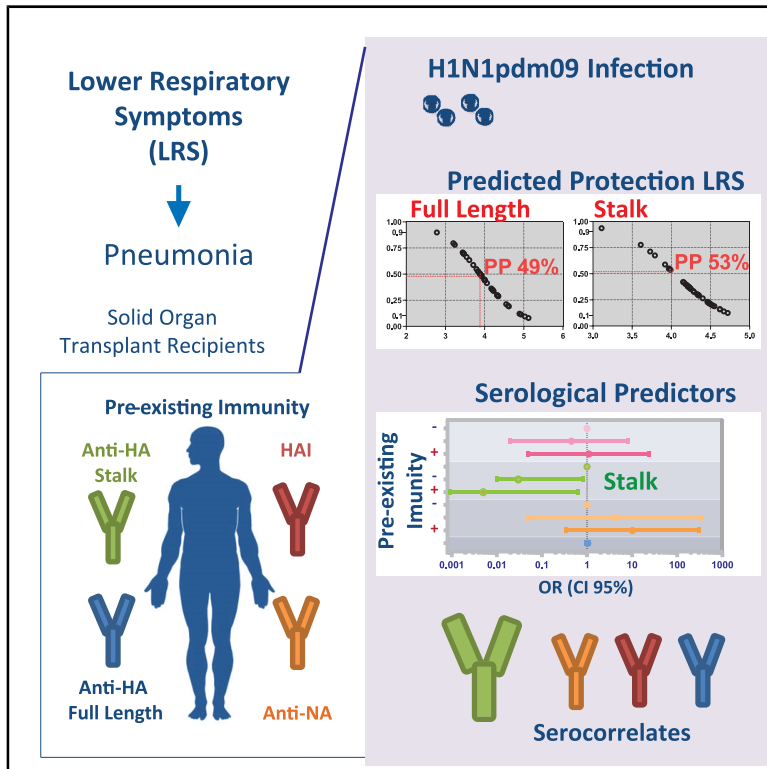


Pre-existing Hemagglutinin Stalk Antibodies Correlate with Protection of Lower Respiratory Symptoms in Flu-Infected Transplant Patients

Graphical Abstract



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In Brief

Aydillo et al. identify lower respiratory symptoms (LRSs) as a predictor of influenza pneumonia in a cohort of transplant recipients. When pre-existing immunity was characterized, the levels of anti-HA stalk antibodies correlated independently with protection from lower respiratory infection.

Highlights

- Solid organ transplant recipients (SOTRs) had low levels of HAI antibodies at baseline
- SOTRs have high levels of pre-existing, broadly cross-reactive anti-HA stalk antibodies
- Anti-HA stalk antibodies correlate with lack of lower respiratory symptoms in SOTRs
- Presence of lower respiratory symptoms is associated with influenza pneumonia



Report

Pre-existing Hemagglutinin Stalk Antibodies Correlate with Protection of Lower Respiratory Symptoms in Flu-Infected Transplant Patients

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SUMMARY

Hemagglutination-inhibitory antibodies are usually highly strain specific with little effect on infection with drifted or shifted strains. The significance of broadly cross-reactive non-HAI anti-influenza antibodies against conserved domains of virus glycoproteins, such as the hemagglutinin (HA) stalk, is of great interest. We characterize a cohort of 40 H1N1pmd09 influenza-infected patients and identify lower respiratory symptoms (LRSs) as a predictor for development of pneumonia. A binomial logistic regression of log₁₀ pre-existing antibody values shows that the probability of LRS occurrence decreased with increased anti-HA full-length and stalk antibody ELISA titers. However, a multilevel logistic regression model adjusted by other potential sero-correlates demonstrates that only antibodies directed against the stalk of HA correlate with protection from lower respiratory infection, limiting disease progression. Our predictive model indicates that a threshold of protective immunity based on broadly cross-reactive HA stalk antibodies could be feasible.

INTRODUCTION

The effect of influenza epidemics on society, including morbidity, mortality, and economical consequences, is still a major challenge and a public health concern.^{1,2} The World Health Organization (WHO) estimates that about 3–5 million cases of severe disease and 290,000–650,000 deaths annually are caused by influenza disease. Vulnerable populations, such as solid organ transplant recipients (SOTRs), are at high risk of severe outcomes with mortality rates from 5%–8% and complications in 5%–20% of cases.^{3,4} Although annual influenza vaccination can help reduce the burden of influenza disease,^{4,5} its efficacy

varies widely.^{6–8} Currently licensed influenza vaccines are formulated to mainly induce strain-specific antibody responses against the major surface glycoprotein of influenza virus, hemagglutinin (HA). However, influenza viruses have the ability to accumulate yearly mutations in HA, favoring emergence of antigenic variants in a phenomenon known as antigenic drift. This leads to the regular need to update influenza vaccines according to the circulating viruses in humans.⁹ Although cross-reactivity between antigenically related influenza viruses can happen, no protection would be provided by seasonal influenza vaccines in the case of emergence of a shifted pandemic strain, like what happened in 2009.^{10,11} Although the globular HA head domain



is immunodominant, eliciting potent neutralizing antibodies that can be measured by a hemagglutination inhibition (HAI) assay,¹² it is rather tolerant and permissive to mutations,¹³ enabling seasonal influenza viruses to escape pre-existing immunity in humans. Paradoxically, the HA head is the main target of influenza vaccines, and HAI titers in serum are traditionally used as a correlate of influenza vaccine-induced protection; a HAI titer of more than 40 is considered to provide protection in humans.¹⁴ In contrast, antibodies targeting other conserved regions of the HA surface protein, such as the stalk domain, can bind to a variety of influenza subtypes.^{12,15} In addition to being cross-reactive, HA stalk antibodies can also mediate Fc-Fc receptor (FcR) effector functions, such as antibody-dependent cell cytotoxicity (ADCC), potentially contributing to protection from disease through Fc-FcR interactions and engagement that results in apoptosis of infected cells and secretion of antiviral cytokines and chemokines.^{16,17} Antibodies targeting neuraminidase (NA), the second surface glycoprotein of influenza viruses, might also be more cross-protective than traditional HAI antibodies.^{18,19}

A growing interest regarding development of a universal influenza vaccine capable to provide long-term protection in humans against multiple strains and subtypes of the virus, including potential emerging pandemic strains, is guiding intense research efforts to identify new vaccine candidates and, therefore, new correlates of protection for influenza infection and disease.^{9,20–22} Several approaches have been proposed, including targeting conserved regions of the HA surface protein, such as the stalk domain.^{15,21,23} Although a few human studies have suggested that HA stalk and NA antibodies are additional independent correlates of protection from influenza infection, different from HAI antibodies,^{18,22,24} more clinical and epidemiological data are needed to demonstrate antibody association with severe/mild disease versus infection and transmission. Studies in SOTRs might provide valid information regarding correlates of immune protection against influenza disease. First, severe disease outcomes are more frequent in such patients,^{3,4} which reduces the number of individuals in the cohort required to identify a significant number of cases with different disease outcomes. Second, the need to constantly monitor the health status of SOTRs allows frequent clinical assessment of infectious diseases, including influenza virus infection, and monitoring of immune responses to influenza vaccine and/or infection at baseline and post-vaccination/infection.

Baseline antibody responses in humans depend on the first encounter with influenza virus antigens, usually at an early age, and on repeated exposure during the lifetime through repeated infection and vaccination.^{25,26} Because of the age of SOTRs, one expects diverse antibody responses in these individuals against different viral strains because of their differences regarding virus and vaccine exposure. Antibody responses induced by natural infection compared with vaccination can differ, with induction of broader and longer-lived antibody responses in the first. Here we aimed to identify whether antibodies against conserved or non-conserved antigens of influenza virus glycoproteins (HA head, HA stalk, and NA) correlate with the burden of influenza H1N1pdm09 disease in a clinical cohort of naturally influenza virus-infected SOTRs. We sought to identify

anti-influenza antibody levels of protection by characterizing risk factors of severe outcomes, such as development of lower respiratory infection in SOTRs and determining how broadly and non-broadly pre-existing specific influenza antibodies correlate with clinical outcome. Last, we investigated which antibody levels can predict protection from severe clinical outcomes after adjustment for confounders, including other potential serocorrelates.

RESULTS

SOTRs Show High Levels of Pre-existing Anti-HA Antibodies

A total of 127 SOTRs were included in the naturally influenza infected cohort between 2010 and 2013 (Figure S1A). Of the 127 SOTRs, 118 had a positive RT-PCR for influenza virus, and a total of 74 (62.7%) had paired serum samples at enrollment (baseline) and convalescent time points. Of those, 40 (54%) were H1N1pdm09, 23 (30%) were H3N2, and 11 (16%) were influenza B virus infections. The levels of pre-existing HAI active antibodies at onset of the infection in the influenza A cases were 14 (35%) and 12 (53%) for H1N1pdm09 and H3N2, respectively. For this report, only H1N1pdm09-infected SOTRs with a full set of serum samples were considered. High levels of HAI antibodies in SOTRs with H3N2 infection limited assessment of the potential role of non-HAI and NA antibodies; low levels of HAI in H1 influenza cases allowed us to investigate the effect of other potential serocorrelates. No influenza B cases were included because of the limited sample size and change of predominantly circulating influenza B virus strains between seasons. Serum samples from 9 SOTRs with suspected influenza virus infection (but who tested negative by RT-PCR) were included for comparison. No follow-up samples were collected from these patients. Most of the H1N1pdm09-infected cohort represented individuals enrolled in the 2010–2011 post-pandemic season (34, 85%); four (10%) and two (5%) patients were enrolled in the 2011–2012 and 2012–2013 influenza seasons, respectively.

More SOTRs were men (57.7%), and the median age was 57 years (interquartile range, 27–77 years). The type of transplant was kidney in 22 cases (55%), liver in 10 (25%), and heart in 6 (15%). Only 2 (5%) had a lung transplantation. Comorbidities such as diabetes or other chronic diseases were present in 72.5% of cases (Figure S1B). Baseline antibody titers (Figure 1A) demonstrated high levels of pre-existing anti-influenza stalk and full-length HA; only a few SOTRs (9, 22.5%) had HAI titers of 40 or higher. Interestingly, pre-existing antibody titers against the HA and NA protein showed high associated ADCC activity (Figure 1B).

HA Stalk Antibodies Correlate with Protection against LRSs in Influenza Virus-Infected SOTRs

Twenty-five (62.5%) SOTRs required hospitalization. All naturally influenza-infected SOTRs received antiviral therapy with oseltamivir. Most of them (33, 82.5%) started treatment within 48 h of onset of symptoms (mean; 95% confidence interval [CI], 1.7; 0.2–3.1 days). A high proportion of patients reported upper respiratory and systemic symptoms, such as headache, fever, or

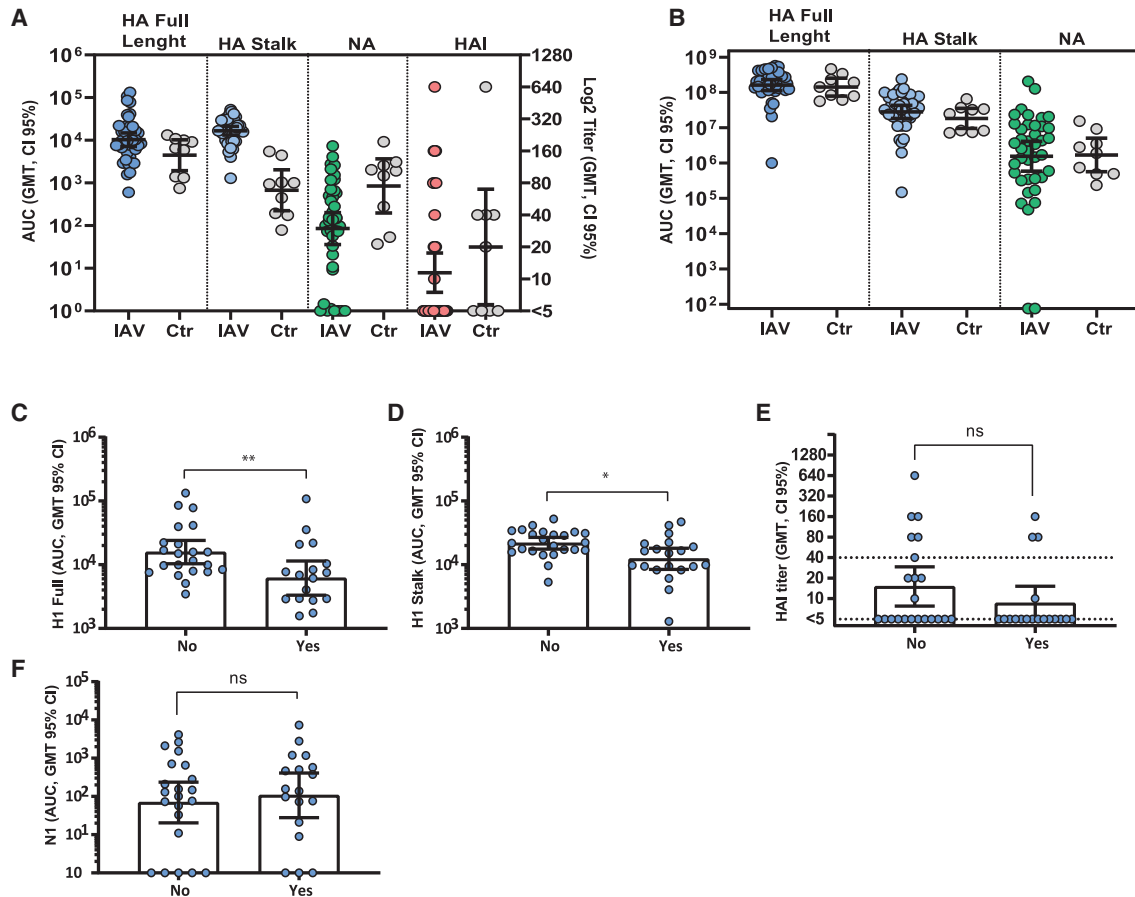


Figure 1. Immunological Features in the H1N1 Influenza-Infected SOTR Cohort

(A and B) Pre-existing antibody titers in SOTRs with H1N1pdm09 influenza virus infection ($n = 40$) compared with non-infected matched SOTRs controls ($n = 9$). The antibody level for each patient, GMT, and 95% CI are shown (A) for the indicated ELISA assay on the left y axis (full-length HA, stalk HA, and NA recombinant proteins as antigen) and HA-inhibiting (HAI) antibody titers on the right y axis. The HAI assay was performed using the whole H1N1pdm09 reference strain virus. (B) shows antibody-dependent cellular cytotoxicity (ADCC), measured by a reporter assay for full-length HA, stalk HA, and NA. (C–F) Pre-existing antibody responses in SOTRs with LRSs at baseline. The antibody level for each patient and time point, GMT, and 95% CI are shown for (C) full-length HA ELISA, (D) stalk HA ELISA, (E) HAI, and (F) NA ELISA (ns, not significant; *** $p = 0.01$; ** $p = 0.006$; * $p = 0.03$). Experiments were performed in triplicates.

myalgia (26, 65% and 25, 62.5%, respectively) at enrollment. Acute lower respiratory symptoms, such as dyspnea or tachypnea, were noted in 18 (45%) SOTRs. Twelve (30%) patients developed pneumonia. Of those, four (33.3%) required admission to an intensive care unit (ICU), and two (16.6%) died. Co-infection was detected in one patient with pneumonia and one without: *Streptococcus pneumoniae* and *Pseudomonas aeruginosa*, respectively. Clinical characteristics, treatment, and outcomes of the naturally influenza virus-infected SOTRs are shown in Figure S2A. The presence of upper respiratory and systemic symptoms was less common in pneumonia influenza virus-infected SOTRs. Conversely, SOTRs with acute LRSs had a higher risk of worsening influenza virus infection and pneumonia development; 50% (9 of 18) SOTRs with LRSs had pneumonia, and only 13.6% (3 of 22) of SOTRs without LRSs developed viral pneumonia (odds ratio [OR] 95% CI, 6.3; 1.3–29.2; $p = 0.01$). No other risk factors for development of pneumonia were found

(Figure S2B). Bivariate analysis of the multiple serological surrogates demonstrated no statistical differences in levels of circulating pre-existing antibodies at baseline in SOTRs with upper respiratory or systemic symptoms, but an inverse correlation was found when we compared geometric mean titers (GMTs) in SOTRs with LRSs. As shown in Figures 1C–1F and Table 1, patients with LRSs had lower GMTs against the full-length (6,170 versus 15,746; $p = 0.006$) and stalk (12,834 versus 21,558; $p = 0.03$) HA protein, as measured by ELISA. More importantly, the currently accepted correlate of protection, HAI titer, was similar in patients regardless of symptomatology. Although 25 patients (62.5%) received the corresponding season-matched influenza vaccine at least 6 weeks before the episode of influenza virus infection, only 14 (56%) of the influenza vaccinated SOTRs had pre-existing detectable levels of HAI antibodies, and only 9 (36%) of them were scored as seroprotected (HAI titers ≥ 40) (Figure 1A).

Table 1. Pre-existing Antibody Levels in SOTRs According to Symptomatology

	Yes GMT (95% CI)	No GMT (95% CI)	p Value
Upper Respiratory Symptoms			
HAI	10 (6–16)	15 (6–40)	0.62
HA hull length ELISA	8,918 (5,598–14,208)	13,568 (6,759–27,238)	0.45
HA stalk ELISA	16,325 (11,729–22,724)	18,545 (13,575–25,334)	0.68
NA ELISA	134 (48–368)	37 (7–196)	0.22
Systemic Symptoms			
HAI	12 (7–20)	12 (6–25)	0.9
HA full length ELISA	10,136 (5,700–18,023)	10,571 (63,04–17,725)	0.84
HA stalk ELISA	14,713 (10,371–20,873)	20,470 (15,030–27,877)	0.14
NA ELISA	109 (45–263)	63 (12–340)	0.77
Lower Respiratory Symptoms			
HAI	8 (5–15)	15 (7–29)	0.21
HA full length ELISA	6,170 (3,327–11,440)	15,746 (10,341–23,974)	0.006
HA stalk ELISA	12,834 (8,380–19,655)	21,558 (17,165–27,075)	0.03
NA ELISA	106 (27–408)	70 (21–237)	0.67

We then selected presence of LRSs at baseline as a predictor of disease outcome in SOTRs to discriminate HA full length and stalk cutoff points correlating with protection from lower respiratory tract influenza virus infection. The plotted predicted probability curve for HA full length and stalk (OR, 0.13; 95% CI, 0.02–0.74; $p = 0.02$ and OR, 0.06; 95% CI, 0.004–0.85; $p = 0.03$, respectively) demonstrated that the probability of developing LRSs decreased with increasing log₁₀ values for titers, and $\sim 10^4$ full-length and stalk antibodies showed $\sim 50\%$ predicted protection in SOTRs (Figures 2A and 2B). The fitted curves of the relationship between log₁₀ assay values and the probability of protection were next used to estimate protective thresholds and intervals of antibody titers for HA full length and stalk as 0–10,000, 10,000–25,000, and more than 25,000. We then defined intervals of protection according to the proportion of patients with LRSs in those selected cutoffs. As shown in Figures 2C and 2D, only 25% of SOTRs (3 of 12) with HA stalk antibodies higher than 25,000 had LRSs; most SOTRs (80%, 8 of 10) with antibodies against the HA stalk lower than 10,000 presented LRSs at onset of the influenza episode. This was similar for antibodies against HA full length higher than 25,000. Receiver operating characteristic (ROC) curve analysis was also performed to estimate the sensitivity and specificity of HA full length and stalk ELISA titers for predicting LRSs. The results showed an area under the curve (AUC) of 77% and 76% for HA full length and stalk, with a sensitivity and specificity of predicting LRS of 60% and 70%, respectively ($p < 0.005$).

We next performed a multivariate logistic regression analysis including our potential serological predictors (HA full length and stalk antibody titers) and adjusted by HAI and NA ELISA titers to assess the balance of the antibody responses that correlated independently with the protection afforded. Age, type of transplant, and influenza vaccination status were also included in the model to control variability between SOTRs (Figure S3). Immunosuppressive drugs were included in an alternative model, but no predictive value was found (data not shown). In

this multilevel analysis, we categorized antibodies for HA full length and stalk antibody titers in the levels of protection observed (0–10,000; 10,000–25,000, and >25,000). Because mean NA antibody levels were 10 times lower than those for the HA protein at baseline (Figure 1A), we defined the intervals as 0–1,000, 1,000–2,500, and more than 2,500. HAI titer was categorized at two levels: less than 40 (no seroprotection) and 40 or more (seroprotection). The adjusted model shown in Figure 2D demonstrates that anti-HA stalk antibodies were associated independently with protection from development of acute LRSs (10,000–25,000; OR, 0.03; 95% CI, 0.01–0.83; $p = 0.03$ and >25,000; OR, 0.005; 95% CI, 0.00–0.62); no protection was observed for stalk titers lower than 10,000. Likewise, HA full length pre-existing titers showed no significant protection when adjusted by levels of stalk titers. A previous history of vaccination was also associated with lower risk of LRSs (OR, 0.02; 95% CI, 0.001–0.52; $p = 0.01$). Remarkably, a linear regression analysis demonstrated good correlation between values of anti-HA stalk antibodies measured by ELISA and stalk-mediated ADCC activity (Figure 2E; $p < 0.0001$), an important mechanism of antibody-mediated protection in humans.²⁷ Despite the fact that whole HA- and HA stalk-binding antibodies at baseline correlated inversely with LRSs and that LRS symptoms at onset were the best predictors of pneumonia, when the analysis of serocorrelates was restricted to prevention of development of pneumonia during the course of influenza virus infection, we found no direct association of any of the potential serological surrogates, suggesting that some other events, such as colonization with pathogenic bacteria, might also influence the outcome of pneumonia. Alternatively, our study did not have enough power to find correlations associated with development of pneumonia. Nevertheless, there was a clear correlation between HA stalk antibody titers and lack of LRSs. To determine whether influenza vaccination can also induce HA stalk antibodies in SOTRs, we compared the effect of vaccination versus infection in a second cohort of seasonal influenza-vaccinated

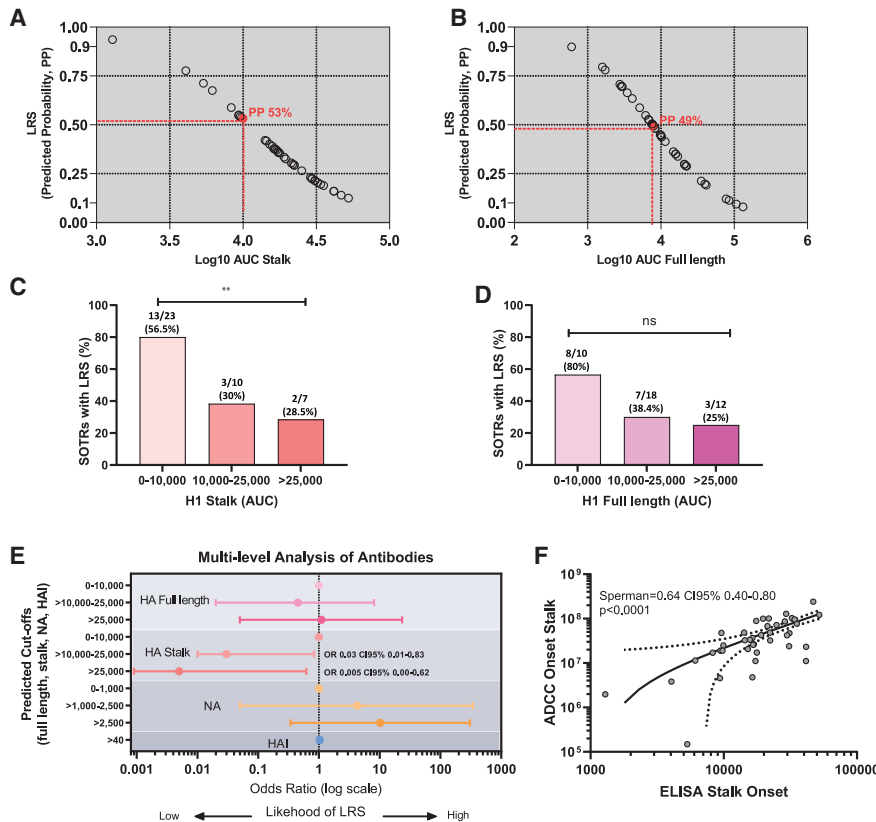


Figure 2. Anti-Stalk HA Antibodies as an Independent Correlate of Protection against Development of LRSs

(A and B) Binomial logistic regression of log₁₀-transformed values of full-length and stalk HA: fitted curves of the relationship between log₁₀ assay values and the probability of protection. A red dot indicates predicted protection of ~50%. (C and D) Bars shows the proportion of SOTRs with LRSs according to defined antibody cutoffs. Each bar shows the number and percentage of SOTRs with LRSs among the total number of SOTRs with each antibody interval. **p = 0.02.

(E) Multivariate logistic regression model of factors correlated with LRSs at baseline on SOTRs with influenza infection. The model is adjusted by potential confounding factors to estimate the independent effect of antibody titers on disease progression and the serological predictors (Figure S3). The adjusted odds ratio (OR; 95% CI) for each assay and each level are shown.

(F) Linear regression of log₁₀-values of anti-stalk antibodies measured by ELISA and anti-stalk antibodies with mediated ADCC activity measured in a bioreporter assay. ADCC experiments were performed in triplicate.

SOTRs enrolled during the 2010–2011 and 2011–2012 influenza seasons and randomly selected 20 seasonal influenza-vaccinated SOTRs for both influenza seasons (10 each). Both cohorts matched in age, type of transplant, comorbidities, immunosuppressive drug regimen, and time from transplantation. Overall, the humoral immune response was greater after natural influenza virus infection compared with vaccination (Figure S4). Although infection induced HAI activity, full-length HA and stalk binding, NA binding, and ADCC responses against influenza virus antigens, vaccination failed to significantly induce stalk- and NA-specific antibodies.

SOTRs Show a Significant Increase of Anti-Influenza Antibodies after Infection

To characterize the immune response after influenza disease, we also measured HAI and non-HAI antibody levels and associated ADCC activity in convalescent sera of SOTRs. As expected, a significant increase compared with baseline levels was shown after influenza infection for all potential serocorrelates (Figure 3A). To quantify induction of *de novo* antibody response according to disease outcome, we calculated fold induction log₁₀ values in SOTRs. Overall, patients with LRSs, and likely lower respiratory infection, at hospital admission had higher induction of anti-influenza antibodies (Figures 3B–3E), although this was only statistically significant for *de novo* NA antibodies produced after influenza virus infection (Figure 3E). These antibodies showed a good correlation with NA-mediated ADCC activity (Figure 3F). In contrast, SOTRs that had HAI titers of 40 or higher

at baseline had lower levels of *de novo* antibodies against the HA and NA protein of influenza virus. Unexpectedly, a negative correlation between pre-existing antibodies against the HA head with HAI activity and induction of full-length and stalk HA and NA titers after influenza virus infection was found (Figures 4A and 4B). Although HAI titers higher than 40 at hospital admission did not prevent severe disease in SOTRs, we found that SOTRs scored as seroprotected (HAI ≥ 40) at baseline had lower induction of HA (full length and stalk) and NA antibodies, measured by ELISA (Figures 4C and 4D) after the influenza episode.

DISCUSSION

Influenza virus HA-inhibitory activity has been traditionally acknowledged as a correlate of protection for influenza vaccine efficacy. However, head-specific HA-neutralizing antibodies have been only modestly associated with protection against infection with drifted and shifted influenza viruses in humans, and the role of heterosubtypic antibodies capable of inducing additional immune functions is not clear. Although the influenza virus continues to evolve every year because of antigenic drift, humans face new exposure to the influenza virus through infection and repeated vaccination, making immune responses complex and multifaceted. Here we present a detailed analysis of humoral immunological correlates associated with protection from disease progression in a clinically characterized cohort of 40 SOTRs naturally infected with the H1N1 influenza virus. We analyzed the levels of anti-influenza virus antibodies targeting the two surface glycoproteins HA and NA and the HA stalk domain in patient sera and their associated ADCC activity. Our

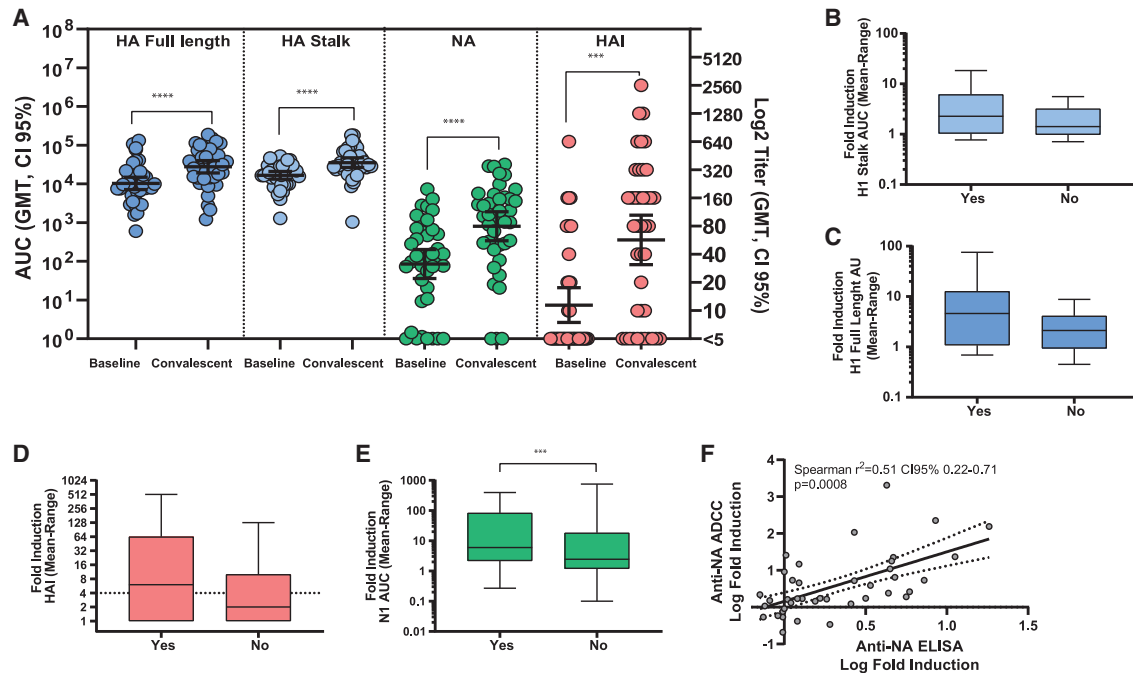


Figure 3. Antibody Responses after Influenza Infection in SOTRs

(A) Paired antibody levels for each patient, GMT, and 95% CI are shown at baseline and convalescence for the indicated ELISA assay on the left y axis (full-length HA, stalk HA, and NA) and hemagglutination inhibition (HAI) antibody titers on the right y axis.

(B–E) Fold induction log values after influenza virus infection according to presence of lower respiratory symptoms (LRSs; yes or no), GMT, and 95% CI are shown for (B) stalk HA, (C) full-length HA, (D) HAI, and (E) NA.

(F) Linear regression of log fold induction values of anti-NA antibodies measured by ELISA and anti-NA antibodies with ADCC activity.

Experiments were performed in triplicate.

results showed that pre-existing HA-binding antibodies (ELISA) were associated with a lower risk of development of LRSs in the setting of influenza virus infection; as the antibody titer increased, the frequency of symptoms decreased (Figures 2B and 2C). Indeed, our predictive model suggests that, among non-HAI antibodies, a stalk antibody titer higher than 10^4 , as measured by ELISA, would suffice to protect at least 50% of patients (as shown in the predicted probability curve in Figures 2A and 2B). Even though the presence of LRSs at hospital admission was a potent predictor of development of pneumonia during the influenza episode, no differences in antibody titers were found when comparing pneumonia and SOTRs without pneumonia. It is likely that progression to pneumonia, especially in SOTRs with substantial morbidity and chronic immunosuppression, involves other immunological or predisposing factors that are not accounted for in our study, such as T cell immunity or bacterial coinfection. In fact, 3 patients (13.6%) developed pneumonia regardless of the absence of LRSs at the enrollment. Likewise, the rate of previous influenza vaccination was more than 60% among SOTRs, but we found no evidence that HAI antibodies protected from disease burden, perhaps in part because of the titers being in the low range, suggesting that, when HAI antibodies fail to confer protection from symptomatic influenza infection, pre-existing HA stalk antibodies protect against severe disease in SOTRs. Our results agree with recent data where HA stem antibodies correlated with protection from infection in natu-

rally exposed individuals and experimental human challenges independent of HAI antibodies.^{24,28–31}

In addition to direct antiviral activity, some broadly influenza cross-reactive antibodies can mediate FcR functions against infected cells. Our data showed a good correlation (Spearman correlation coefficient, $r^2 = 0.64$; Figure 2F) between levels of anti-HA stalk antibodies and stalk ADCC activity, measured by a bioluminescence reporter assay,²⁷ suggesting that the ability to recruit cell-mediated functions can also contribute to protection from LRSs and, therefore, disease progression. A higher increase in HA and NA antibodies with ADCC activity was also noted after influenza virus infection compared with after vaccination (Figures 3C–3E), which was expected because natural infection is known to induce anti-stalk antibodies whereas vaccination with currently licensed vaccines does not.¹² Although current influenza vaccinations are potent inducers of anti-HA head antibodies⁹ with anti-agglutinating function, our data show limited induction of antibodies with other specificities by the seasonal influenza vaccine (Figure S4). This is not necessarily surprising because influenza vaccines are optimized for HA content, and the HA head is immunodominant. Nonetheless, a previous history of vaccination was associated independently with a lower incidence of LRSs in multivariate logistic regression (Figure S3); however, no differences were found in antibody titers between patients with influenza vaccination compared with unvaccinated ones (Figure S5). Whether this is due to the need

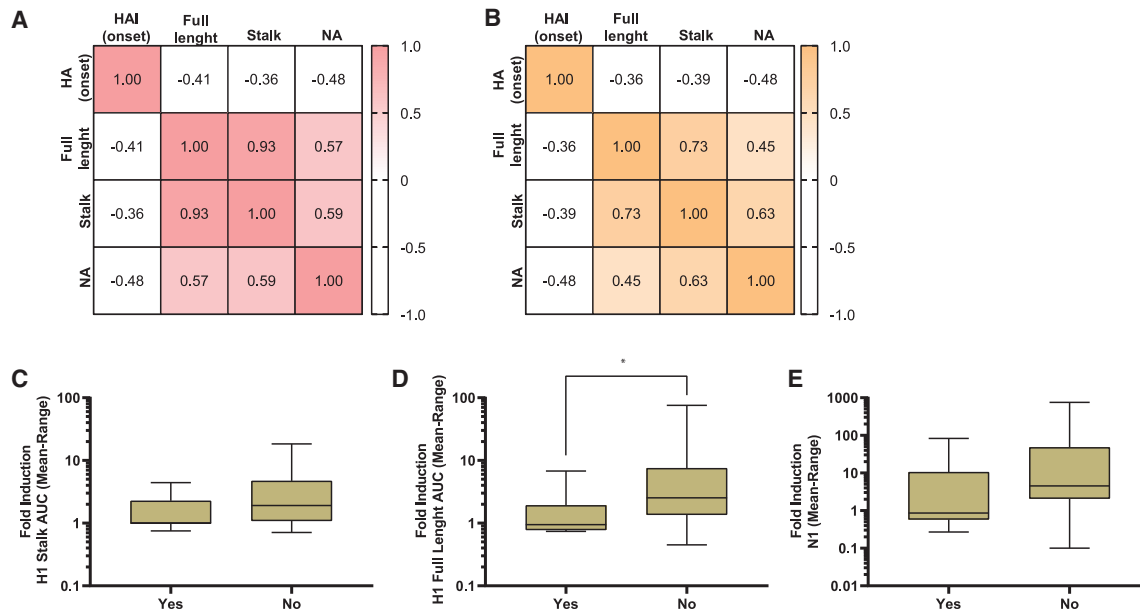


Figure 4. Influence of Pre-existing Immunity on Subsequent Immune Response in Influenza-Infected SOTRs

(A and B) Correlation matrix showing Spearman's correlation coefficient between levels of HAI antibodies at onset and fold induction ELISA titers and ADCC activity, respectively, at convalescence after influenza infection. Strength of the association of HAI at the onset with ELISA full-length HA ($p = 0.02$), ELISA stalk HA ($p = 0.01$), ELISA NA ($p = 0.002$), ADCC full-length HA ($p = 0.009$), ADCC stalk HA ($p = 0.02$), and ADCC NA ($p = 0.002$). * $p = 0.04$. (C) Fold induction log₁₀ values after influenza infection. GMT and 95% CI are shown for patients with a pre-existing HAI titer of 40 or higher (seroprotection) in comparison with those with an HAI titer of less than 40 (no seroprotection). Experiments were performed in triplicate.

for additional immunological assays to further characterize the correlates of protection associated with the seasonal influenza vaccine or due to the sample size of our study, which might be underpowered to address this question, remains unanswered.

In summary, our results have significant implications for prevention and control of influenza virus infection. We provide a more granular characterization of the specificity of antibodies after natural influenza virus infection and vaccination in SOTRs. In addition, we provide evidence that supports the ability of cross-reactive antibodies in limiting clinical disease progression in naturally influenza virus-infected individuals. We also confirmed that natural infection is a better inducer of cross-reactive protective antibodies than seasonal vaccination, underscoring the need for improved influenza vaccines. This study contributes to a better understanding of the mechanisms that mediate the pathogenesis of influenza disease and induction of broad humoral immune responses, which will aid the design of better, perhaps even universal influenza vaccines. Our data indicate that a threshold of protective immunity based on broadly cross-reactive anti HA-stalk antibodies could be feasible.

Limitations of Study

Some limitations of our study need to be acknowledged. First, the number of subjects with available paired serum samples at the end of the enrollment period is relatively small compared with the total SOTR cohort included in the study. Different influenza virus strains circulated during three consecutive seasons: pandemic H1N1pdm09 and seasonal H3N2 and influenza B.

As expected, the levels of pre-existing HAI active antibodies against the H1 pandemic strain were low compared with the seasonal H3 strain. Indeed, only 35% of SOTRs with pandemic influenza infection had some detectable levels of HAI active antibodies, which allows assessment of the protective role of other non-HAI cross-reactive antibodies in disease burden. On the other hand, the numbers of H3N2 and influenza B cases were low compared with H1N1pdm09, limiting conclusions regarding the role of influenza A group 2 and influenza B stalk antibodies. Our study shows that the presence of stalk antibodies against H1N1pdm09 group 1 HA correlated with lack of LRSs in SOTRs with influenza infection. Whether group 2 HA or influenza B stalk antibodies have a protective effect on influenza disease outcome is still not clear. Second, we defined stalk antibodies levels of protection in a complex population of SOTRs with different ages, types of transplants, and immunosuppressive drug therapies. Although immunosuppression has been associated with a lower immune response after influenza infection and vaccination in SOTRs,^{3,7,32} no correlation was found among the different combinations of drug therapies or individual treatments and disease outcome. It is likely that the complexity of treatment, with concomitant drugs according to time from transplantation, variability of combinations, and different drug doses, limits possibilities to assess the influence of specific therapies (e.g., mammalian target of rapamycin [m-TOR] inhibitors). Although our results cannot be fully translated to other populations, such as healthy adults, they provide a better understanding of new serocorrelates that can mediate influenza disease protection in

immunosuppressed subjects, especially important because of the high frequency of severe outcomes. Last, it is likely that some SOTRs with very mild or asymptomatic influenza infection did not go to the hospital despite having been advised to seek care in the case of influenza-compatible respiratory symptoms. Cases of influenza infection that required hospital admission were included in this study, allowing characterization of the immune response and risk factors of severe outcome in SOTRs with influenza infection.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.xcrm.2020.100130>.

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AUTHOR CONTRIBUTIONS

T.A., E.C., and A.G.-S. conceived and designed the study. T.A. collected samples and data, performed experiments, analyzed data, and wrote the report. A.E. performed experiments. S.S., S.A., J.A., and F.K. provided reagents, methods, and expertise. P.P.-R., C.R.-O., M.M., J.G., P.M., F.L.-M., J.S.-C., and J.C. collected samples and data. T.A., E.C., and A.G.-S. supervised the study. All authors reviewed and edited the paper.

DECLARATION OF INTERESTS

A.G.-S. is an inventor of patents owned by the Icahn School of Medicine at Mount Sinai in the field of influenza virus vaccines. The A.G.-S. lab has received research funds from Avimex, GSK, and 7Hills to investigate novel influenza virus vaccines. F.K. is a named inventor of technology in the field

of human influenza vaccines and a named co-inventor on patents to develop chimeric HA technology to develop a universal influenza vaccine.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Peroxidase-conjugated anti-human IgG (Fc-specific) monoclonal antibody	Sigma	Cat# A0170, RRID:AB_257868
Human monoclonal antibody CR9114	Florian Krammer's laboratory stock	N/A
Human monoclonal antibody 1000-3C05	Florian Krammer's laboratory stock	N/A
Bacterial and Virus Strains		
A/California/7/2009-H1N1	Adolfo García-Sastre's laboratory stock	ATCC, Cat# VR-1894
Biological Samples		
Human Sera	This study	N/A
Chemicals, Peptides, and Recombinant Proteins		
cH6/1 HA	Florian Krammer's laboratory stock	N/A
H1N1 A/California/4/2009 NA	Florian Krammer's laboratory stock	N/A
A/California/4/2009 HA	Florian Krammer's laboratory stock	N/A
Receptor-destroying enzyme	Denka Seiken	Cat#370013
Fluorescent Treponemal Antibody hemagglutination buffer	BD Biosciences	Cat# 211248
Phosphate Buffered Saline	GIBCO	Cat# 10010023
Turkey red blood cells	Lampire Biologicals	Cat#7209403
Tween-20	Fisher Scientific	Cat#BP337-100
Goat serum	GIBCO	Cat#16210072
Peroxidase-conjugated anti-human IgG (Fc-specific)	Sigma	Cat# A0170
Sulfuric acid solution	Fisher Science	Cat#S25898
3,3',5,5'-Tetramethylbenzidine	Rockland	Cat# TMBM-100
Non-fat powdered milk	Boston Bioproducts	Cat#P-1400
Opti-MEM (Minimal Essential Medium) Reduced Serum Medium	GIBCO	Cat# 31985062
Roswell Park Memorial Institute (RPMI) 1640 medium	GIBCO	Cat# 11875093
Critical Commercial Assays		
ADCC Reporter Bioassay	Promega	Cat# TM387
Experimental Models: Cell Lines		
MDCK	ATCC	Cat#CCL-34
Recombinant DNA		
pCAGGS	Adolfo Garcia-Sastre's laboratory stock	N/A
Software and Algorithms		
GraphPad Prism v8.3.1	Graph Pad Software	N/A
IBM SPSS Statistics (version 21)	IBM	N/A

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Adolfo García-Sastre (adolfo.garcia-sastre@mssm.edu).

Materials Availability

MDCK cell lines expressing cH6/1 HA, Cal09 HA and Cal09 NA generated in this study can be distributed upon request.

Data and Code Availability

The published article includes all datasets generated or analyzed during this study.

EXPERIMENTAL MODEL AND SUBJECTS DETAILS

A multicenter prospective human cohort study was carried out by the Spanish Network for Research on Infectious Diseases (REIPI, Spain) during three consecutive influenza seasons (2010–2013). This study was designed with two primary objectives, to evaluate the clinical characteristics, outcomes and immune responses after natural influenza virus infection^{3,4} and to evaluate the immunological response to seasonal influenza vaccination in SOTRs.³² For this all suspected SOTRs ≥ 16 years admitted to the hospitals with influenza-like syndrome, fever, exacerbation of pulmonary disease and/or pneumonia (new pulmonary infiltrate in the chest X-ray), without known cause were evaluated by infectious diseases medical staff. Symptomatic SOTRs that seek care in emergency room or had other consultations at the hospital as a part of the transplantation routine care were also screened. A positive case was defined as the presence of influenza-like illness with laboratory-confirmed influenza infection by reverse-transcription polymerase chain reaction (RT-PCR). A second cohort of SOTRs (≥ 16 years old) who received the seasonal influenza vaccine was enrolled during two consecutive influenza vaccine campaigns (2010–2012). In this cohort, SOTRs were excluded if they received the transplant less than one month prior immunization, if allergic to chicken egg proteins or any vaccine component, and if pregnant. Collection of data on both cohorts included demographics, (age and gender), co-morbidities (chronic obstructive pulmonary disease (COPD), diabetes mellitus, chronic heart, liver or kidney disease), history of influenza vaccination, type of transplant, time from transplantation and immunosuppressive drug regimen. Clinical signs and symptoms (lower respiratory (LRS): dyspnea, tachypnea; upper respiratory (URS): rhinorrhea, throat ache; and systemic: headache, fever, myalgia), chest X-ray findings, concomitant and secondary bacterial-fungal infections (if isolated from sputum, bronchoalveolar lavage and/or blood), and/or positive urinary antigen detection, and outcomes, including mortality were recorded in the naturally infected SOTR. Adverse effects after influenza vaccination, including graft rejections and mortality, were recorded in the vaccinee cohort. Serum samples were collected at enrollment and approximately 28 days post-enrollment and cryopreserved. In the case of naturally influenza virus-infected patients, an initial sample was collected within the inclusion in the study (baseline). A pre-vaccination (pre-TIV) sample was collected before influenza vaccination in the SOTR vaccination cohort. A subsequent visit took place in both cohorts and samples were collected for each participant 28 days after inclusion (convalescence and post-TIV, respectively). All patients or their legally authorized representatives provided informed consent. The study protocol was approved by the institutional review board of University Hospital Virgen del Rocío, Seville, Spain and related participant hospitals and by the Icahn School of Medicine at Mount Sinai, New York. This study was carried out strictly following ethical regulations of the Helsinki Declaration and the guidelines on good clinical laboratory practice. Vaccinated SOTRs received the trivalent non-adjuvant inactivated vaccine recommended for each influenza seasons (2010–2011 and 2011–2012; Gripavac, Sanofi-Pasteur MSD, Madrid, Spain) as part of the standard of care on immunosuppressed patients.³³ A summary of demographic parameters and clinical characteristics of SOTR with influenza infection is included in [Figures S1](#) and [S2](#).

METHOD DETAILS

Hemagglutination Inhibition (HAI) Assay

SOTRs serum samples were incubated overnight with receptor-destroying enzyme (RDE; Denka Seiken) for 16–18 h in a 37°C water bath. Three volumes (relative to serum) of 2.5% sodium citrate solution was added and RDE were heat inactivated at 56°C in a water bath (30 minutes). Final serum dilutions were adjusted to 1:10 in PBS. Reference virus strain A/California/7/2009-H1N1 was diluted to a final concentration of 8 HA units/50 μ L in Fluorescent Treponemal Antibody (FTA) hemagglutination (HA) buffer (BD Biosciences). Two-fold dilutions of RDE treated serum (25 μ L) were incubated with equal amount of the virus at 8 HA units/50 μ L (30 minutes, room temperature). Turkey red blood cells (RBCs) (Lampire Biological) at 0.5% in HA buffer (50 μ L) were added and incubated 45 minutes at 4°C. The HAI titer was determined by taking the reciprocal dilution of the last well in which serum inhibited the hemagglutination of RBCs. Sera were considered positive based on international criteria of seroprotection if neutralizing titers measured were ≥ 40 .^{7,22}

Enzyme-linked Immunosorbent Assay (ELISA)

The recombinant proteins cH6/1³⁴ HA [containing an H6 head domain (H6N1 virus A/mallard/Sweden/ 81/02) from wild bird origin, and hence no specific antibodies should be present in the SOTRs sera, in combination with an H1 stalk domain (H1N1 A/California/4/2009)], full length H1 HA and N1 NA (H1N1 A/California/4/2009) were generated in insect cells by using a baculovirus expression system using a previously described protocol.^{35,36} Flat-bottom 96-well plates (Immulon 4 HBX; Thermo Fisher Scientific) were coated with 6 μ g/ml of cH6/1 HA, full length H1 HA or N1 NA proteins in PBS (GIBCO) and incubated at 4°C overnight. Next, plates were washed 3 times with washing buffer (PBS containing 0.1% Tween-20; Fisher Scientific). Plates were incubated 1.5 hours at room temperature with blocking solution (washing buffer containing 0.5% non-fat powdered milk, Boston BioProducts, and 3% goat serum, GIBCO). Blocking solution was removed and two-fold dilutions of serum (starting 1:800) were added to each well and incubated for 1.5 hours at room temperature. Plates were then washed 3 times with washing buffer and a peroxidase-conjugated anti-human IgG (Fc-specific) monoclonal antibody (Sigma) was added at a final concentration of 1:20,000 in blocking solution. After washing, 100 μ L of peroxidase substrate (3,3',5,5'-Tetramethylbenzidine, TMB, Rockland) was added and incubated at

room temperature for 30 min. The reaction was stopped with 1 N sulfuric acid solution (Fisher Science). The absorbance was measured at 450 nm with a plate spectrophotometer (Synergy H1 hybrid multimode microplate reader, Biotek). Optical density (OD) for each well was calculated by subtracting the average background plus three standard deviations. Area under the curve (AUC) was computed using GraphPad Prism software.

Antibody-dependent Cell Cytotoxicity (ADCC) Bioassay²⁷.

Madin-Darby canine kidney (MDCK) stably expressing cH6/1 chimeric HA,³⁴ full length A/California/4/2009 (Cal09) HA and Cal09 NA were generated. For this the open reading frames of cH6/1, Cal09 HA and Cal09 NA were cloned into a pCAGGS mammalian expression vector.³⁷ A stable cell line was then generated as described before^{38,39} and chosen based on immunofluorescence with human monoclonal antibody CR9114¹⁵ for HA and 1000-3C05⁴⁰ for NA. Antigen-specific MDCK cells were seeded in sterile white flat bottom polystyrene tissue culture-treated 96-well plates (Corning) at a density of 35,000 cells/well. Twenty-four hours later, cells were washed with Opti-MEM (Minimal Essential Medium) Reduced Serum Medium (GIBCO) and 25ul of Roswell Park Memorial Institute (RPMI) 1640 medium (GIBCO) was added to each well. Three-fold dilutions of sera (starting 1:75) and ADCC effector cells (Jurkat T cells stably expressing Nuclear factor of activated T cells (NFAT)-luciferase reporter and human Fc γ R1IIa, Promega) adjusted at 7×10^4 cells in RPMI 1640 medium were added to each well and incubated 6 h at 37°C. Bio-Glo Luciferase assay reagent (Promega) was added, and luminescence was measured using a Synergy H1 hybrid multimode microplate reader (Biotek). The average background plus two standard deviations was used to discriminate between positive and negative values. Data were plotted in GraphPad Prism software to calculate AUC.

QUANTIFICATION AND STATISTICAL ANALYSIS

Demographics and clinical characteristics were compared using the chi-square test or Fisher exact test for categorical variables, and the t test, Mann-Whitney U test or Kruskal Wallis, for continuous variables, when appropriate. All immune assay values were log₁₀-transformed to improve linearity. Geometric mean titers (GMT) and 95% confidence intervals (CI) were computed by taking the exponent of the mean and the 95% CI of the log₁₀-transformed values.⁷ Paired samples across time points were compared using the Wilcoxon signed rank test to assess the immunological response after infection and vaccination. A scaled logit model^{41,42} modeling disease outcome among SOTRs was performed, with modifications. Briefly, a binomial logistic regression of log₁₀-transformed values was used to model a parametric protection curve against acute lower respiratory symptoms at the inclusion and hospital admission. The fitted curves of the relationship between log₁₀-assay values and the probability of protection was used to estimate protective thresholds of antibody titers. Number and proportion of SOTRs who developed LRS in each interval was calculated to quantify the protection from disease among SOTRs. We used a multivariate logistic regression model adjusted by potential co-founding factors to estimate the independent effect of antibody titers on disease progression. ELISA HA values were included as categorical variables of protection defined by the calculated protection curve. Contrasts among the levels of the serological predictors and type of transplant were performed as a set of K-1 variables internally to avoid co-linearity between groups. HAI titers were dichotomized according to international criteria for seroprotection⁷. Goodness of fit of the model was assessed by using the Hosmer and Lemeshow test. The seroconversion ratio was calculated as log₁₀-assay values at convalescence normalized by the log₁₀-assay values at the onset of the infection. The Spearman's rank correlation coefficient was calculated to measure the association between different assay readouts in their original scale. All the analysis was performed with IBM SPSS Statistics (version 21). Statistical significance was established at $p < 0.05$. All reported p values are based on two-tailed tests.