NATURAL T CELL MEDIATED PROTECTION AGAINST SEASONAL AND PANDEMIC INFLUENZA

RESULTS OF THE FLU WATCH COHORT STUDY

Online Data Supplement

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SUPPLEMENTARY METHODS

The Flu Watch T cell Cohort

We designed Flu Watch as a community level household cohort study to observe the clinical behaviour of seasonal influenza virus infection and to test the hypothesis that T cell immunity can protect against natural influenza virus infection at the population level. The broad approach was to: recruit successive cohorts each year (via random selection of households from general practice registers across England); to take blood prior to the circulation of influenza virus in order to measure baseline antibody and T cell responses; to follow participants up intensively over the influenza season to determine who develops respiratory illness (weekly follow up from late autumn to late spring using automated telephone calls or emails); to collect self-administered nasal swabs, which participants were asked to submit on day two of any respiratory illness and send through the post in viral transport medium; to test nasal swabs by RT-PCR for influenza (A subtypes H1, H3 and from 2009 onwards A(H1N1)pdm09 and influenza B) and a panel of other respiratory viruses including Respiratory Syncytial virus, Rhinovirus, Coronavirus, Adenovirus, Human Metapneumo Virus and Parainfluenza virus, and take a post-season blood sample (May-July) to assess rises in antibody titre indicative of influenza virus infection.

Households with 1-6 members were included. Participants could be of any age. Only households where all members agreed to participate were included. Willingness to provide a baseline and follow up blood sample was a condition of inclusion in adults aged ≥ 16. Additional exclusion criteria included terminal illness, severe mental illness or incapacity and heavy involvement in other on-going research. Blood samples were collected during evening home visits and were voluntary in children

aged <16 and not requested from children under 5 years. Samples were transported by courier to the laboratory in Oxford for processing early the following morning, less than 10-14 hours after collection. This timing was chosen on the basis of preliminary experiments that showed minimal loss of T cell recovery and function in samples stored for 12 hours.

Serology

Each year pre- and post-season serum samples were frozen and batch-tested using hemagglutinin inhibition assays (HIA) for antibodies to strains of influenza that had circulated that season. Serum was separated from cellular components and stored at -20°C. Sera were screened at the HPA Colindale laboratory by haemagglutination inhibition assay using standard methods as described previously.[E1-E3] Serum samples were stored at and analysed in batches by HI using appropriate egg-grown seasonal strains of choice. Strains used for the 2006/07 season were influenza A/New Caledonia/20/99; influenza A/Wisconsin/67/05; influenza B/Malaysia/2506/04 (H1N1, H3N2 and FluB respectively); for the 2007/08 season, influenza A/Solomon Islands/03/06; influenza A/Brisbane 10/07; influenza B/Florida/04/06; for the 2008/09 season influenza A/Brisbane/59/07; influenza A/Brisbane/10/07 and influenza B/Malaysia/2506/04; and finally for the 2009/10 and 2010/11 seasons a reverse-genetics derivative of influenza A/England/195/09 (A(H1N1)pdm09) was used. In 2010/11 A/Perth/16/09 and B/Brisbane/60/08 were also used. Serum specimens were tested in a two-fold serial dilution series with an initial dilution of 1:8 and ending at 1:1,024. Titres were expressed as a reciprocal of the highest serum dilution that fully prevented haemagglutination. Serum specimens with no reactivity in the first dilution (<8; considered negative) were assigned a titre of 4; serum specimens that

showed titres >1,024 were assigned a numerical value of 1,024 for statistical analysis. Four-fold rises in titre in individuals who were not vaccinated between baseline and follow up samples were taken as indicative of infection.

The participants included in the T cell analysis were unvaccinated participants with baseline serology and T cell data and clinical follow up data. Vaccinated participants were excluded as it is not possible to reliably interpret serological titre rises in this group.

Full details of the cohort design have been submitted for publication elsewhere (Fragaszy et al).

Synthetic peptides

We designed 18-amino-acid peptides overlapping by ten amino acid residues. Peptides spanning hemagglutinin, neuraminidase of H3N2 (A/New York 388/2005) and internal proteins of H3N2 (A/New York 232/2004), as well as nucleoprotein and matrix protein of pandemic H1N1 (A/California/04/2009(H1N1) were generated using the Los Alamos National Library web-based software PeptGen (http://www.hiv.lanl.gov/content/sequence/PEPTGEN/peptgen.html). Peptides were pooled together to allow the calculation of T cell response to each influenza antigen and individual peptide concentration was at 2μg ml-1.

Ex vivo interferon-γ enzyme-linked immunospot

Peripheral blood mononuclear cells (PBMC) from heparinized blood (17 IU/ml sodium heparin) were separated by Lymphoprep™ density gradient centrifugation using Leucosep™ tube.[E4,E5] ELISpot assay was performed using the human IFN-γ

ELISpot kit (Mabtech) and 250,000 freshly isolated PBMCs were used per well. Final peptide concentrations were 2μg/ml and 250,000 PBMC per well were tested in triplicate. During the 2009 pandemic we additionally tested nucleoprotein (NP) and matrix (M) peptides matched to the A(H1N1)pdm09 virus (Table S1). Because of good sequence conservation in internal virus proteins (% homology – 86%NP, 87%M) (Table S2), which are the primary targets for both CD8 and CD4 T cell responses,[E6,E7] we used overlapping peptide sets, matched to A/H3N2/New York 232/2004 in the ELISpot assay. Related A(H3N2) viruses were prevalent in 2006/7 and 2008/9 (Table S1) and in the previous decade. Acute infection rapidly boosts T cell responses but these return to near baseline levels within weeks.[E8] Since including patients with high T cell responses due to recent infection could lead to a spurious protective effect we excluded any subject with >800 spot forming units per million (SFU/M) PBMC for any protein pool.

Intracellular cytokine staining assay

To confirm influenza specific T cells detected in ex vivo ELISpot assays and determine whether these T cells were CD4+ or CD8+, T cells were stimulated with peptides (2μg ml^{-1}) then cultured for 13 to 14 days with IL-2 supplement every 3-4 days. Culture expanded T cells were washed and rested for 30 hours then cultured with or without peptides overnight in the presence of anti-CD28 (BD Bioscience, catalogue no. 340975, 1μg ml^{-1}), anti-CD49d (BD Bioscience, catalogue no. 340976,1μg ml^{-1}) and Brefeldin A (Sigma, catalogue no. B-7651, 10μg/ml). Antibodies against human CD3 (conjugated to APC-H7, BD Bioscience, catalogue no. 641397), CD4 (conjugated to PE, DAKO, catalogue no. R0805), CD8 (Conjugated to FITC, DAKO, catalogue no. F0765) and human IFN-γ (conjugated to

APC, BD Bioscience, catalogue no. 341117) were used to distinguish IFN-γ producing CD4+ or CD8+ influenza specific T cells. Live/Dead cell staining solution (invitrogen, catalogue no. L34955) was used to exclude dead cells. A dump channel composed of Pacific Blue or eFluor® 450 conjugated antibodies against human CD56 (Cambridge Bioscience, catalogue no. 304629), CD14 (e-Bioscience, catalogue no. 48-0149-42) and CD19 (e-Bioscience, catalogue no. 48-0199-42) was used to exclude Non-T cell lineage. Influenza specific IFN-γ T cell responses > two times of the no peptide control were included in the analysis.

Analysis of T cell data

If either PHA responses was >200 SFU/M or FEC responses was > 40 SFU/M, then the assay passed the positive control criteria. If a test well (influenza peptide response) presented >200 spots/well (800 SFU/M), we could not count accurately, then we excluded those from the analysis; they may also have been infected at the time of bleeding.

Only those assays that passed these biological pass/fail criteria were included in independent statistical analysis. The quality of the assays was independently assessed by examining the distribution of spot counts in negative control wells and the consistency of results in replicate test wells. For negative control wells the values were consistently zero or very low (median 1, IQR 0-3, 1^{st}-99^{th} percentile 0-20, maximum 77.5). The degree of concordance between replicate wells was assessed using Lin's correlation coefficient (Table S3). There was a very high degree of correlation between replicate test wells (for NP and M Lins correlation coefficient was > 0.90, $p < 0.001$) allowing data from replicate wells to be pooled (Table S3). We defined positive responses to individual proteins as those where the counts from the

pooled replicate wells had significantly more SFU/M PBMC than negative control wells ($p<0.05$ based on the negative binomial distribution) and had at least 20 SFU/M PBMC more than the negative control wells (i.e. > than the 99th percentile of the negative control well distribution). All reported responses are background subtracted SFU/M PBMC.

Person time denominators for rates in Table S4 were measured using “person seasons” (the time from first isolation of PCR-positive influenza in the cohort to the last isolation of PCR-positive influenza is defined as 1 person season). We used logistic regression to investigate the independent effect of NP response on odds of PCR-positive disease in those with four-fold titre rises and poisson regression to investigate the effect of NP response on rates of respiratory illness in those infected with four-fold titre rises. All covariates of interest (age, gender, homotypic baseline antibody titre and year) were tested as potential confounders (as each were potentially associated both with NP response and with the outcomes of interest) and retained them in the final regression models if they independently predicted the outcome or made an appreciable impact on the final NP effect estimate. Robust standard errors were used with STATA cluster commands accounting for lack of independence between repeated measurements in the same individual. Stata testparm commands were used to assess interactions. We assessed the effect of response to NP separately for pandemic and seasonal influenza even if there was no evidence of interaction as it was of a priori interest to know whether the protective effect was present in both. We also tested whether there was an independent effect of response to M protein by including this within regression models with NP. For comparability with previous work we also used regression models to test whether overall response to internal proteins was protective.

For participants who completed daily illness diaries scoring a range of symptoms (fever, feeling feverish, headache, muscle aches, sore throat, runny nose cough, blocked nose and sneezing) from 0 (absent) to 3 (severe) we used ordinal logistic regression of daily symptom scores over the first week of illness adjusting for clustering at the illness episode level to assess whether symptom scores significantly differed between those with and without detectable NP response. Bonferonni adjustments were made to take account of multiple significance testing.

The main results of the analysis are presented in Table 1 of the paper. There were no significant interactions between the effect of NP response and age, gender, year or baseline antibody (<32 vs ≥32) suggesting there is protective effect of T cells across all categories of these variables. The protective effect against PCR-positive disease in those infected was not significant for total internal protein response (at cut-offs of 30 or above - adjusted OR 0.59, 95% CI 0.27-1.30, p=0.194) and there was no independent effect of response to M after controlling for the effect of NP response (adjusted IRR 1.15, 95% CI 0.49-2.69, p=0.75).

To ensure the findings were not due to high NP responses caused by very recent influenza infection we conducted a sensitivity analysis excluding those with an NP response of > 50. The protective effect of NP response against PCR-positive disease remained significant (adjusted IRR 0.16, 95% CI 0.37-0.68, p=0.013).

After adjusting for NP response there was no independent effect of T cell response specific for total internal proteins or M. However, since NP and M responses are highly correlated (correlation coefficient for log transformed NP and M responses =0.68 (95% CI 0.64-0.71), p<0.001), assessment of their independent effects was not possible.

SUPPLEMENTARY TABLES

Table E1. Cohort Characteristics

T cell cohort subset headings	Unvaccinated participants* in T cell cohort with baseline T cell and serology data							
			Subset of above who have follow-up serology					
				Subset of above who are infected (four-fold titre rise to dominant strain)				
					Subset of above with respiratory illness			
							Subset of above with nasal swab	
	Number of baseline observations* (person seasons)	# Virus shedders (# PCR-positive per 100 person seasons)**	Number of baseline observations* (person seasons)	# infections (# per 100 person seasons)**	# respiratory illness (# per 100 infections)	# of illnesses with fever > 37.8C (# per 100 illnesses)	# respiratory illnesses with swabs (% of illnesses)	# (%) of swabs with PCR-positive influenza A**
Baseline T cell response to nucleoprotein SFU/M PBMC								
NP < 20	973 (846)	32 (3.78)	869 (762)	126 (16)	96 (76)	23 (24)	75 (78)	29 (39)
NP ≥20	730 (647)	11 (1.70)	630 (562)	79 (14)	47 (59)	21 (45)	37 (79)	6 (16)
Variables tested for potential confounding effect in multivariable regression models – Adjusted results are shown in Table 1 of the main paper								
2006	321 (315)	6 (1.90)	294 (289)	51 (18)	30 (59)	11 (37)	26 (87)	6 (23)
2007	404 (386)	9 (2.33)	351 (339)	40 (12)	25 (63)	6 (24)	18 (72)	8 (44)
2008	322 (248)	16 (6.45)	285 (221)	67 (30)	51 (89)	20 (39)	44 (86)	12 (27)
2009	656 (543)	12 (2.21)	569 (475)	47 (9.9)	37 (79)	7 (19)	24 (65)	9 (38)
Male	795 (699)	25 (3.58)	692 (612)	85 (14)	61 (72)	17 (28)	48 (79)	19 (40)
Female	908 (794)	18 (2.27)	807 (712)	120 (17)	82 (68)	27 (33)	64 (78)	16 (25)
Age 5-15	146 (128)	4 (3.13)	105 (94)	25 (27)	23 (92)	9 (39)	14 (61)	2 (14)
Age 16-45	570 (500)	16 (3.2)	499 (440)	77 (18)	53 (69)	17 (32)	44 (83)	14 (32)
Age 45-64	804 (709)	23 (3.24)	734 (651)	89 (14)	62 (70)	18 (29)	50 (81)	19 (38)
Age 65+	183 (155)	0 (0.00)	161 (139)	14 (10)	5 (36)	0 (0.0)	4 (80)	0 (0.0)
Baseline titre**								
<16	1328 (1163)	41 (3.53)	1170 (1033)	186 (18)	129 (69)	39 (30)	103 (80)	34 (33)
16-32	232 (206)	2 (0.98)	206 (183)	18 (9.8)	14 (78)	5 (36)	9 (64)	1 (1.1)
64-128	108 (94)	0 (0.00)	89 (78)	1 (1.3)	0 (0.0)	n/a	n/a	n/a
>128	35 (30)	0 (0.00)	34 (29)	0 (0.0)	n/a	n/a	n/a	n/a

* Some individuals participated in more than one season and are therefore counted more than once in the table (except when results are stratified by year). We therefore report the number of baseline observations (N=1703) instead of the number individuals (N=1414).

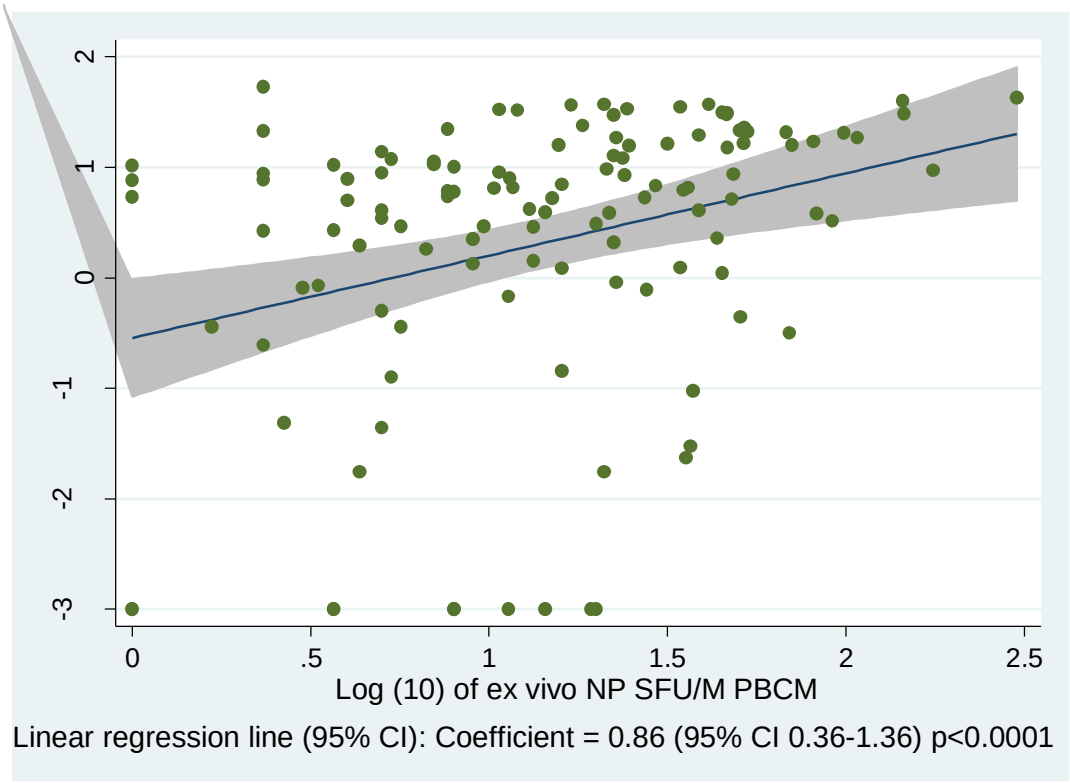
**Refers to dominant influenza strain: H3N2 in 2006/7 and 2008/9; seasonal H1N1 in 2007/8 and A(H1N1)pdm09 in 2009/10. Each year one strain of influenza A dominated: In 2006/7 there were 6 PCR-positive cases of H3N2; in 2007/8 there were 9 PCR-positive cases of seasonal H1N1 and one of H3N2; in 2008/9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

Table E2. Comparison of design and results of community cohort studies of effect of T cells on natural influenza infection.

	Sridhar et al	Hayward et al
Population group	Healthy adults recruited in University setting (n=342)	General population. All age groups, including those with chronic illness (n=1414 with 1703 observation sets)
Follow up	Baseline T cell responses and serology. 3 weekly reporting of symptoms. Self-sampling using nasal swabs for PCR confirmation. End of season serology Follow up over pandemic period only.	Baseline T cell responses and serology. Weekly contact for reporting of symptoms. Self-sampling using nasal swabs for PCR confirmation. End of season serology Follow up over seasonal influenza and pandemic periods.
Identification of infection	4 fold rise in titre or PCR (n=43)	4 fold rise in titre excluding titre rises due to vaccination (n=205)
Protective effect of T-cells against infection	None observed	None observed
Infected group in whom effect of T cells studied	Those infected with influenza excluding those with multiple illnesses during follow up (n=25)	Those infected with influenza (n=205)
Approach to analysis of T cell data	Continuous variable	Binary variable (≥20 SFU/M). - Data distribution could not be transformed to meet assumptions of statistical tests + no evidence of quantitative effect above this threshold.
Protective effect of total T cell response	Higher baseline T cell results in those with no fever (n=12 - p=0.03), no symptoms (n=3, p=0.02) and those with lower symptom scores (p=0.05). No difference in those who were PCR positive (n=11)	No effect on PCR positive disease or symptoms
Protective effect of conserved internal proteins	Higher baseline levels of cellular responses to highly conserved CD8 epitopes from the immunodominant internal PB1, NP and M1 proteins in those with no fever (n=12 p =0.02) no ILI (n= 15 p=0.04) and those with lower symptom scores (p=0.01). Higher baseline levels of IFN-γ+IL-2− T cells specific for conserved CD8 epitopes in those with low symptom scores (p=0.004) and those who were PCR negative (p=0.05).	Positive baselineT cell response to immunodominant NP protein associated with reduced odds of PCR positive disease (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)),. Positive responses also associated with asymptomatic disease during the pandemic. No association with total symptom scores (although used lower weights for severe symptoms compared to Sridhar et alt).
CD4 or CD8?	Protective response associated with CD8 epitopes. Cannot exclude additional CD4 response	Protective effect associated with NP response that was dominated by CD8 response. Cannot exclude additional CD4 effect.

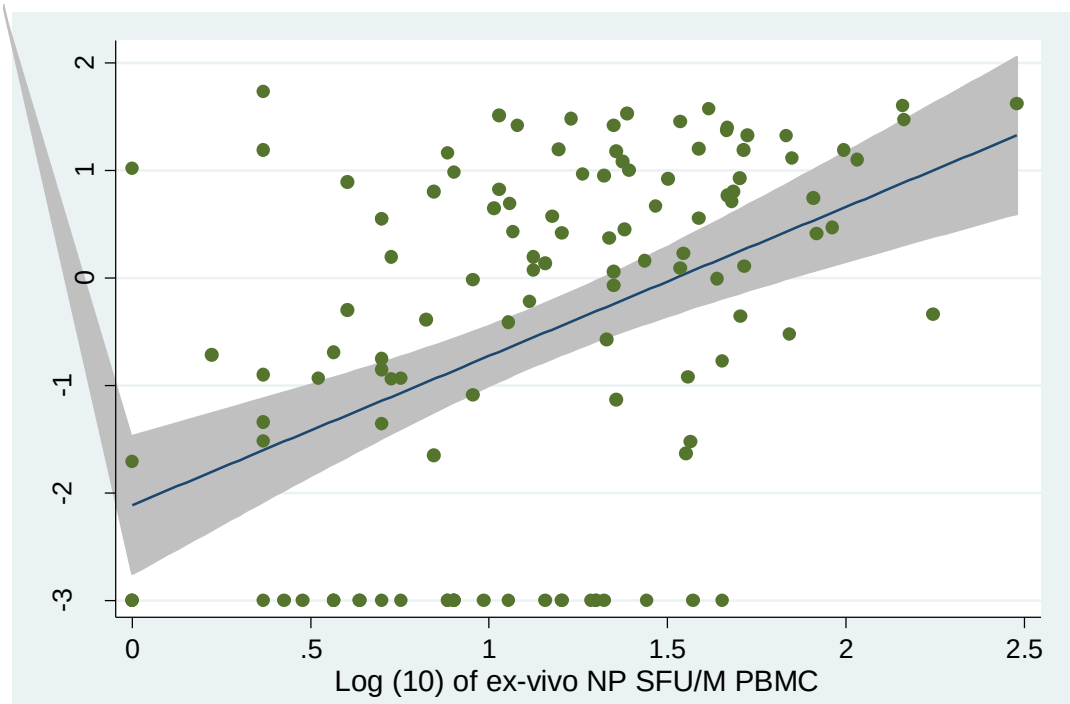
SUPPLEMENTARY FIGURES

Figure E1A



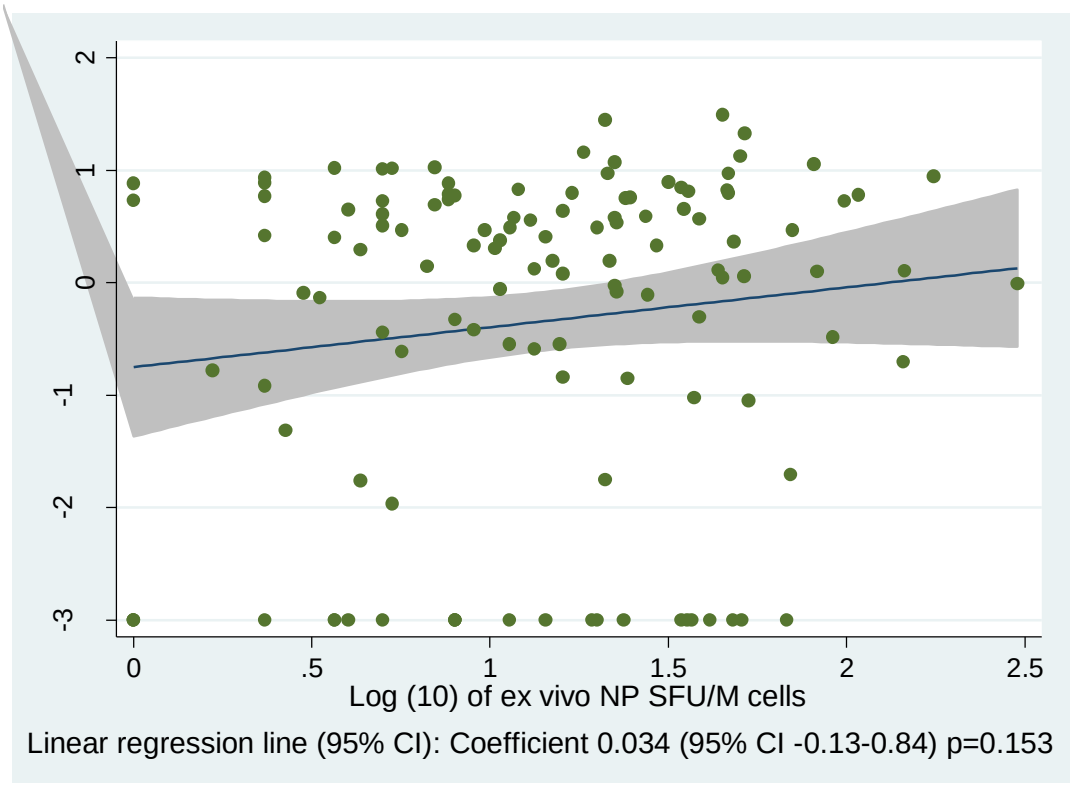
Linear regression line (95% CI): Coefficient = 0.86 (95% CI 0.36-1.36) p<0.0001

Figure E1B



Linear regression line (95% CI): Coefficient 1.38 (95% CI 0.878-1.89) p<0.0001

Figure E1C



Each dot represents one Flu Watch participant with the log_{10} transformed *ex vivo* frequency of H3N2 NP specific T cells, detected by IFN-γ ELISpot, shown on the X axis. In Figure E1A, The Y axis shows the culture expanded H3N2 NP specific T cells (CD3+ IFN-γ+) detected by intracellular cytokine staining and flow cytometry (log_{10} transformed). In Figure E1B, the Y axis shows the culture expanded H3N2 NP specific CD8+ T cells (CD3+CD8+ IFN-γ+) detected by intracellular cytokine staining and flow cytometry. In Figure E1C, the Y axis shows the culture expanded H3N2 NP specific CD4+ T cells (CD3+CD4+ IFN-γ+) detected by intracellular cytokine staining and flow cytometry.

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