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Cellular immune correlates of protection against symptomatic pandemic influenza

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The role of T cells in mediating heterosubtypic protection against natural influenza illness in humans is uncertain. The 2009 H1N1 pandemic (pH1N1) provided a unique natural experiment to determine whether crossreactive cellular immunity limits symptomatic illness in antibody-naive individuals. We followed 342 healthy adults through the UK pandemic waves and correlated the responses of pre-existing T cells to the pH1N1 virus and conserved core protein epitopes with clinical outcomes after incident pH1N1 infection. Higher frequencies of pre-existing T cells to conserved CD8 epitopes were found in individuals who developed less severe illness, with total symptom score having the strongest inverse correlation with the frequency of interferon-γ (IFN-γ)$^+$ interleukin-2 (IL-2)$^-$ CD8$^+$ T cells ($r = -0.6$, $P = 0.004$). Within this functional CD8$^+$IFN-γ$^+$IL-2$^-$ population, cells with the CD45RA$^+$ chemokine (C-C) receptor 7 (CCR7)$^-$ phenotype inversely correlated with symptom score and had lung-homing and cytotoxic potential. In the absence of crossreactive neutralizing antibodies, CD8$^+$ T cells specific to conserved viral epitopes correlated with crossprotection against symptomatic influenza. This protective immune correlate could guide universal influenza vaccine development.

The constant evolution of influenza-virus surface proteins by point mutations or genetic reassortments enables evasion from strain-specific protective humoral immunity. When humoral immunity is inadequate or is circumvented during seasonal epidemics or pandemics by a reassortant virus such as pH1N1, limiting disease severity acquires great public health importance. Epidemiological evidence from past pandemics has suggested that previous seasonal influenza infections in individuals are associated with a decreased risk of symptomatic illness with newly emerged pandemic strains[1,2], but the mechanism of this heterosubtypic immunity is unknown.

In mouse and nonhuman primate models, heterosubtypic immunity is mediated primarily by crossreactive cytotoxic CD8$^+$ T cells[3–9]. We and others have recently shown that CD4$^+$ and CD8$^+$ T cells induced by seasonal influenza can crossrecognize internal proteins of pH1N1 (refs. 10–15). Such crossreactive T cells, which recognize epitopes that are conserved across viral subtypes[16–20], might potentially mediate this heterosubtypic immunity and confer protection against symptomatic illness in individuals lacking pre-existing humoral immunity to the pandemic virus. Experimental challenge in humans lacking pre-existing antibodies has identified an inverse association between influenza-specific T cells and viral shedding and symptomatology among infected individuals, providing proof-of-concept support for the protective capacity of T cells against influenza illness[21,22]. The seminal human challenge experiment[21] raised the key question of whether crossreactive T cells influence the symptomatic outcomes of influenza infection in naturally exposed populations, a question that remains unanswered 30 years later.

Addressing this fundamental question requires a natural experiment involving the spread of an antigenically shifted influenza virus through a population lacking protective antibodies[23]. Such a unique opportunity was presented by the emergence of pH1N1. We therefore recruited and followed a community cohort of healthy adults lacking pH1N1-neutralizing antibodies through the pandemic to define the relationship between crossreactive T cells and illness severity. We determined the crossreactive cellular immune correlates of favorable clinical outcomes of influenza infection to help inform the rational design and evaluation of broadly protective T cell–inducing vaccines.

RESULTS

Study design and cohort

We designed a prospective cohort study in which we recruited 342 eligible participants before the onset of the second UK pandemic wave. The epidemiology of pH1N1 in the UK over the 2009–2010 and subsequent 2010–2011 influenza seasons was characterized by three distinct waves: the first was from April to August 2009, the second was from September 2009 to April 2010 and the third was from August 2010 to April 2011. Participants were followed through the two consecutive influenza seasons, 2009–2010 and 2010–2011, during which pH1N1 was the predominant circulating strain in the UK (**Fig. 1**).

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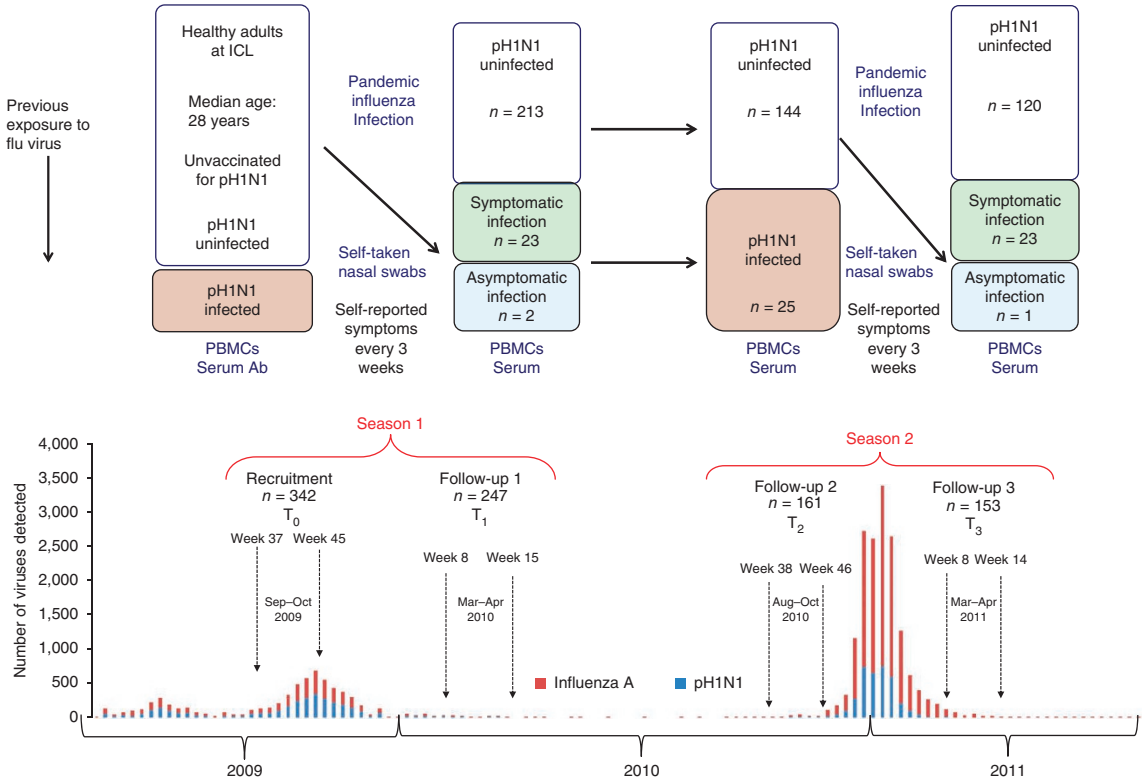


Figure 1 Study outline and timeline of the 2009–2010 and 2010–2011 pandemic waves in the UK. Shown is the outline of the study (top) in the context of the timeline of the evolving pH1N1 pandemic (bottom). The bar chart shows UK influenza virological surveillance data from the World Health Organization's FluNet (http://www.who.int/influenza/gisrs_laboratory/flunet/en/) highlighting the study recruitment and follow-up time points for seasons 1 and 2 in relation to influenza activity in the UK during 2009–2011. Red bars indicate influenza A, and blue bars indicate the number of cases of pH1N1 detected by virological national surveillance. Week number was measured from the start of each year. Healthy adults recruited just after the first wave of the pandemic had passed in the UK were followed over two influenza seasons (T_0–T_3), with PBMCs and serum samples collected before and at the end of each winter influenza season. During each influenza season, symptom questionnaires were emailed to participants every 3 weeks with automated weekly reminders. Nasal swabs were collected by participants if they were symptomatic and were returned to the laboratory. Infection was defined by detection of pH1N1 virus in the returned nasal swabs or a fourfold rise in pH1N1 hemagglutination-inhibition titer in paired serum samples. Arrows between the boxes denote the longitudinal progression of individuals during the study; white boxes indicate individuals not infected with pH1N1, pink boxes indicate pH1N1-infected individuals, green boxes indicate symptomatic infection, and blue boxes indicate asymptomatic infection. ICL, Imperial College London; Ab, antibodies.

51 individuals lacking neutralizing antibodies to pH1N1, as determined by standard hemagglutination-inhibition and microneutralization assays (**Supplementary Table 1**), developed incident pH1N1 infection, 43 of whom had complete clinical data and viable baseline peripheral blood mononuclear cells (PBMCs) (**Fig. 2**). The median age of the 43 subjects was 34.5 years (interquartile range (IQR) 27–40 years) (**Supplementary Table 2**), and there were no differences in age or gender between the individuals included and excluded from the analysis.

Incidence of infection and outcomes

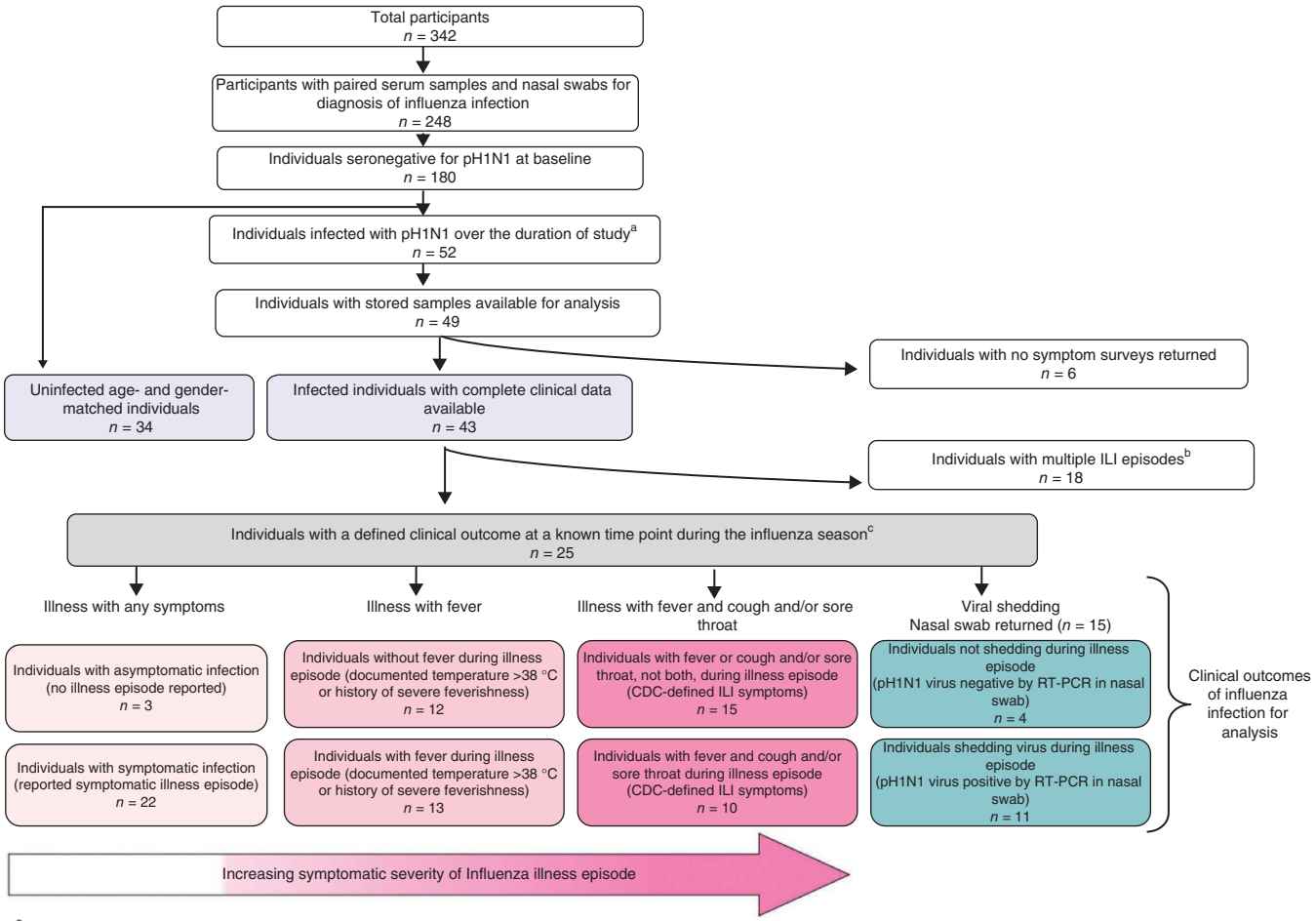
Of the 43 incident cases included in the analysis, 32 were diagnosed by antibody seroconversion and 11 were diagnosed by detection of virus in nasal swabs; all of the subjects except one had antibody seroconversion. In 25 of these individuals, we reliably determined the date, symptoms and symptom score for the clinical episode during which influenza infection occurred, whereas in the remaining 18 infected individuals, the presence of more than one reported symptomatic episode precluded this determination. We grouped these 25 individuals according to their clinical outcomes for analysis (Online Methods and **Fig. 2**). Of these 25 subjects, 15 returned nasal swabs during their illness episode, 11 of whom shed virus (pH1N1-positive nasal swab determined by RT-PCR), and 4 individuals did not shed virus despite having antibody seroconversion.

Pre-existing crossreactive T cells and illness severity

In all 43 individuals seronegative for pH1N1 antibodies at the time of recruitment, we detected pre-existing crossreactive T cells to pH1N1 (**Supplementary Fig. 1**) that contained a predominance of cells with an IFN-γ^+IL-2^- cytokine-secreting profile, as has been reported previously[12]. The frequency of crossreactive T cells was independent of age.

We first assessed the association between pre-existing T cells and risk of subsequent pH1N1 infection. The frequencies of pre-existing crossreactive total cytokine-secreting T cells or cytokine-secreting T cell subsets at baseline did not differ between individuals who became infected ($n = 43$) and age- and gender-matched individuals who did not acquire pH1N1 infection ($n = 34$) (**Supplementary Fig. 2**).

Among pH1N1-infected individuals, we determined the relationship between the frequencies of pre-existing crossreactive cytokine-secreting T cells before infection with subsequent development of symptomatic outcomes (**Fig. 3**). We first assessed the global CD4 and CD8 response to antigens that were conserved between



[a]Infection was defined by detection of pH1N1 virus in returned nasal swab or a fourfold rise in pH1N1 hemagglutination inhibition titre in paired serum samples.

[b]pH1N1-infected individuals with antibody seroconversion between the start and end of the influenza season but negative RT-PCR in nasal swab who reported multiple illness episodes precluding definitive ascertainment of the illness episode in which the individual was infected.

[c]Single illness episode in those with seroconversion or positive nasal swab temporally associated with symptomatic illness.

Figure 2 Study flow chart of sample selection for the analysis of heterosubtypic T cell correlates of protection against clinical outcomes of infection. A total of 342 participants were enrolled in the study. During the study period, 51 individuals were infected (identified by seroconversion or a pH1N1-positive RT-PCR result in a self-administered nasal swab), 43 of whom were included in the final analysis. Individuals were excluded from the analysis because of any of the following factors: unavailability of paired serum samples for diagnosis of infection ($n = 94$); being seropositive for pH1N1 at baseline, which indicates a previous encounter with pH1N1 and thus would prevent analysis of heterosubtypic T cell responses ($n = 68$); remaining uninfected through the study ($n = 128$); not returning any symptom survey ($n = 6$); or unavailability of viable stored PBMCs ($n = 3$). Of these 43 individuals, definitive clinical outcomes were ascertained in 25 individuals. Following the analysis plan, the 25 infected individuals were grouped on the basis of increasing severity of symptomatic outcomes during the influenza illness episode. Colored boxes represent the different groups compared in the analysis. Light pink boxes represent individuals with asymptomatic or symptomatic influenza infection, pink boxes represent individuals with fever (bottom) or without fever (top) during the illness episode, and dark pink boxes represent individuals with fever and cough or sore throat (defined by the US Centers for Disease Control and Prevention as symptomatic illness). Those individuals who shed (blue box, bottom) or did not shed (blue box, top) virus were also compared.

the pandemic strain and influenza A virus strains that had circulated previously by quantifying T cells responding to live virus. We detected higher frequencies of pre-existing crossreactive total cytokine-secreting T cells to live pH1N1 virus ($P = 0.03$) in individuals who developed illness without fever ($n = 12$) compared to those whose illness was accompanied by fever ($n = 13$) (**Fig. 3a**). We determined the quantitative relationship between the frequency of virus-specific T cells and the risk of developing influenza illness (**Supplementary Fig. 3**). Three individuals with completely asymptomatic infection had higher frequencies of pre-existing total cytokine-secreting crossreactive T cells to pH1N1 virus ($P = 0.02$) as compared to the 22 individuals with symptomatic infection (data not shown).

We next tested our specific hypothesis that $CD8^+$ T cells to highly conserved viral epitopes mediate a protective response by assessing cellular responses to highly conserved CD8 epitopes from the immunodominant internal PB1, NP and M1 proteins (**Supplementary Table 3**). We observed higher frequencies of total cytokine-secreting T cells to conserved CD8 epitopes in individuals who developed illness without fever ($P = 0.02$, $n = 12$) or symptoms of influenza-like illness (ILI) ($P = 0.04$, $n = 15$) as compared to individuals with fever ($n = 13$) or ILI symptoms ($n = 10$) (**Fig. 3b**). No differences in the frequencies of total cytokine-secreting T cells between the different symptomatic groups was observed for responses specific to control cytomegalovirus (CMV) antigen (**Fig. 3c**). $CD8^+$ T cell depletion abrogated the response to these epitopes, confirming that the responses were mediated by $CD8^+$

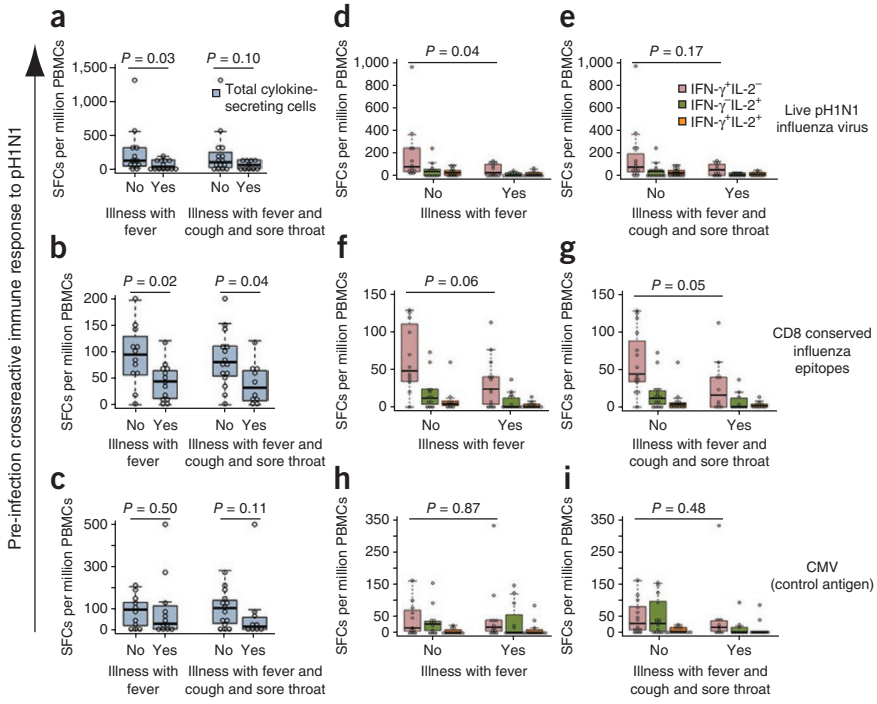


Figure 3 The frequencies of pre-existing crossreactive T cells are inversely associated with illness severity in infected individuals. (**a**–**i**) Responses to live pH1N1 virus stimulation (**a**,**d**,**e**), summed responses to conserved CD8 epitopes from PB1, M1 and NP proteins (**b**,**f**,**g**) and responses to CMV lysates (control antigen) (**c**,**h**,**i**) of total (blue), IFN-γ+IL-2− (pink), IFN-γ−IL-2+ (green) and IFN-γ+IL-2+ dual (orange) cytokine-secreting cells quantified by FLISpot. The total frequency of cytokine-secreting T cells represents the summed frequencies of the three functional subsets, IFN-γ+IL-2−, IFN-γ−IL-2+ and IFN-γ+IL-2+. Cellular immune responses to live pH1N1 virus, CD8 conserved influenza epitopes and CMV lysates were determined in individuals ($n = 25$) developing an illness with fever ($n = 13$) compared to those with no fever ($n = 12$) and in individuals with fever with cough or sore throat ($n = 15$) compared to those without fever and cough or sore throat ($n = 10$). P values were estimated by Mann-Whitney nonparametric test. In the box plots, the box represents the third centile (75%) and first centile (25%), with the horizontal line representing the median (50%). The whiskers represent 1.5 times the IQR, with outliers shown. Each circle represents the frequency of cellular responses for an individual. SFCs, spot-forming cells.

T cells (**Supplementary Fig. 4**). Further enumeration of the three key functional T cell subsets by fluorescence-immunospot (FLISpot) identified only the IFN-γ+IL-2− T cell subset as being associated with reduced risk of developing more severe influenza infection (**Fig. 3d**–**g**).

Total symptom score during the illness episode correlated inversely with the frequency of pre-existing total cytokine-secreting T cells specific for live virus ($r = -0.39$, $P = 0.05$) (**Fig. 4a**), and the inverse correlation was stronger for responses specific to the conserved CD8

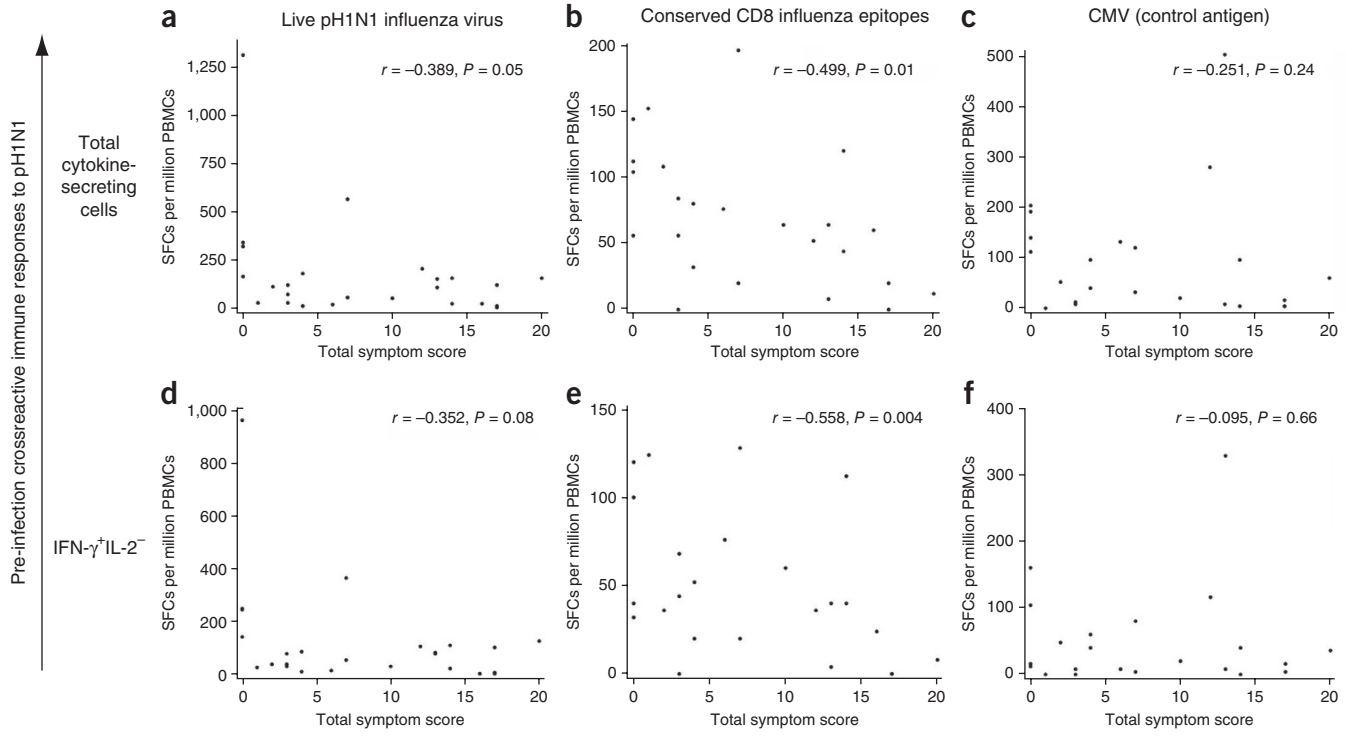


Figure 4 Inverse correlation of crossreactive T cells and symptom score. (**a**–**f**) Correlation between the frequency of total (**a**–**c**) and IFN-γ+IL-2− (**d**–**f**) T cellular responses to live virus (**a**,**d**), the summed responses to conserved CD8 epitopes from PB1, M1 and NP proteins (**b**,**e**) and the responses to CMV lysates (control antigen) (**c**,**f**) quantified by FLISpot and symptom scores. Symptom score was defined by totaling the scores for each of the following symptoms: fever, sore throat, cough, headache and myalgia. r values are the Spearman rank correlation coefficients. Each circle on the plot represents an individual.

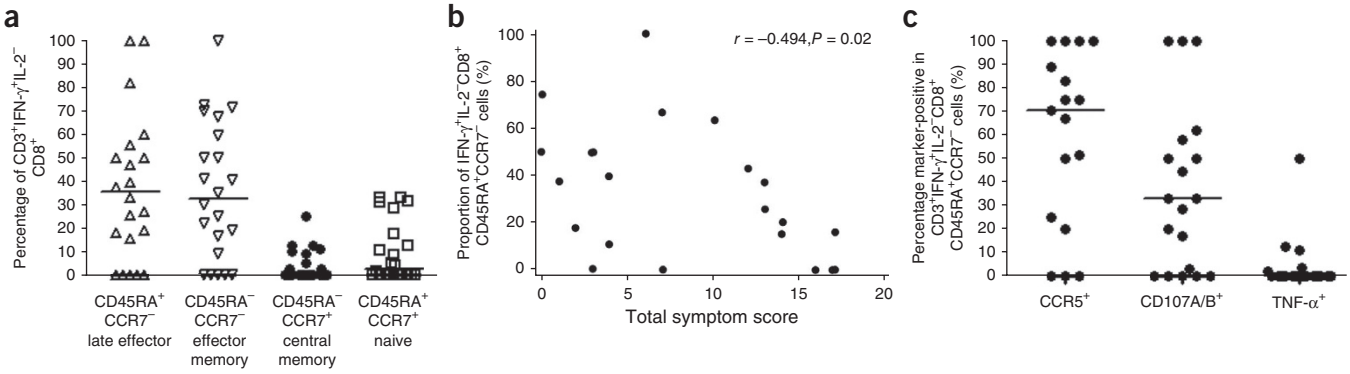


Figure 5 Inverse correlation of pre-existing crossreactive late-effector CD8⁺IFN-γ⁺IL-2⁻ cells and symptom score. Phenotypic characterization was performed using multiparameter flow cytometry of the different memory subsets of influenza virus–specific CD8⁺IFN-γ⁺IL-2⁻ cells on the basis of CCR7 and CD45RA surface expression after overnight stimulation of PBMCs with live pH1N1 virus in pH1N1-infected individuals ($n = 22$; in 3 of 25 infected individuals, samples were of insufficient quantity for flow cytometry analysis). (**a**) The proportions of CD8⁺IFN-γ⁺IL-2⁻ secreting cells that were of the effector-memory (CD45RA⁻CCR7⁻), late-effector (CD45RA⁺CCR7⁻), central-memory (CD45RA⁻CCR7⁺) or naive (CD45RA⁺CCR7⁺) phenotype. (**b**) Correlation between the proportion of pre-existing CD3⁺CD8⁺IFN-γ⁺IL-2⁻ cells of the late-effector CD45RA⁺CCR7⁻ subset and total symptom score. *r* values are the Spearman rank correlation coefficients. (**c**) In individuals with influenza-specific late-effector CD8⁺IFN-γ⁺IL-2⁻CD45RA⁺CCR7⁻ cells ($n = 17$), functional characterization of these cells for expression of CD107A/B, CCR5 and TNF-α was undertaken with multiparameter flow cytometry. Symbols represent the proportion of CD45RA⁺CCR7⁻CD8⁺IFN-γ⁺IL-2⁻ cells expressing CD107A/B, CCR5 and TNF-α for each individual, with the line showing the median response. Each circle on the plot represents responses for an individual.

epitopes of immunodominant core proteins ($r = -0.50$, $P = 0.01$) (**Fig. 4b**). The strongest inverse correlation was between total symptom score and the frequency of IFN-γ⁺IL-2⁻ T cells specific for conserved CD8 epitopes ($r = -0.56$, $P = 0.004$) (**Fig. 4**).

There was no correlation between the frequency of total cytokine-secreting T cells (**Fig. 4c**) or IFN-γ⁺IL-2⁻ T cells (**Fig. 4f**) that were specific for control CMV antigen and total symptom score ($r = -0.33$, $P = 0.13$ and $r = -0.15$, $P = 0.48$, respectively) or decreased risk of more severe illness (**Fig. 3c**,**h**,**i**), suggesting that the T cell responses that were associated with limiting illness severity were influenza specific.

Phenotype of crossreactive CD8⁺IFN-γ⁺IL-2⁻ memory T cells

To pinpoint the specific phenotype of the pre-existing influenza-specific protection-associated CD8⁺IFN-γ⁺IL-2⁻ T cell population, we stratified this population into its constituent memory subsets by multiparameter flow cytometry using the surface markers CD45RA and CCR7 (ref. 24) We hypothesized that the circulating late-effector subset of memory CD8⁺ T cells (cells with this phenotype predominate in the lung[25] and are readily activated to secrete IFN-γ on influenza exposure[23]) would correlate with protection against symptoms. The IFN-γ⁺IL-2⁻ T cell response to live virus was dominated by CD8⁺ T cells, which comprised predominantly CD45RA⁻CCR7⁻ effector-memory and CD45RA⁺CCR7⁻ late-effector T cells (**Fig. 5a**). The proportion of CD8⁺IFN-γ⁺IL-2⁻ T cells in the CD45RA⁺CCR7⁻ late-effector subset was inversely correlated ($r = -0.49$, $P = 0.02$) with total symptom score (**Fig. 5b**). Functional characterization of this protection-associated CD8⁺IFN-γ⁺IL-2⁻CD45RA⁺CCR7⁻ T cell population for lung homing (CCR5)[26–29], degranulation (CD107A and CD107B (CD107A/B))[30] and cytokine secretion (tumor necrosis factor-α (TNF-α)) revealed that 70% of these cells expressed CCR5 and 33% expressed CD107A/B (**Fig. 5c**) in response to live pH1N1 virus, demonstrating a capability for rapid cytotoxicity and lung homing on virus exposure.

Although a recent report in an artificial experimental challenge model implicated CD4⁺ T cells in limiting the severity of influenza illness, we found no association between total symptom score and the proportion of virus-specific CD4⁺IFN-γ⁺IL-2⁻ cells ($r = -0.093$, $P = 0.68$) or the late-effector CD45RA⁺CCR7⁻ subset of CD4⁺IFN-γ⁺IL-2⁻ T cells ($r = -0.012$, $P = 0.96$) (**Supplementary Fig. 5**). We designed our study to test the role of CD8⁺ T cells in heterosubtypic immunity, and therefore we did not use longer peptides containing optimal CD4 epitopes. Thus, our experiments do not rule out a possible correlation of CD4⁺ T cells with illness severity.

Heterosubtypic T cell responses and viral shedding

As evidenced by the decrease in influenza incidence with school closure during the 2009 pandemic[31], limiting influenza transmission is critical for pandemic control. The frequencies of pre-existing live virus–specific IFN-γ⁺IL-2⁻ T cells were significantly higher ($P = 0.05$) in individuals not shedding virus compared to those shedding virus, whereas there was no significant difference in the total frequencies of cytokine-secreting cells between these two groups (**Supplementary Fig. 6**), which is consistent with results of experimental challenge studies showing an association of T cell frequencies with reduced viral shedding[21,22]. Individuals might be misclassified as nonshedders if they collected their nasal swabs at a later time point after the onset of symptoms than nonshedders did; however, there was no statistically significant difference in the time from symptom onset to nasal swab collection between infected individuals that shed virus and those that did not.

DISCUSSION

In influenza pandemics where susceptible populations lack protective antibodies, the most favorable outcome of infection is symptom-free illness. We opportunistically used the natural experiment presented by the 2009 H1N1 pandemic to prospectively identify a cellular immune correlate of protection against clinical illness after natural influenza infection by an antigenically shifted reassortant virus.

In our cohort, a higher frequency of CD8⁺IFN-γ⁺IL-2⁻ crossreactive T cells was associated with decreased risk of fever, fewer ILI symptoms, reduced illness severity score and absence of viral shedding in individuals infected with the pandemic virus. Within this functional CD8⁺ T cell subset, we identified CD45RA⁺CCR7⁻ late-effector T cells as the cellular immune correlate of protection against

community-acquired pandemic influenza illness. These cells have direct antiviral cytotoxic potential, rapidly secreting IFN-γ[23,32] and expressing the degranulation marker CD107 (ref. 30) on recognition of live virus. Further, the majority of these cells express the lung-homing marker CCR5, which is critical for directing $CD8^+$ T cells to the lungs during respiratory viral infection[26], with genetic variants of the CCR5 gene potentially predisposing individuals to risk of severe pH1N1 disease[33]. Thus, the cytokine-secretion profile, capability for rapid cytotoxicity and lung-homing potential displayed by this T cell population are all attributes that are compatible with mediating protection.

Current inactivated influenza vaccines induce antibodies that confer strain-specific, rather than heterosubtypic, immunity against infection, are less efficacious when mismatched with circulating strains[34] and do not maintain a strong T cell immune response[11,12]. Thus, although repeated annual vaccination with the current vaccines prevents infection specifically by matched homologous strains, it may also prevent the induction and maintenance of heterosubtypic cellular immunity[35,36], thereby leaving individuals susceptible to more severe disease with new reassortant viral strains that evade vaccine-induced humoral immunity. Furthermore, a recent comprehensive systematic review revealed that current inactivated influenza vaccines fail to provide consistent high-level protection against both homosubtypic and heterosubtypic strains[37]. There is thus an urgent unmet need for effective crossprotective vaccines[38]. It will therefore now be important to determine whether the live attenuated influenza vaccines that are being rolled out internationally can induce heterosubtypic immunity against new influenza viral strains and, if so, whether this immunity is mediated by $CD8^+IFN\text{-}\gamma^+IL\text{-}2^-$ T cells.

Harnessing crossprotective cellular responses to conserved immunogenic protective epitopes to achieve asymptomatic or mild illness would be a major public health advance. We found that the summed T cell response to highly conserved CD8 epitopes from three immunodominant core proteins, PB1, M1 and NP, was most strongly associated with limiting illness severity. This suggests that $CD8^+$ HLA class I–restricted T cells specific for epitopes conserved across influenza A virus subtypes might confer protection against illness for a broad range of influenza A viruses in the absence of detectable crossreactive neutralizing antibodies. Despite the increasing interest in developing broadly protective universal influenza vaccines, the field is limited by the absence of an immunological surrogate of protection and the identification of protective antigens. Our findings support the development of universal vaccines that induce durable $CD8^+IFN\text{-}\gamma^+IL\text{-}2^-$ T cell responses to these conserved epitopes. Although limited PBMC numbers precluded the quantification of responses to individual epitopes, all conserved epitopes would be necessary to induce protective cellular immunity in HLA-diverse human populations. The correlate of protection identified here provides both the evidence and a surrogate endpoint for the rational development and evaluation of broadly protective T cell–inducing vaccines that are designed to induce heterosubtypic immunity to limit illness severity after infection with new viral strains. Use of the $CD8^+IFN\text{-}\gamma^+IL\text{-}2^-$ signature as a surrogate endpoint in clinical trials of new T cell–inducing vaccines will need to be in combination with robust clinical endpoints.

Our findings, taken together with our recent report of the high prevalence of crossreactive T cells to pH1N1 in healthy adults[12], lead us to hypothesize that cellular heterosubtypic immunity may have in part accounted for the unexplained and unexpectedly mild spectrum of clinical illness during the 2009 pandemic. This heterosubtypic cellular immunity might also partly mitigate the anticipated serious clinical impact of pandemics with highly pathogenic viral strains, such as avian H5N1 influenza[39,40], as a large proportion of individuals possess T cells that recognize H5N1 core proteins[41,42].

A recent human high-dose experimental challenge study using a laboratory-adapted seasonal influenza strain reported an unexpected association between IFN-γ–secreting homosubtypic, not heterosubtypic, $CD4^+$, but not $CD8^+$, T cells, and diminution of clinical symptoms[22]. We found that crossprotective immunity in the clinically relevant setting of naturally acquired infection after community exposure during a pandemic is associated with $CD8^+$ T cells—consistent with results of mouse[4,6] and nonhuman primate challenge studies[8]—and specifically, late-effector $CD45RA^+CCR7^-CD8^+IFN\text{-}\gamma^+IL\text{-}2^-$ T cells. Thus, the protective correlate in real-life, natural aerosol pH1N1 infection was not associated with and was different from that identified in the high-dose experimental challenge model that provided proof of principle for an inverse association between virus-specific T cells and viral shedding and symptomatology.

The experimental challenge model uses direct nasal inoculation of a less virulent serially passaged laboratory-adapted virus strain at ultra-high doses, 10^4–10^6 times higher than the minimum infectious dose, all of which are factors that differ markedly from natural infection that follows human-to-human aerosol transmission. Therefore, whether pre-existing T cells correlate with protection against naturally acquired influenza illness, and whether such protection extends to previously unseen pandemic viruses, remained open questions until this study. In contrast to the experimental challenge models, our cohort represented the clinical spectrum of naturally acquired influenza infection in the community; the majority of the individuals we studied had mild to moderate illness, which enabled us to identify correlates of the most common clinical outcomes of community-acquired influenza infection[43,44].

Our study has limitations that are inherent to conducting real-time population-based research during a rapidly evolving pandemic. By the time ethics approval was in place, the first minor pandemic wave in the UK had passed. We therefore followed individuals through the second and third waves of the pandemic. The community-based nature of the study necessitated stringent criteria to reliably identify individuals with distinct clinical outcomes. Although this reduced the sample of incident events and widened the confidence intervals, the association of heterosubtypic T cells in limiting illness remained consistent across several measures of clinical outcome. Although the findings from this healthy adult cohort are probably generalizable to other healthy adult populations, we cannot directly extrapolate them to individuals at high risk of severe influenza illness. However, it may not be unreasonable to expect a protective effect of T cells that is at least as potent in limiting severe illness and fatality in these high-risk individuals, although it is also possible that the protective correlate against fatal disease may differ.

Our hypothesis addressed specifically the role of cellular immunity in influenza, and accordingly we did not measure mucosal[45] or systemic innate immune responses, which have recently been shown to protect against severe influenza disease[46]. We excluded the contribution of crossreactive neutralizing antibodies, including hemagglutinin stem-specific antibodies that have been implicated in limiting disease severity[47], by purposefully selecting individuals lacking crossprotective neutralizing antibodies to pH1N1 virus using the highly sensitive microneutralization assay in addition to hemagglutination-inhibition assays. However, it is also conceivable that antibodies not detected by the standard assays, such as those to other viral proteins[48,49] or the recently reported non-neutralizing antibodies that can induce antibody-dependent cellular cytotoxicity *in vitro*[50], may have a role

in conjunction with cellular immunity in limiting influenza illness severity. This possibility would be of interest to evaluate prospectively during the next pandemic.

In summary, in a natural infection setting we identified a cellular immunological correlate of protection against symptomatic influenza and demonstrated a potential role for crossreactive CD8$^+$ T cells that are specific for highly conserved epitopes from core proteins in limiting illness severity during a pandemic. Taken together, these data provide the immunological basis for the design of universal influenza vaccines or modification of current influenza vaccines to induce heterosubtypic T cells that could confer protection against influenza-associated illnesses caused by viruses that inevitably evolve to circumvent humoral immunity.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

A.L. conceived the study, and S.S. and A.L. designed the study. S.S. coordinated the study. S.S., S.B., T.B., W.C., W.A., A.B. and K.H. performed the laboratory assays. S.S., A.L. and J.J.D. analyzed and interpreted the data. S.S., S.B. and A.L. drafted the article with critical revisions and important intellectual content provided by J.J.D., K.H. and W.B.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper.

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ONLINE METHODS

Study design and cohort. Healthy adult (>18 years) staff and students of Imperial College London were invited to participate. Individuals already vaccinated for influenza or likely to be offered pandemic vaccination (as per the UK government guidelines in September 2009) were ineligible. Written informed consent was obtained for all participants after study approval by the North West London Research Ethics Committee (study reference number 09/H0724/27).

Participants were recruited between September 13 and November 6 2009 and followed through the 2009–2010 and 2010–2011 influenza seasons, with blood collected at the start and end of each season (**Fig. 1**). Development of any symptomatic illness during each influenza season was recorded by the participants on a web-based symptom questionnaire every 3 weeks. The average response rate for the surveys was 75%. In addition, participants were provided with nasal swab packs and self-swabbing instructions and were requested to record their temperature, take a self sample and return nasal swabs when experiencing any influenza-like symptoms[51].

Laboratory assays. The procedures for the isolation of sera and PBMCs are detailed in the **Supplementary Methods**. Antibody titers to pH1N1 were measured by the hemagglutination inhibition (HI) assay that is used for UK national surveillance[52], with seroconversion being defined as a fourfold rise in HI titer on paired serum samples taken before and after each influenza season. As microneutralization assays are more sensitive than HI assays, we additionally undertook microneutralization assays on baseline serum samples to confirm seronegativity to pH1N1. The presence of virus in nasal swabs was confirmed by a multiplex real-time RT-PCR assay, which is more sensitive (and can detect virus for a longer period after symptom onset) than viral culture[53], using standard methods by the Health Protection Agencies of England[52] and Scotland[54].

Case definitions. Influenza (pH1N1)-infected individuals were defined as pH1N1-unvaccinated individuals with antibody seroconversion or detection of virus in nasal swabs. Among the infected individuals, symptoms reported in the online surveys were used to define the clinical outcomes associated with influenza infection.

Symptomatic infection was defined as having at least one reported episode of symptomatic illness. Asymptomatic infection, by our stringent definition, necessitated an absence of any reported illness episode. A total symptom score for each illness episode was calculated on the basis of an individual's assessment of the severity of their symptoms as none, mild (not interfering with normal daily activities) or severe (affecting normal daily activities or requiring medical attention) in addition to reporting recorded temperatures of ≥38 °C. We attributed a weighted score of 0 for none, 1 for mild and 4 for severe for each of the canonical influenza symptoms (sore throat, cough, headache, myalgia and fever), as has been used in previous clinical studies[55,56], to create a summed total symptom score.

Symptomatic outcomes associated with the illness episode were measured and categorized in four different ways: illness episode with any symptoms; illness episode with fever (recorded temperature ≥38 °C or reported fever rated as severe); illness episode with fever plus cough or sore throat (defined as an ILI by the US Centers for Disease Control and Prevention); and total symptom score for the illness episode.

***Ex vivo* FLISpot assay.** FLISpot (Mabtech AB, Stockholm, Sweden) to simultaneously measure IFN-γ and IL-2 secretion was undertaken as previously described[57] by investigators blinded to the clinical data. PBMCs were stimulated with peptide pools (5 μg ml^{-1}) of conserved class I–restricted 9-mer epitopes from PB1, M1 and NP proteins (n = 91 peptides; **Supplementary Table 1**), live pH1N1 virus (A/England/09/195) or CMV lysate as the control antigen (**Supplementary Methods**). The median background frequencies of the IFN-γ and IL-2 responses were 4 SFCs per million (IQR 0–12) and 16 SFCs per million (IQR 0–32) PBMCs, respectively. The frequencies of antigen-specific cells were calculated by subtracting the average number of SFCs in negative control wells from the number of SFCs in antigen-containing test wells for each donor. The frequency of total cytokine-secreting cells was calculated by summing the frequencies of the three functional subsets, IFN-γ^+IL-2^-, IFN-γ^-IL-2^+ and IFN-γ^+IL-2^+.

Flow cytometry assay. PBMCs were stimulated with RPMI medium (Sigma-Aldrich, USA) (negative control), phorbol myristate acetate (PMA) and ionomycin (positive control), live pH1N1 virus or CMV lysate for 18 h to maintain consistency with the FLISpot assay. Cells were stained for surface markers and intracellular cytokines as previously described[12], with at least 1 million live cells collected for all samples. See **Supplementary Figures 7** and **8** and the **Supplementary Methods** for the details and the gating strategy.

Sample size estimation. We estimated that 100 infected individuals with a prevalence of 60% asymptomatic infection would allow for the detection of a moderate effect size (odds ratio of 4) between symptomatic and asymptomatic infection for each tenfold increase in crossreactive T cell frequency (prevalence of 60%) with 80% power at $P = 0.05$ (two-tailed) significance. Based on 30% incidence of infection during the first wave of the UK pandemic[52], we calculated a sample size of 350 participants to attain 100 infected individuals.

Statistical analyses. Our primary objective was to identify whether individuals developing mild or asymptomatic illness had higher frequencies of crossreactive $CD8^+$ T cells before infection. Our analytical plan was based on our *a priori* hypothesis that crossreactive $CD8^+$ T cells specific for highly conserved virus-internal proteins would be associated with protection against symptomatic pandemic influenza illness. Our predefined analytical strategy was to correlate the frequencies of antigen-specific CD8+ T cells with symptom score and compare these frequencies across symptom groups. The frequencies of antigen-specific T cells between predefined groups categorized by increasing severity of symptoms (**Fig. 2**) were compared using nonparametric Mann-Whitney two-tailed test. Total symptom score reflecting illness severity as a continuous variable was correlated with the frequency of crossreactive T cells using Spearman rank correlation. The association of cellular immunity with infectiousness during an influenza episode was assessed by comparing the frequencies of T cells in individuals with viral shedding to those without viral shedding. Logistic regression was used to model the relationship between T cell frequency (SFCs per million PBMCs) and risk of clinical outcomes. Statistical analyses were undertaken using Stata version 10 (STATA Corp. Texas, USA).

Additional methods. Detailed methodology is described in the **Supplementary Methods**.

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