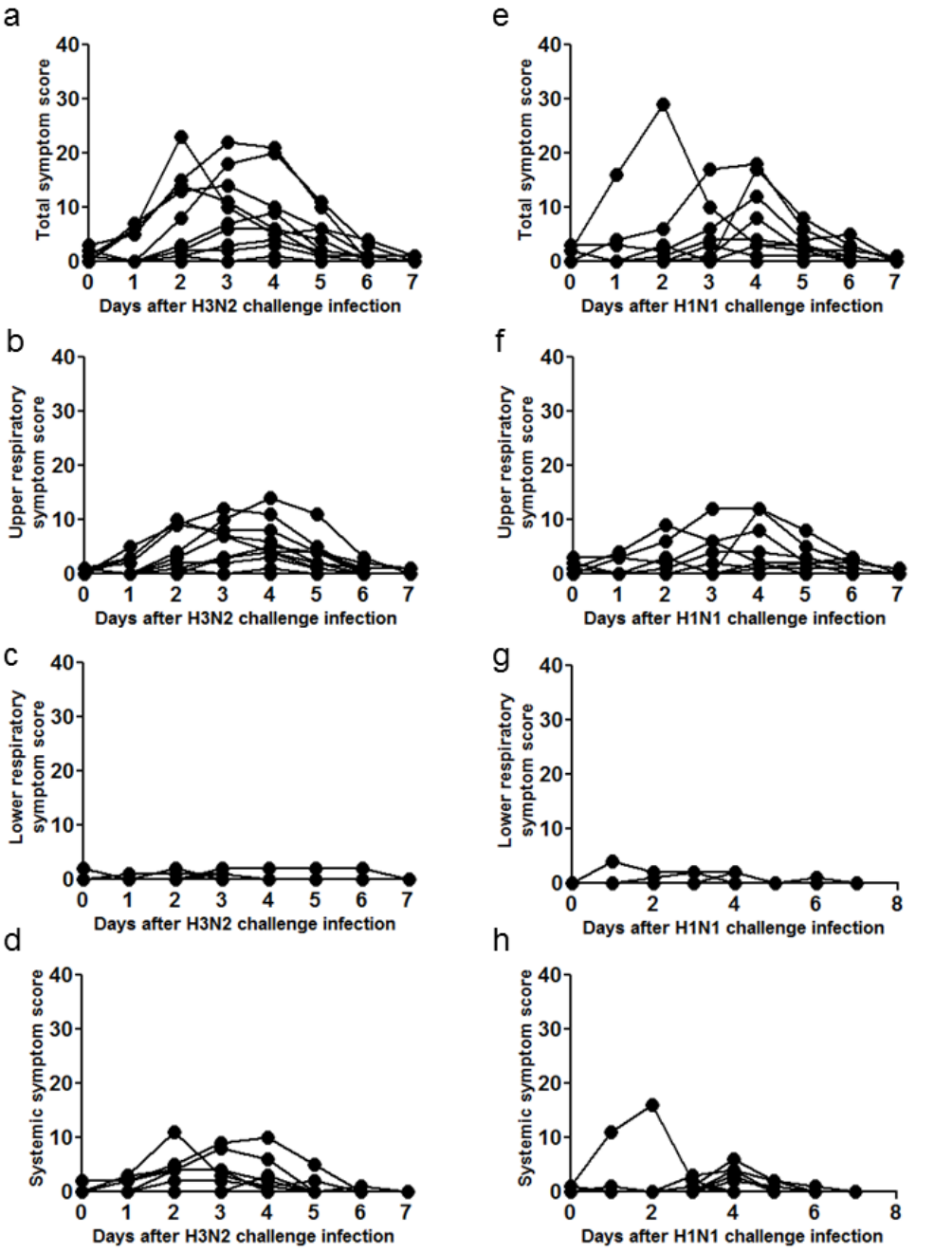
Supplementary Figures

Supplementary Figure 1



Supplementary Figure 1 Symptom scores in each infected volunteers infected with influenza A. (**a**) Total symptom scores in volunteers infected with H3N2 (WS/67/05). (**b**) Upper symptom scores in volunteers infected with H3N2 (WS/67/05). (**c**) Lower symptom scores in volunteers infected with H3N2 (WS/67/05). (**d**) Systemic symptom scores in

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volunteers infected with H3N2 (WS/67/05). (**e**) Total symptom scores in volunteers infected with H1N1 (BR/59/07) virus. (**f**) Upper symptom scores in volunteers infected with H1N1 (BR/59/07) virus. (**g**) Lower symptom scores in volunteers infected with H1N1 (BR/59/07) virus. (**h**) Systemic symptom scores in volunteers infected with H1N1 (BR/59/07) virus.

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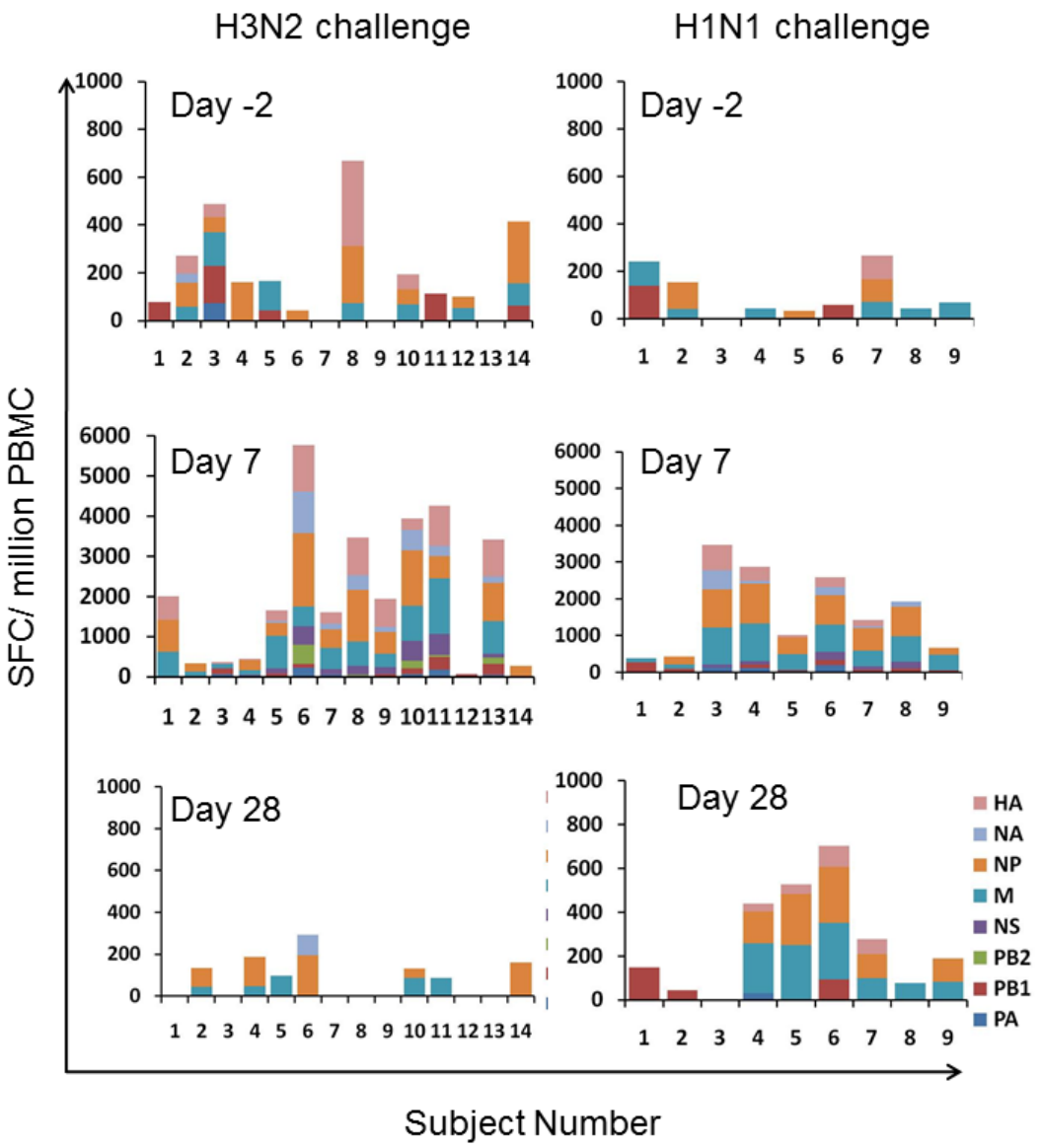
Supplementary Figure 2



Supplementary Figure 2 T cell responses in seronegative healthy volunteers experimentally infected with influenza A virus. Flu-specific T lymphocyte responses were measured from freshly isolated PBMC *ex vivo* from each volunteer by IFN-γ release after stimulation with corresponding peptide pools spanning the entire challenge influenza proteome. Each bar represented the proportion of total T cell responses (per million PBMC) to the entire influenza proteome and each colour box represented the response to each protein. X axes denote subject number.

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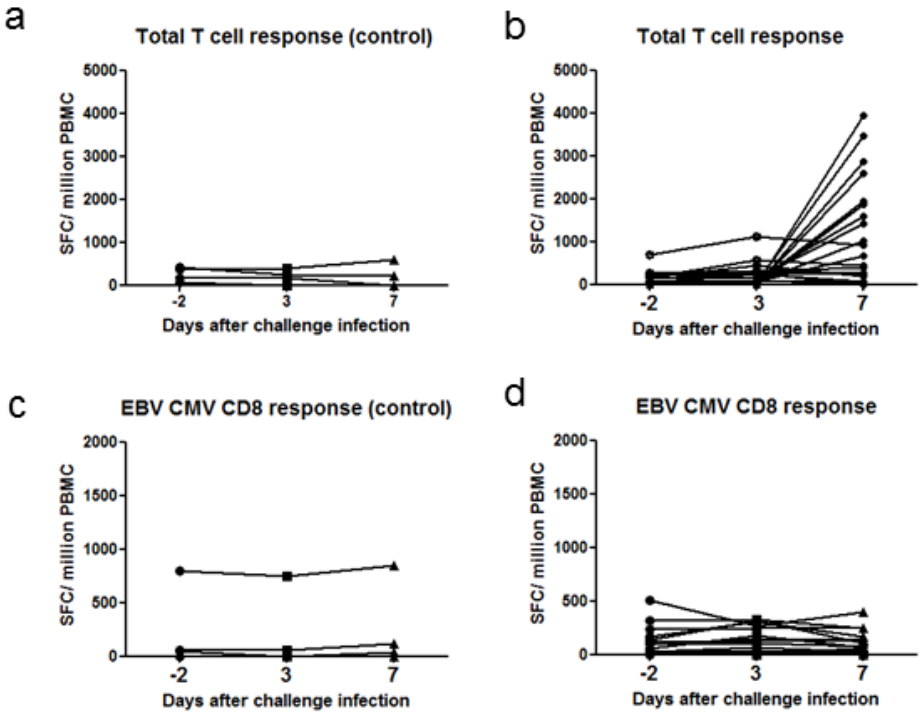
Supplementary Figure 3



Supplementary Figure 3 T cell responses to *ex vivo* stimulation in H1N1 cohort (**a**) Control (negative) in uninfected subjects. (**b**) Influenza peptide stimulation demonstrating expansion of responders by day 7. (**c**) CD8 response to CMV and EBV peptide stimulation in uninfected subjects. (**d**) CD8 responses to CMV and EBV peptide stimulation in uninfected subjects. The lack of expanded response to CMV and EBV during acute influenza infection demonstrates that the T cell response is pathogen specific and bystander activation is minimal in PBMC derived cells.

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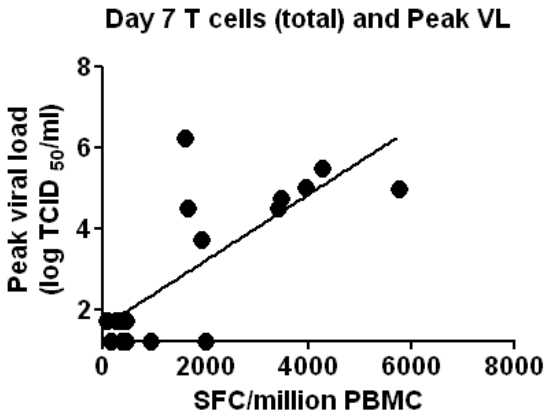
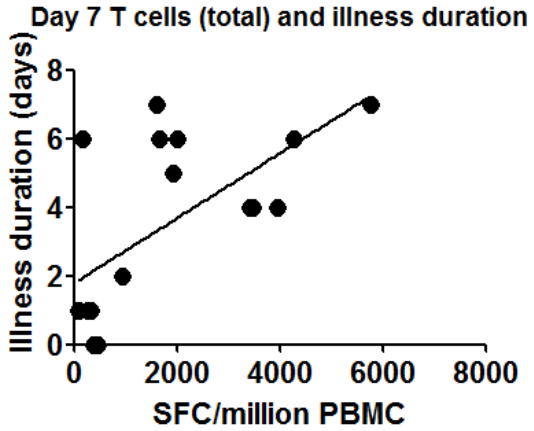
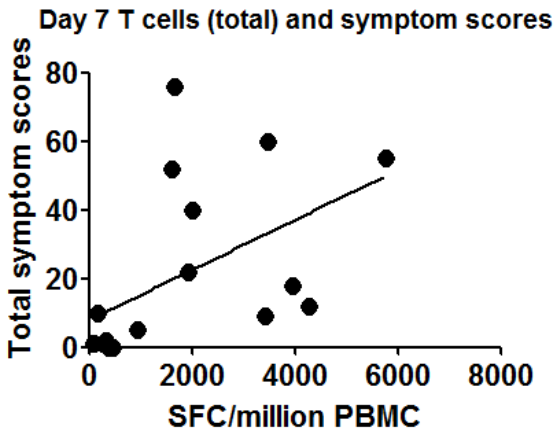
Supplementary Figure 4



r^2=0.5898,
p=0.0003



r^2=0.3932,
p=0.0071



r^2=0.2546,
p=0.0389

Supplementary Figure 4 Relationships in H3N2 cohort between viral shedding, total symptom score and illness duration against total T cells responsive to influenza proteins at Day 7.

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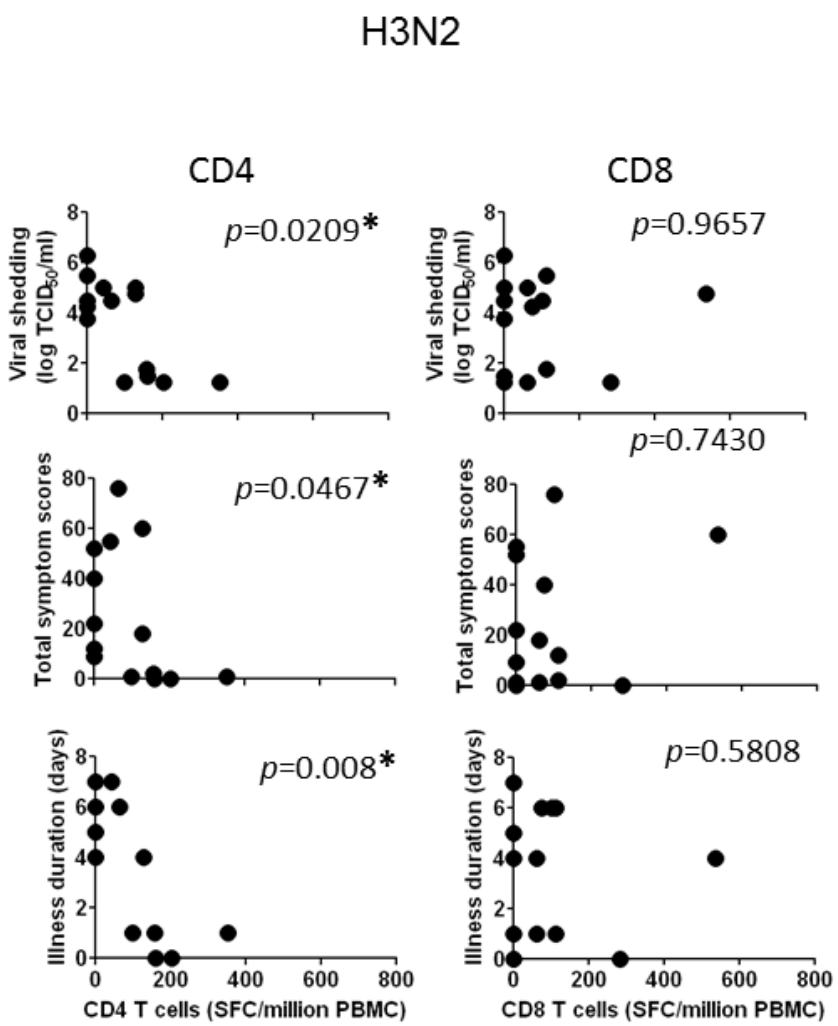
Supplementary Figure 5a



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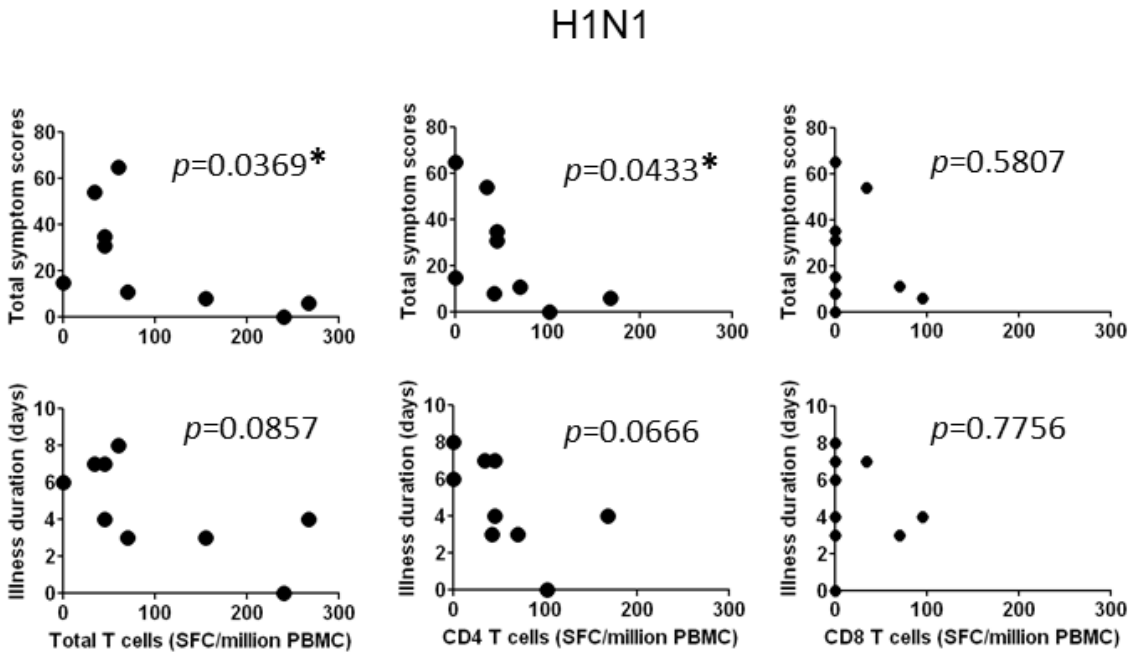
Supplementary Figure 5b



Supplementary Figure 5 (**a**) Relationships in H3N2 cohort between viral shedding, total symptom score and illness duration against pre-existing $CD4^+$ and $CD8^+$ T cell responsive to immunodominant proteins (NP+M). (**b**) Relationships in H1N1 cohort between total symptom score and illness duration against pre-existing total T cell and $CD4^+$ and $CD8^+$ T cells responsive to immunodominant proteins (NP+M).

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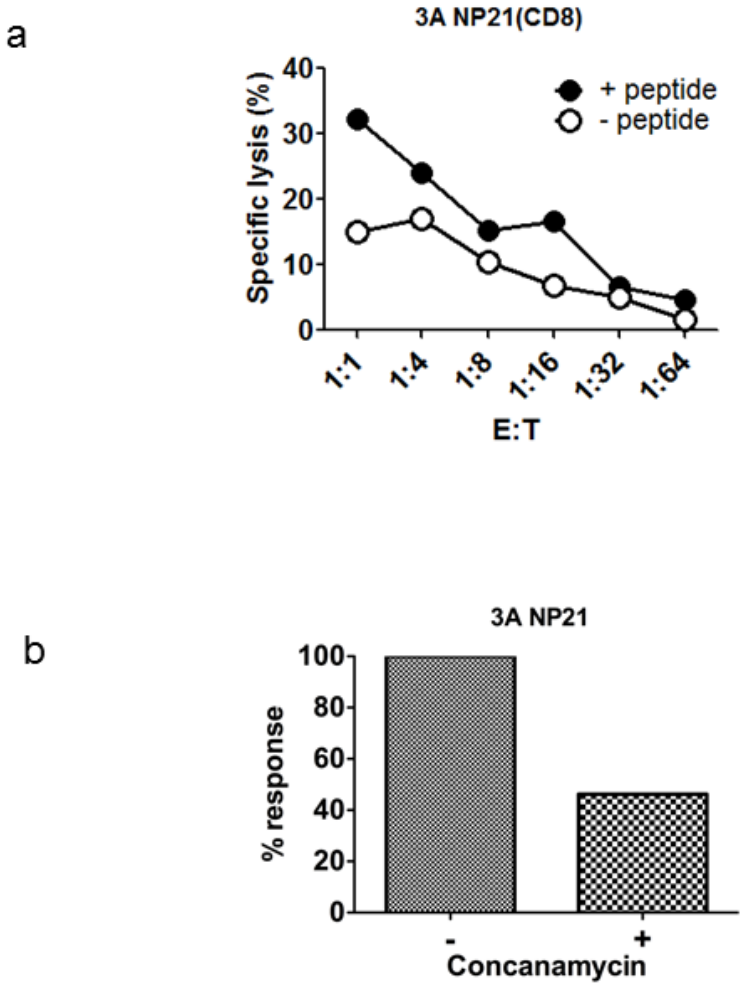
Supplementary Figure 6



Supplementary Figure 6 (**a**) Killing function of $CD8^+$ T cell lines from the same baseline sample upon recognition of autologous NP expressing target cells line was measured by chromium release assay. (**b**) Perforin-dependent cytotoxicity was confirmed by sensitivity to concanamycin.

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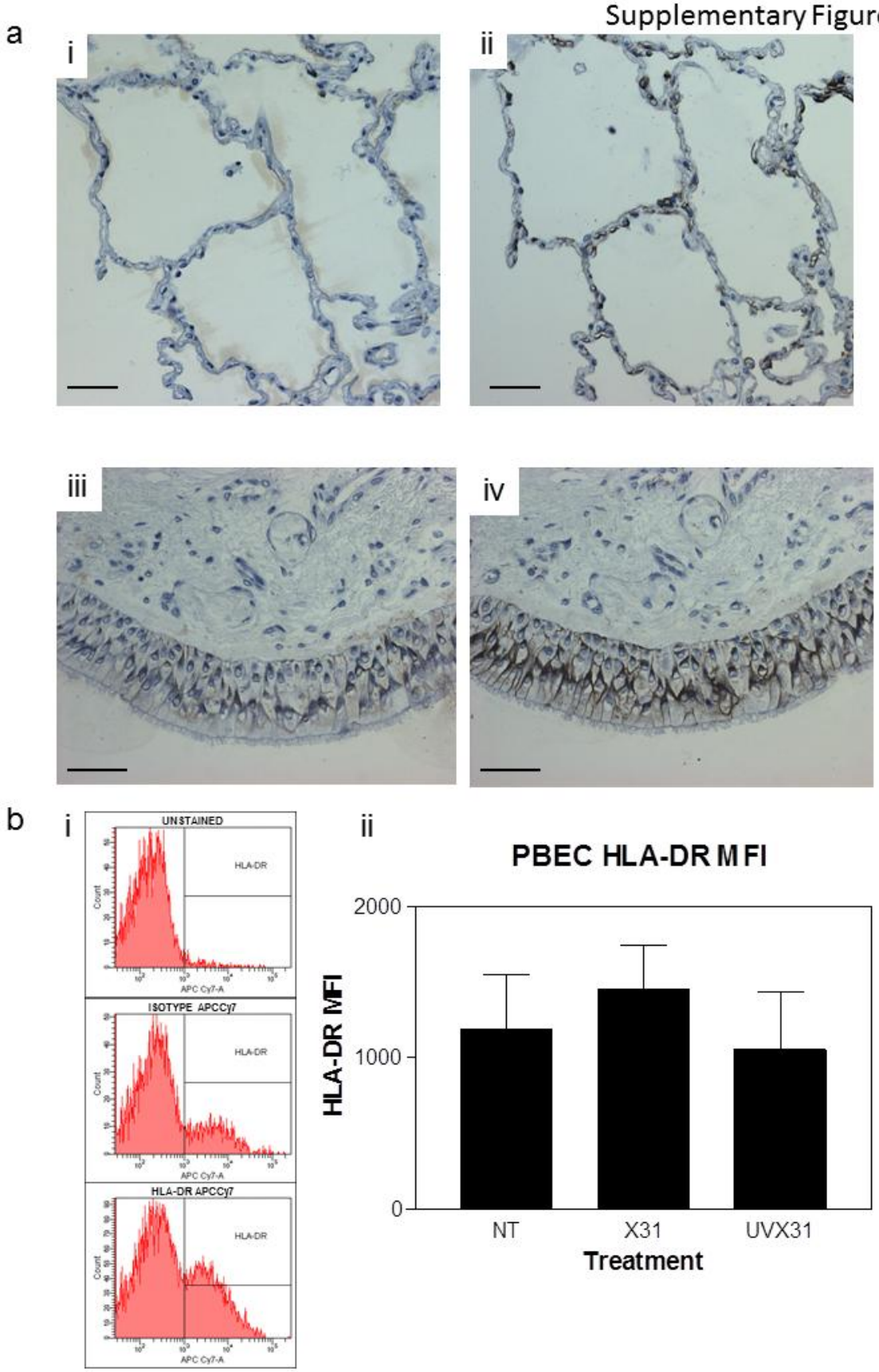
Supplementary Figure 7



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Supplementary Figure 7 (**a**) Human parenchymal (i) and (ii) and bronchial tissue stained (iii and iv) for MHC II (HLA-DR) 2mm sequentially cut sections and immunostained using isotype control monoclonal antibodies (i) and (iii) or antibodies specific for HLA-DR (ii) and (iv) at the same concentration. Signal was amplified using the ABC system, and colour developed using DAB stain. Specific staining is shown in brown, haematoxylin counterstain is shown in blue. Size bar represents 50 μm. (**b**) (i) Representative histograms showing specific staining of HLA-DR expression on primary bronchial epithelial cells (PBECs) by flow cytometry using cells incubated in the presence or absence of HLA-DR APCCy7 or IgG2a APCCy7 (isotype). (ii) Graph of mean fluorescence intensity of HLA-DR expression on PBECs using flow cytometry. NT – non treated control, X31 influenza infected cells, UVX31-UV inactivated viral control. HLA-DR is constitutively expressed on primary respiratory epithelial cells, there is a small rise in expression following *in vitro* infection of these cells with influenza virus which was significant in comparison to stimulation with UV-treated (inactivated) virus. This confirms that respiratory epithelial cells are potential target cells for cytotoxic $CD4^+$ T cells.

H3N2 Challenge Study (A/Wisconsin/67/05) T cell Elispot layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	HA			NA			NP			Matrix		NS
	Pool 1	Pool 2	Pool 3	Pool 4	Pool 5	Pool 6	Pool 7	Pool 8	Pool 9	Pool 10	Pool 11	Pool 12
B	NS	PB2					PB1					PA
	Pool 13	Pool 14	Pool 15	Pool 16	Pool 17	Pool 18	Pool 19	Pool 20	Pool 21	Pool 22	Pool 23	Pool 24
C	PA			Second dimension								
	Pool 25	Pool 26	Pool 27	Pool 28	Pool 29	Pool 30	Pool 31	Pool 32	Pool 33	Pool 34	Pool 35	Pool 36
D	Second dimension											
	Pool 37	Pool 38	Pool 39	Pool 40	Pool 41	Pool 42	Pool 43	Pool 44	Pool 45	Pool 46	Pool 47	Pool 48
E	2nd				No peptide control x 4				Flu CD8	EBV + CMV (CD8)		PHA
	Pool 49	Pool 50	Pool 51	Pool 52								

H1N1 Challenge Study (A/Brisbane/59/07) T cell Elispot layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	HA			NA			NP			Matrix		NS
	Pool 1	Pool 2	Pool 3	Pool 4	Pool 5	Pool 6	Pool 7	Pool 8	Pool 9	Pool 10	Pool 11	Pool 12
B	NS	PB2				PB1				PA		
	Pool 13	Pool 14	Pool 15	Pool 16	Pool 17	Pool 18	Pool 19	Pool 20	Pool 21	Pool 22	Pool 23	Pool 24
C	PA	PB1-F2	Second dimension									
	Pool 25	Pool 26	Pool 27	Pool 28	Pool 29	Pool 30	Pool 31	Pool 32	Pool 33	Pool 34	Pool 35	Pool 36
D	Second dimension											
	Pool 37	Pool 38	Pool 39	Pool 40	Pool 41	Pool 42	Pool 43	Pool 44	Pool 45	Pool 46	Pool 47	Pool 48
E	2nd			No peptide control x 4				Flu CD8		EBV + CMV (CD8)		PHA
	Pool 49	Pool 50	Pool 51									

Supplementary Figure 8 Elispot layout of experimental influenza A infection in humans. (**a**) H3N2 challenge study. (**b**) H1N1 challenge study. In each well, freshly isolated 300,000 PBMC were put into each well and stimulated with peptide pool at 2 µg/ml for 18-24 h.

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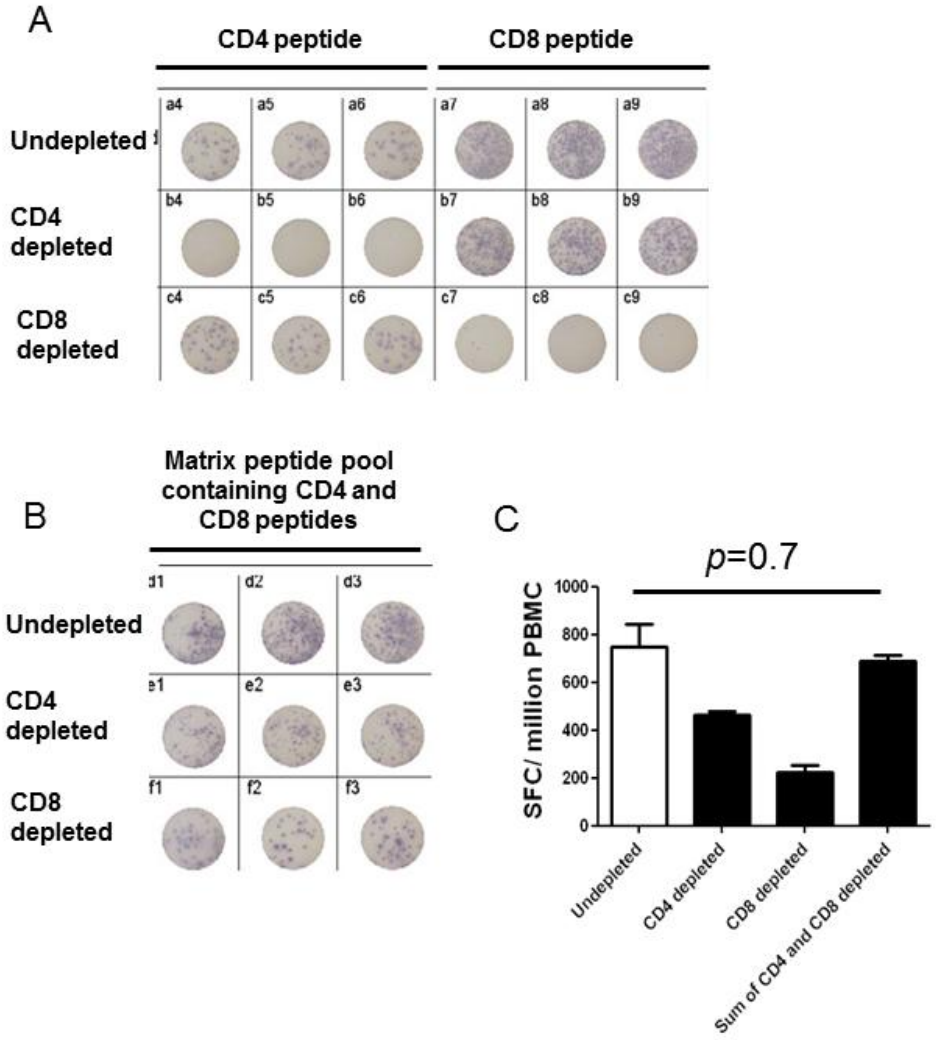
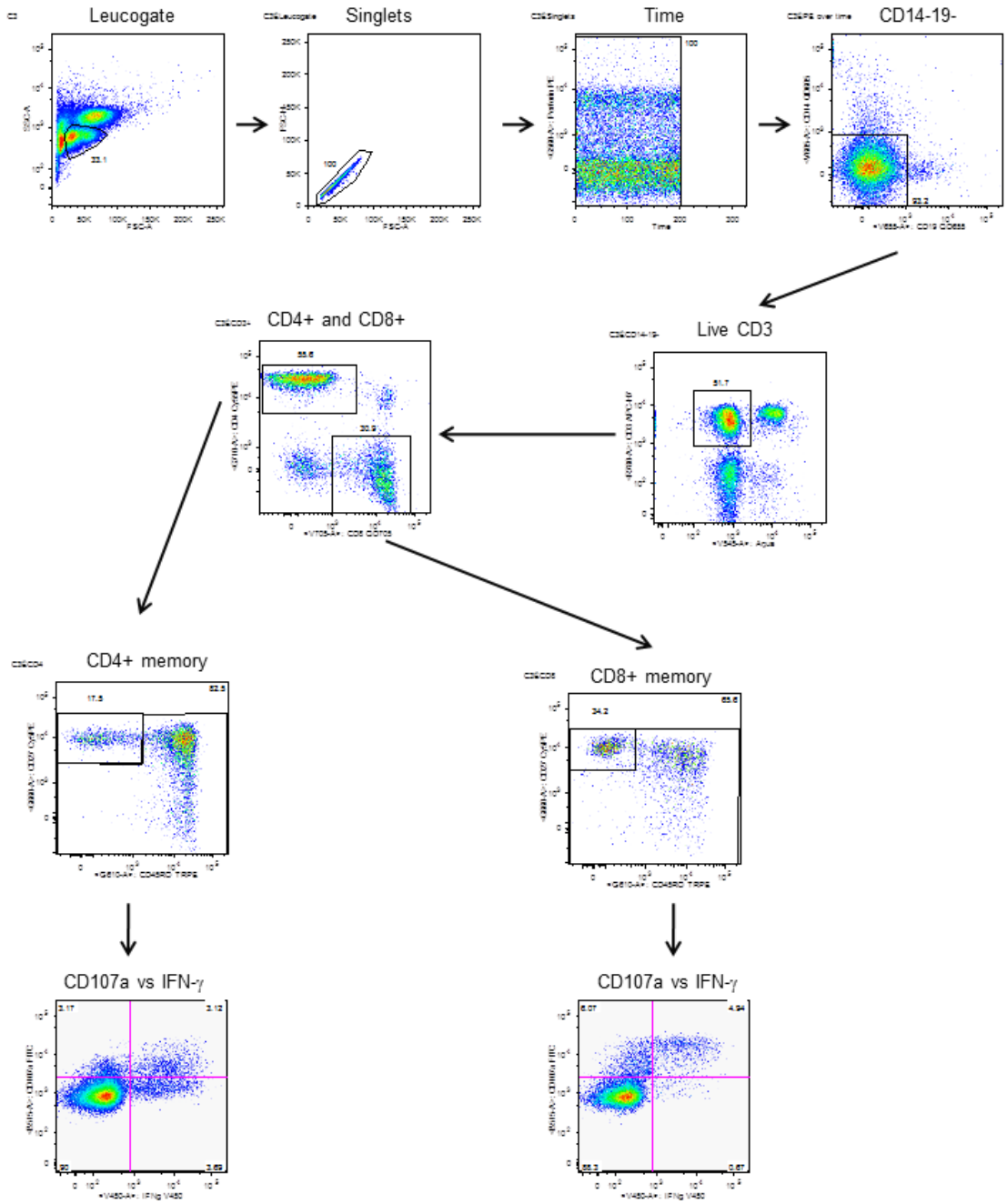
Supplementary Figure 9



Supplementary Figure 9. *Ex vivo* IFN-γ Elispot with cell depletion. (**a**) Undepleted PBMC, $CD4^+$ depleted and $CD8^+$ depleted PBMC from the same flu positive subject were stimulated with either flu $CD4^+$ or $CD8^+$ peptides and the response was measured by IFN-γ Elispot. (**b**) Undepleted PBMC, $CD4^+$ depleted and $CD8^+$ depleted PBMC from the same subject were stimulated with flu matrix peptide pools containing a mixure of $CD4^+$ and $CD8^+$ peptides and the response was measured by IFN-γ Elispot . (**c**) IFN-γ Elispot response in undepleted, $CD4^+$ depleted , $CD8^+$ depleted fractions. Comparison of between groups was determined by Mann-Whitney U test.

Supplementary Figure 10



Supplementary Figure 10 The gating strategy from a representative subject: PBMC from a healthy volunteer were stimulated with SEB for 6 hours in the presence of BFA and monensin and then stained for T cell functions (CD107a, IFN-γ) along with lineage (CD14, CD19, CD3, CD4, CD8), memory (CD27, CD45RO)and live/dead (aqua) markers.

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Supplementary Tables

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Supplementary Table 1 Demography, virus shedding and antibody titre of the study groups

	H3N2 challenge group				H1N1 challenge group			
	Group 1	Group 2	Group 3	Group 4	Group 1	Group 2	Group 3	Group 4
N	4	4	4	5	6	6	6	6
Age-yr								
Mean±SD	26±3	28±5	25±2	29±8	25±5	25±5	27±4	23±3
Median	26	27	25	28	24	24	26	22
Range	23-29	25-35	22-27	22-41	20-32	22-35	23-31	19-27
Sex-no.(%)								
Female	2(50)	2(50)	2(50)	2(40)	2(33)	1(17)	2(33)	2(33)
Male	2(50)	2(50)	2(50)	3(60)	4(67)	5(83)	4(67)	4(67)
Virus shedding no. (%)								
	1(25)	4(100)	2(50)	2(40)	1(17)	3(50)	1(17)	1(17)
HAI titre on Day 28								
Positive	3(75)	2(50)*	1(25)**	1 (25)	2 (33)	2 (33)	2 (33)	1(17)
GMT	96	33	0.6	0	1	5	0	0
Mean symptom scores								
Mean±SD	10.5±19.7	60.8±10.7	13.8±14	4.6±5.5	11.2±7.7	39±23.2	14.5±14.1	8.3±15.2
Median	1	57.5	7.7	1	11.5	44.5	14.5	0.5
Range	0-40	52-76	5-22	0-9	0-22	3-65	0-31	0-38

* One subject was unavailable for D28 visit. **Two subjects were unavailable for D28 visit.

We ran correlation tests (Spearman rank correlation test, Prism 5) to see if the magnitude of influenza-specific CD4+ or CD8+ cells were correlated to virus shedding and disease severity as indicated by total symptom scores and length of illness duration in both H3N2 and H1N1 challenge studies. The results clearly showed that the magnitude of the CD4 response against immunodominant nucleoprotein (NP) and matrix (M) proteins was inversely correlated to peak virus shedding, symptom scores and illness durations (Table 1). Moreover, correlation was independent of the size of the influenza-specific CD8 response. Similarly, in a separate H1N1 challenge study, total symptom scores were inversely correlated to the magnitude of the flu-specific CD4 response against NP and M and were independent of flu-specific CD8 responses

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Supplementary Table 2

Supplementary Table 2a. Correlation of pre-existing CD4 or CD8 cell responses to immunodominant proteins with control of virus shedding and symptom development in **H3N2** challenge infection.

	Peak viral load ($TCID_{50}$/ml)		Symptom scores		Illness duration (days)	
Protein	Correlation coefficient	P-value	Correlation coefficient	P-value	Correlation coefficient	P-value
Total	-0.3330	0.2447	-0.2257	0.4379	-0.5740	0.0318
NP and M	-0.4972	0.0704	-0.3402	0.2340	-0.6918	0.0061
NP and M (CD4)	-0.6087	0.0209	-0.5390	0.0467	-0.7886	0.0008
NP and M (CD8)	0.0127	0.9657	0.09640	0.7430	-0.1617	0.5808

Supplementary Table 2b. Correlation of pre-existing CD4 or CD8 cell responses to influenza proteins with control of virus shedding and symptom development in **H1N1** challenge infection.

	Symptom scores		Illness duration	
Protein	Correlation coefficient	P-value	Correlation coefficient	P-value
Total	-0.7113	0.0369	-0.6102	0.0857
NP and M	-0.7280	0.0311	-0.6695	0.0589
NP and M (CD4)	-0.6908	0.0433	-0.6468	0.0666
NP and M (CD8)	-0.2079	0.5809	-0.1053	0.7756

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Supplementary Table 3 T cell responses in H1N1 challenge study.

Subjects	Peptide ID	Amino acid position	Amino acid sequence	CD4 or 8 dependency	SFC/million PBMC		
					Days after challenge		
					-2	7	28
B017(2C)	NP22	169-186	GSTLPRRSGAAGAAVKGV	8	28	53	10
B017(2C)	NP52	409-426	QPTFSVQRNLPFDKTTIM	4	26	76	10
B017(2C)	M13	97-114	VKLYRKLKREITFHGAKE	4	52	316	33
B015 (7C)	NP14	105-122	VRELVLYDKEEIRRIWRQ	4	15	154	17
B015 (7C)	M14	105-122	REITFHGAKEIALSYSAG	4	10	221	17
B021 (8C)	M14	105-122	REITFHGAKEIALSYSAG	4	34	166	10
B006 (14C)	M12	89-106	DPNNMDRAVKLYRKLKRE	4	30	681	0
B005 (15C)	NP27	209-226	GENGRKTRIAYERMCNIL	8	30	157	10
B005 (15C)	NP28	217-234	IAYERMCNILKGKFQTAA	8	40	67	10
B005 (15C)	M12	89-106	PKKTGGPIYKRVDGKWVR	4	46	80	10
B005 (15C)	M13	97-114	YKRVDGKWVRELVLYDKE	4	30	70	10
B004(19C)	M8	57-74	KGILGFVFTLTVPSERGL	4	15	49	30
B004(19C)	M27	209-226	ARQMVQAMRAIGTHPSSS	4	46	69	20
B009 (20C)	NP09	65-82	RMVLSAFDERRNKYLEEH	4	38	187	113
B009 (20C)	M27	209-226	GENGRKTRIAYERMCNIL	4	28	97	30

Published epitopes were underlined.

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SUPPLEMENTARY METHODS

Study design

Between October 2008 and October 2009, two separate prospective, randomised, and double blinded, parallel group clinical studies of experimental human influenza A infections were undertaken in a single site in Cambridge, UK. The two studies were carried out 9 months apart. The H3N2 challenge study was carried out between 24th Oct and 24th Nov 2008 whereas H1N1 challenge study was carried out between 18th August and 18th September 2009. Healthy, non-pregnant adults between the ages 18 and 45 were eligible for the enrolment. Exclusion criteria included health care workers, history of acute respiratory illness, chronic illness or medications. In H3N2 challenge study, a total of 17 healthy adult volunteers, which are haemagglutination-inhibition (HI) titres less than 1:8 to influenza A/Wisconsin/67/05, were enrolled in the study. Whereas, in H1N1 challenge study, a total of 24 healthy adult volunteers with HI titres less than 1:8 to influenza A/Brisbane/59/07 were enrolled in the study.

Both studies were conducted in compliance with Good Clinical Practice guidelines (CPMP/ICH/135/95) and the Declaration of Helsinki. Additional samples for the immunohistochemistry analysis of human lung biopsies and flow cytometric analysis of cultured respiratory epithelial cells were collected as part of an established protocol at Southampton University Hospitals NHS Trust.

All protocols were approved by East London and City and the Southampton and Southwest Hampshire ethics review committees. Written informed consent was obtained from each participant with an ethics committee approved form. No medications, except acetaminophen for treatment of severe symptoms, were permitted. Subjects were compensated for their participation of the study.

Study outline

Screening assessments began within 45 days of the scheduled viral inoculation. Volunteers were confined to individual rooms in an isolation unit 2 d before the day of inoculation, and remained in isolation for 7 days thereafter. They were randomised into 4 groups and each group of the participants were inoculated intra-nasally with different doses of influenza A virus on day 0. The dose of the virus was designated as 1:10 (high), 1:100 (medium-high), 1:1000 (medium-low) and 1:10,000 (low) from the original virus stock. Group 1 received high dose, Group 2 received medium-high dose, Group 3 received medium-low and Group 4 received low dose of virus. Nasopharyngeal swab were collected daily from baseline day 0 during the quarantine period for virus isolation. Serum samples were taken daily for serum cytokine and biomarker study. Fresh whole blood for cellular assays was taken on day -2 or 0, 7 and day 28. An additional time point day 3 was taken for H1N1 study.

Oral temperatures were measured four times daily. Fever was defined as an oral temperature >37.7°C. Symptom assessments were performed by the volunteers twice daily on a four-point scale (0-3 corresponding to absent to severe) (Hayden et al., 1998). The symptoms assessed were nasal stuffiness, runny nose, sore throat, cough, sneezing, earache/pressure, breathing difficulty, muscle aches, fatigue, headache, feverish feeling, hoarseness, chest discomfort, and overall discomfort. The total symptom score for each day was obtained by adding the individual symptoms scores for that particular day including morning and evening sessions. The individual symptoms contributing to the total symptoms scores were divided into three subgroups: systemic symptoms (muscle aches, fatigue, headache, and fever), upper respiratory symptoms (nasal stiffness, ear ache/pressure. runny

nose, sore throat, and sneezing) and lower respiratory symptoms (cough, breathing difficulty, hoarseness and chest discomfort).

Viruses

In both challenge studies, GMP grade viruses were manufactured and processed by GlaxoSmithKline, UK. The stock virus were diluted to four different inoculum titres and prepared in individual aliquots intended for single use and then administered. The titre of the stock virus was 10^7 $TCID_{50}$ infectious dose. They were ten-fold diluted and the titre were ranged from high titre (1:10), medium-high (1:100), 3 medium-low titre (1:1,000) and low titre (1:10,000). Subjects were observed for potential allergic reactions for 30 min following inoculation. In H3N2 challenge study, tissue culture grown A/Wisconsin/67/05 virus was used. In H1N1 challenge study, egg grown A/Brisbane/59/2007 virus was used.

Virus titration by $TCID_{50}$ (Tissue Culture Infectious Dose 50%) assay

Viral load in the nasopharyngeal samples were determined by $TCID_{50}$ assay as described by the WHO manual of Animal Influenza Diagnosis and Surveillance 2002[2]. Serial ten-fold dilutions of virus-containing samples were inoculated into 96-well microtitre plates seeded with Madin-Darby canine kidney (MDCK) cells, and incubated for 5–6 days at 37°C. Cytopathic effects in individual wells were determined via light microscopy. A titre greater than 1:5 was considered positive.

Hemagglutination Inhibition (HI) Assay

Haemagglutinin-specific antibody titers against H1N1 (A/Brisbane/59/2007) or H3N2 (A/Wisconsin/67/05) in the serum samples were determined by HI assay using chicken erythrocytes as described in the WHO manual[2] .

Synthetic peptides

18-mer peptides overlapping by 10 amino acid residues and spanning the full proteome of the H1N1 and H3N2 influenza A viruses were designed using the Los Alamos National Library web-based software PeptGen (http://www.hiv.lanl.gov/content/sequence/PEPTGEN/peptgen.html) and synthesized (purity >70%; PEPscreen; Sigma-Aldrich) using the sequences of the following strains: A/Brisbane/59/2004 (H1N1), A/New York 388/2005 (H3N2) (surface proteins), and A/New York 232/2004 (H3N2) (internal proteins). In H3N2 peptides, the amino acid sequence homology between challenge Wisconsin strain and New York strain was greater than 99%. The total numbers of peptide used in detecting antigen-specific responses for H1N1 and H3N2 were 554 and 601 respectively. In each influenza ELISPOT assay, all overlapping peptides in each individual were simultaneously tested using 2-dimensional matrices with a total of 50 pools (1^{st} D = 25 pools; 2^{nd} D = 25 pools; up to 25 peptides/pool) so that each peptide was present in two different pools (see Supplemental Figure 8 for ELISPOT layout). Peptides were used at a final concentration of 2 μg/ml each. The putative peptide from each positive response well could be deconvoluted from a 2-dimensional matrix system where each peptide only appeared once in each dimension. The putative peptides were then confirmed individually in the second ELISPOT assay with the same input cell number per well.

Ex vivo IFN-γ ELISPOT

Peripheral mononuclear cells (PBMC) were separated from 50 ml heparinised blood by density gradient centrifugation using Lymphoprep (Axis-Shield, Norway) and Leucosep tube (Greiner, UK) within 3-6 h of each bleed (Li et al., 2008). To detect influenza-specific effector memory cells (CD45RO+), PBMC were added into 96-well ELISPOT Multiscreen plates (MAIPS4510, Millipore) at 300,000 cells/well and cultured with peptide pools for 18-

24h incubation at 37°C and 5% CO_2. The end concentration of each peptide in each well was 2 μg/ml, for both peptide pools and individual peptides. All ELISPOT assays were performed using the human IFN-γ ELISPOT kit (Mabtech) according to the manufacturer's instructions. The internal negative control was no peptide in quadriplicate, and positive controls were EC (a mixture of EBV and CMV T cell epitope peptides) or PHA (10 μg/ml). The spots in each well were counted using an automated ELISPOT reader and AID ELISPOT 3.1.1 HR software (Autoimmune Diagnostika). In pool responses, wells containing spot numbers greater than the mean + 4 SD of three negative control wells (no peptide) were regarded as positives in each individual, provided that the total was greater than 50 spot forming cells (SFC)/million PBMC, to rule out false positives where background was very low. In all assays, background values of no peptide control wells were determined at 1.8 ± 4.6 SFC/million PBMC from 150 healthy subjects and 2 ± 5.7 SFC/million PBMC from 150 influenza-exposed subjects. Values of T cell responses were all background subtracted and presented as SFC/million PBMC. To determine whether T cells were CD4 or CD8, in the second ELISPOT assay, cell depletion was conducted using Dynal CD8 beads, as described in the manufacturer's instructions (Invitrogen, UK), before the ELISPOT assay. Undepleted PBMC served as positive controls. The assay was validated by comparing the response between undepleted PBMC control and single CD4 or CD8 peptide control and peptide pool control. The response of cell depletion by magnetic bead sorting was complete as described by manufacturer's instruction (FACS data not shown). The sum of response from CD4 depleted and CD8 depleted fraction was comparable to undepleted PBMC control. For single peptide confirmation ELISPOT assay, responses greater than 10 SFC/million PBMC was considered positive after background subtraction.

Generation of short-term T cell lines

Short-term T cell lines were generated to confirm influenza peptides and the $CD4^+$ or $CD8^+$ property of each peptide by ICS and flow cytometry, as described previously[35]. In brief, frozen samples of PBMC were thawed and rested for 2h before stimulating with 10 µg/ml of each peptide at final concentration for 1 h. Cells were cultured in RPMI 1640 supplemented with 10% human serum (National Blood Services, UK) and 25 ng/ml IL-7 (PeproTech) for 3 d, and then 100 U of IL-2/ml (Proleukin, Novartis UK) was added every 3 to 4 d thereafter. On day 14, cells were washed three times with sterile PBS and then rested in fresh RAB-10 for 25 to 35 h at 37°C, 5% CO_2.

FACS staining assay

Activated (CD38+) and proliferating (Ki67+) cells in freshly isolated PBMC were stained by were stained with mAbs against human Ki67-FITC (Clone B56, BD Biosciences), DR-PE (clone TU36, BD) CD38-APC (clone HB7, BD), CD4-pacific blue (Clone MT130, DakoCytomation), and CD8-PE-Cy5 (Clone SK1, BD). Cytotoxicity as measured by expression CD107a (clone H4A3, BD) and IFN-γ (clone XMG1.2, BD) in both CD4 and CD8 memory cells were also studied *ex vivo* using frozen PBMC as described previously[2]. PBMC (1 million per stimulation) were stimulated with peptide pools for 6 h in the presence of brefeldin A and monensin. For each stimulation condition, at least 500,000 total events were acquired using LSRII (BD immunocytometry Systems, San Jose, California). Data analysis was performed using FlowJo (version 8.8.4; TreeStar, Ashland, Oregon). Response greater than 3 times background was considered positive. The gating strategy is illustrated in Supplementary figure 9.

Chromium Release Assay

A standard ^{51}Cr release assay was used as described previously[6]. T cell lines generated from PBMC samples were used as effector cells and their autologous EBV-transformed B cell lines were used as target cells. Inhibition of perforin-mediated cytotoxicity was obtained by incubating the CD4+ T cells for 2 h with 100 nM concanamycin (Sigma) before the assay. Specific ^{51}Cr release was calculated from the following equation: ([experimental release-spontaneous release]/[maximum release-spontaneous release])x 100%.

Epithelial Cell MHC Class II Expression

Immunohistochemistry

Lung explants were harvested from lung tissue recovered from patients undergoing routine thoracic surgery under additional consent. Human parenchymal and bronchial tissue was fixed in acetone prior to embedding in GMA resin. Two millimetre sections were cut sequentially and immunostained using isotype control monoclonal antibodies or antibodies specific for MHC II (HLA-DR) at the same concentration.. Signal was amplified using the ABC system, and colour developed using DAB stain. Specific staining is shown in brown, haematoxylin counterstain is shown in blue.

Flow cytometry

Primary bronchial epithelial cells (PBECs) were obtained from subjects undergoing research bronchoscopies in the Wellcome Trust Clinical Research Facility at Southampton General Hospital. Bronchial brushings were cultured in Bronchial Epithelium Growth Media (BEGM), (Lonza, Wokingham, UK) in collagen coated flasks (PureCol™, Inamed Biomaterials, California, USA) and incubated in a humidified atmosphere at 37 °C, 5 % CO_2. The collection and use of these samples was approved by the Southampton and South West Hampshire Research Ethics Committee (REC No: 06/Q1701/98 & 08/H0504/138).

Influenza A virus strain X31 was supplied at a concentration of 4 x 10^7 pfu/ml (a kind gift of 3VBiosciences). Inactivated virus (UVX31) was prepared by exposure to an ultra-violet (UV) light source for 2 h.

PBECs were seeded at 1 x 10^5 cells per well onto a collagen-coated 24 well plate and left at 37 °C, 5 % CO_2 for 24 h. Cells were then growth media starved for 24 h in 0.5ml Bronchial Epithelium Basal Media (BEBM) supplemented with 1 mg/ml BSA, insulin, transferrin and selenium (BEBM+ITS). Cells were incubated for 2 h with no virus, or 2 x 10^3 pfu of X31 or UVX31. Cells were then washed three times with BEBM+ITS and incubated for a further 20 h at 37 °C, 5 % CO_2 in 0.5ml of BEBM-ITS. Cells were dispersed by trypsinisation and prepared for flow cytometric analysis as previously described {Vijayanand, 2007}.

Samples were incubated on ice in the dark for 30 min with Allophycocyanin-Cyanine 7 (APC-Cy7)-conjugated anti-HLA-DR (BD Biosciences, Oxford, UK) or appropriate isotype control (IgG2a BD Biosciences Oxford, UK). After washing, intracellular staining for viral nucleoprotein (NP)-1, was performed using BD Cytofix/Cytoperm kit according to manufacturer's instructions, and AlexFluor 488 (AF488)-conjugated anti-NP-1 antibody (HB-65, a kind gift of 3VBiosciences). Flow cytometric analysis was performed on a FACSAria using FACSDiva software v5.0.3 (all BD).

Statistics

All graphs were presented by GraphPad Prism (version 5) and statistical analysis was done by GraphPad Prism and SPSS. Magnitude of T cells response was presented by SFC/million PBMCV and breadth of T cell response was defined by the number of proteins recognized by each subject. To study the role of T cell in the virus shedding (viral control) and symptom development (immunopathology), correlation was run between pre-existing T

cells and measures of infection and illness (virus titre, symptom assessments, temperature) by Spearman rank correlation analysis. Correlation analysis was based on data collected from all infected (culture positive and/or four fold or greater rise in HI antibody titre) individuals.

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