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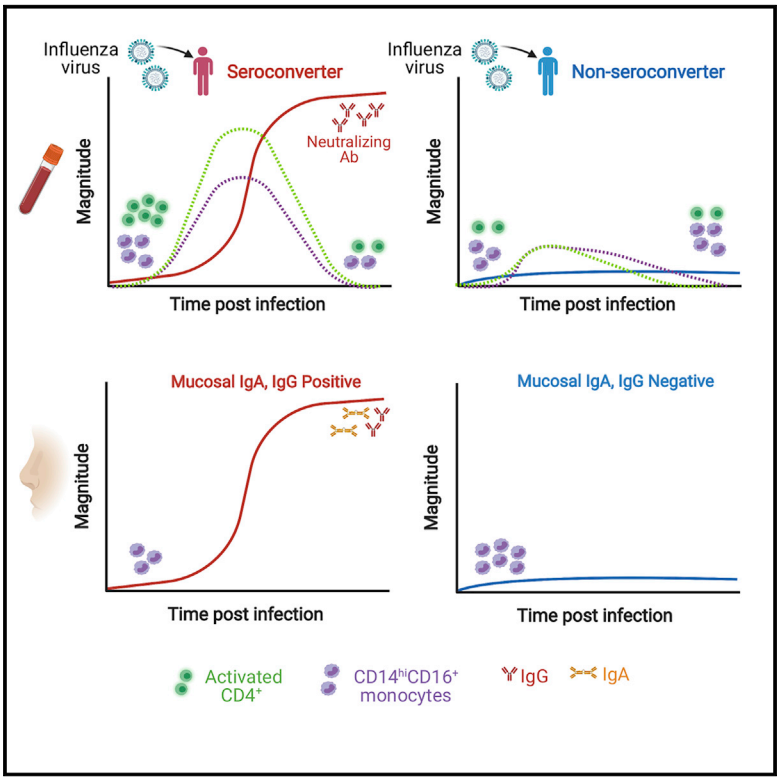
Report

Activated CD4^{+} T cells and CD14^{hi}CD16^{+} monocytes correlate with antibody response following influenza virus infection in humans

Graphical abstract



Highlights

- Post-infection seroconversion is associated with severity of influenza virus infection
- Seroconverters have early proliferation and activation of CD4^{+} T cells
- CD8^{+} T cells are unaffected
- CD14^{hi}CD16^{+} monocytes in the blood and nasal mucosa is associated with antibody response

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In brief

Wong et al. show that antibody responsiveness after influenza virus infection is associated with CD4^{+} T cells and CD14^{hi}CD16^{+} monocytes. CD14^{hi}CD16^{+} monocytes are also important in the mucosal antibody response. This demonstrates that seroconversion failure after infection is a definable immunological phenomenon, an important consideration for diagnostics and epidemiological studies.

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Report

Activated CD4^{+} T cells and CD14^{hi}CD16^{+} monocytes correlate with antibody response following influenza virus infection in humans

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SUMMARY

The failure to mount an antibody response following viral infection or seroconversion failure is a largely underappreciated and poorly understood phenomenon. Here, we identified immunologic markers associated with robust antibody responses after influenza virus infection in two independent human cohorts, SHIVERS and FLU09, based in Auckland, New Zealand and Memphis, Tennessee, USA, respectively. In the SHIVERS cohort, seroconversion significantly associates with (1) hospitalization, (2) greater numbers of proliferating, activated CD4^{+} T cells, but not CD8^{+} T cells, in the periphery during the acute phase of illness, and (3) fewer inflammatory monocytes (CD14^{hi}CD16^{+}) by convalescence. In the FLU09 cohort, fewer CD14^{hi}CD16^{+} monocytes during early illness in the nasal mucosa were also associated with the generation of influenza-specific mucosal immunoglobulin A (IgA) and IgG antibodies. Our study demonstrates that seroconversion failure after infection is a definable immunological phenomenon, associated with quantifiable cellular markers that can be used to improve diagnostics, vaccine efficacy, and epidemiologic efforts.

INTRODUCTION

An increase in antigen-specific antibody titer in the serum, known as seroconversion, has long been accepted to be a serological hallmark of a recent infection or antigen exposure. However, the advent of molecular diagnosis has led to the observation that some infections do not always result in the subsequent production of detectable antibodies, particularly those with neutralizing and protective activity. This has been documented in infections with influenza virus,[1–5] human coronaviruses,[6] the Middle East respiratory syndrome (MERS) coronavirus,[7] and the recently emerged severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2)[8,9] infections. The immunological mechanisms underlying seroconversion failure are not well understood, but a better understanding is important, particularly for vaccine design.

The production of high-affinity, durable antibody and B cell memory responses requires the initiation of a germinal center (GC) response in secondary lymphoid organs, which is a multistep process involving multiple innate and adaptive immune cells and cytokine signals.[10–12] In humans, the majority of our understanding of what is required for the generation of a robust antibody response have been derived from vaccination studies. Such studies have the advantage of having temporally defined pre- and post-antigen exposures that facilitate sample and data acquisition and have used targeted or systems-wide

approaches to identify correlates of robust antibody production. These studies have generally found that the activation of B cell maturation pathways and engagement of innate immunity are critical for robust antibody production after vaccination.[13–17] The early proliferation of antigen-specific plasmablasts was another characteristic that was found to precede the development of vaccine-induced serum antibody responses and in limited studiesof influenza and dengue virus infections.[18–20] However, given the differences in antigenic composition and exposure route, post-vaccination responses may not necessarily reflect the post-infection immune response, particularly for respiratory viral infections.

For influenza viruses, antibodies that target the major surface viral glycoproteins, hemagglutinin (HA) and neuraminidase (NA) are induced after infection. In terms of seroresponses to influenza virus infection, HA antibodies measured in a hemagglutination-inhibition (HAI) assay are considered to be the gold standard. Antibodies detected by HAI assay primarily bind to the HA globular head, which contains the receptor-binding domain and the major antigenic sites. These antibodies confer high antigenic specificity and can provide potent protection from infection in humans and animal studies.[21,22] The HAI assay does not strictly measure virus neutralizing activity, but its relative ease of use and established correlation with protection[21–23] have justified its use as a major serological endpoint in influenza vaccine and infection studies. Serum HAI-antibody titers $\geq$40 have been shown to be associated with protection from seasonal influenza virus infections[21] and have been adopted as the minimal immunogenicity requirement for the licensure of seasonal influenza vaccines.[24] Compared to HA, NA antibodies are less well studied, although they have also been recently identified as a potential additional correlate of protection from severe influenza disease.[4,25,26]

The failure to seroconvert by the standard HAI assay after laboratory-confirmed influenza virus infection has been reported in seroepidemiological,[1,2] vaccination,[27] and even challenge studies.[3–5,28] A review of human challenge studies showed that between 50% and 90% of individuals with PCR confirmation of influenza virus infection failed to seroconvert by HAI assay.[3–5,28] The underlying immunologic factors driving the magnitude of the antibody response following influenza virus infection is still, however, poorly defined.

Here, we detail the cellular immune profile of individuals following natural influenza virus infection to identify cell populations that are involved in mounting a subsequent antibody response, as indicated by seroconversion in an HAI assay. We used paired lymphocytes and serum samples collected from participants with laboratory-confirmed influenza enrolled in The Southern Hemisphere Influenza and Vaccine Effectiveness, Research and Surveillance (SHIVERS) study based in Auckland, New Zealand. We showed that robust antibody production after infection was associated with the early proliferation of $CD4^+$ T cells and engagement of $CD14^{hi}CD16^+$ monocytes. We further validated the role of the $CD14^{hi}CD16^+$ monocytes in the development of mucosal antibodies in the airways using samples from a separate cohort, FLU09, based in Memphis, Tennessee. Our findings reveal an immunological basis to antibody responsiveness after infection that shed light on why some people fail to seroconvert after respiratory viral infections.

RESULTS

Description of seroconverters (SCs) and non-seroconverters (non-SCs) in the SHIVERS cohort

In the SHIVERS cohort, participants were enrolled according to the World Health Organization (WHO) case definition for influenza-like illness (ILI) or severe acute respiratory illness (SARI) from general practices or hospitals in Auckland between August and October 2013, which coincided with the peak influenza season in New Zealand.[29] Of those with PCR-confirmed infection (N = 66), 21 (32%) participants met the definition of HAI seroconversion (at least a 4-fold increase in antibody titer in the paired sera). No significant associations of seroconversion with age (Figure 1A), gender, ethnicity, or virus subtypes were detected. Hospitalization was, however, associated with more robust responses, with SARI participants more likely to seroconvert than ILI participants (86% versus 14%, respectively, p = 0.0012; Table S1). Since the time of sampling and baseline titers are important factors associated with seroconversion, we also examined these variables in our cohort. Although the time from symptom onset to first sample was shorter (median days, interquartile range [IQR]: 9–13.5 versus 12–13.5) (Figure 1B) and the average first sera HAI titer was lower (geometric mean titer [GMT], 95% confidence interval [CI]: 36.9, 18.3–74.4 versus 39.4, 24.6–63.4) for SCs than for non-SCs (Figure 1C), these differences were not statistically significant. The large variance within these two datasets suggests that the failure to seroconvert is likely due to multiple factors.

To examine the cellular immune responses associated with seroconversion, we restricted our cohort to (1) individuals with low baseline titer-HAI titer of <40 in the first sera and (2) those whose first sera were sampled within 14 days post-symptom onset. By definition, SCs showed a 4-fold increase in HAI titer in the second sera, while non-SCs did not, effectively representing a failure to mount neutralizing anti-HA antibodies. Individuals who did not have paired blood samples were excluded. This resulted in N = 9 non-SCs and N = 7 SCs. For ease of description, the first sera were considered acute and the second sera were considered convalescent.

In this subcohort, SCs had a 16-fold median increase in HAI titer (range: 4- to 128-fold), resulting in significantly higher convalescent HAI titers compared to non-SCs (GMT, 95% CI: 320, 120.2–852 versus 12.6, 7.9–20.0, respectively; Figure 1D). SCs showed a more modest increase in the neuraminidase inhibition (NAI)- and influenza-specific immunoglobulin G (IgG)-antibody titers between the two time points, with 4- and 2-fold median increases, respectively (range: 2- to 64-fold for NAI and 1- to 128-fold for HA-IgG, respectively) (Figures 1E and 1F). Non-SCs had no significant increases in either HAI-, NAI-, or influenza-specific IgG titers by convalescence, showing similar trends across antibody measures. By these measures, SCs showed a robust HAI-dominant antibody response by convalescence compared to non-SCs.

SCs have more proliferating $CD4^+$ T cells in the periphery than non-SCs

To identify the cellular correlates of seroconversion, we examined innate and adaptive immune cell populations in

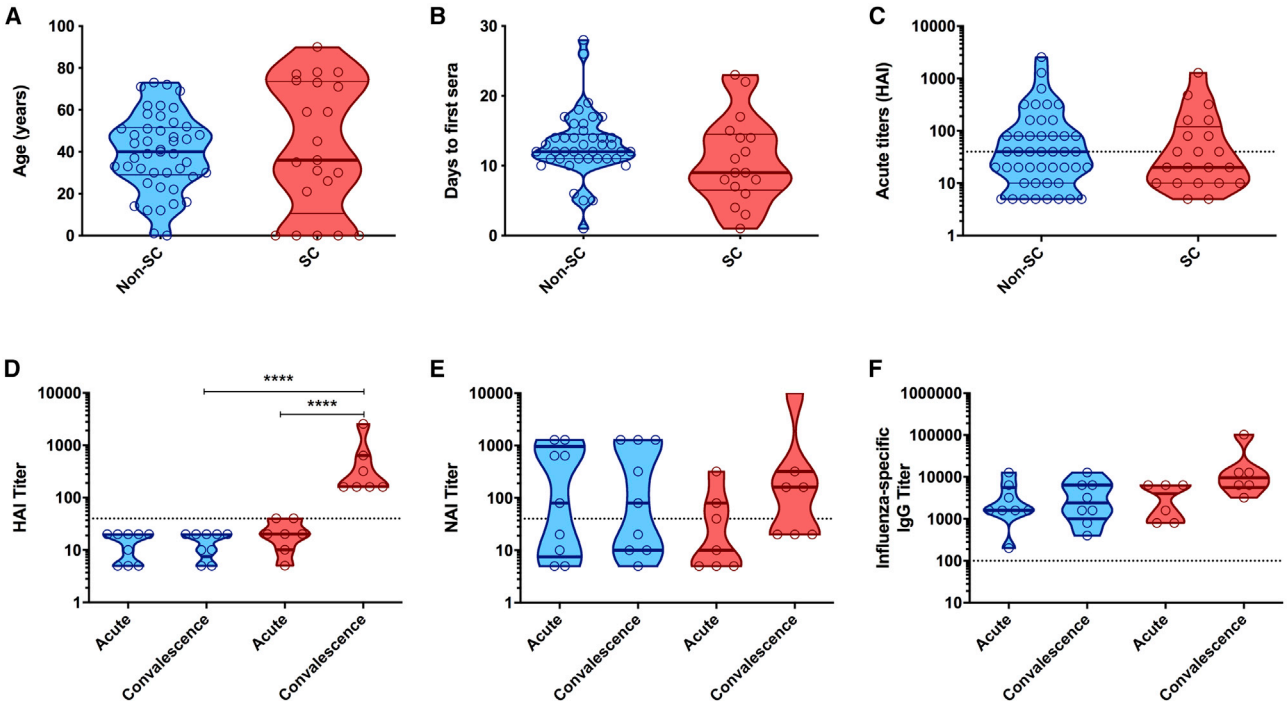

Figure 1. Characteristics of seroconverters (SCs) and non-seroconverters (non-SCs) in the SHIVERS cohort

(A–C) Distribution of (A) age, (B) time-to-first-sera (days), and (C) first sera hemagglutination-inhibition (HAI) titers in the SCs (red, n = 21) and non-SCs (blue, n = 45) in the SHIVERS cohort. Serology profile of SCs (red, n = 7) and non-SCs (blue, n = 9) in the subcohort with HAI titers <40 within 14 days of enrollment that were selected for further analysis of their cellular immune profile.

(D–F) HAI titer (D), (E) neuraminidase inhibition (NAI) titer, and (F) influenza-specific IgG titer.

Dashed line in (C)–(E) indicates a titer of 40 and in (F) a titer of 100. ****$p < 0.0001$, by 1-way analysis of variance, with Bonferroni corrections applied for multiple comparisons using log-transformed data. Serum samples were tested in single wells at least twice for HAI and NAI assays, while samples were run in duplicate wells in the ELISA assays. Lines and error bars indicate the median and interquartile range.

the acute and convalescence samples. The percentage and number of $CD4^+$ T cells in the peripheral blood of the SCs tended to be lower than those in non-SCs in acute samples ($p = 0.06–0.09$; Figures 2A and 2B). However, a significantly higher percentage of these cells in the SCs were actively proliferating (being $Ki67^+$ and $Bcl2^-$) at this time point (Figure 2C). There was also a trend toward higher frequencies of activated $CD4^+$ T cells that were human leukocyte antigen-DR isotype-positive ($HLA\text{-}DR^+$) and $CD38^+$ in the SCs compared to the non-SCs, although this difference was not statistically supported, likely due to the presence of two high responders in the non-SC group (Figure 2E). The frequencies of these cell populations had normalized and were comparable between the two groups by convalescence (Figures 2D and 2F). Notably, there were no significant differences in the frequencies, absolute numbers, or proliferative or activation states of the $CD8^+$ T cell populations between SC and non-SC (Figures S1A–S1F), suggesting that the cytotoxic $CD8^+$ T cell response was independent of factors regulating the antibody response. We also did not detect any differences between SCs and non-SCs in the frequencies of interferon γ-positive ($IFN\gamma^+$) or tumor necrosis factor α-positive ($TNF\text{-}\alpha^+$), $CD4^+$, or $CD8^+$ T cells (Figures S2 and S3).

Dynamics of the $CD14^{hi}CD16^+$ monocyte population is associated with seroconversion

We next examined the concordance of the innate immune cell populations and seroconversion. Here, we found differences in the peripheral monocyte and myeloid dendritic cell (mDC) subpopulations between the SC and non-SC groups. Three monocyte subpopulations have been described based on the relative expression of CD14 and CD16 surface markers (Figure 3A). The differentiation of "classical" monocytes ($CD14^{hi}CD16^-$) to either "inflammatory" ($CD14^{hi}CD16^+$) or "patrolling" ($CD14^{lo}CD16^+$) monocytes can indicate the extent of inflammation.[30] During the acute phase, there was greater conversion of the classical $CD16^-$ monocytes to $CD16^+$ monocytes in the SC group than the non-SC group ($p < 0.05$; Figure 3B). By convalescence, however, the $CD14^{hi}CD16^+$ subpopulation appeared smaller in the SC compared to the non-SC (Figure 3C). In a side-by-side comparison, SCs showed greater depletion of $CD14^{hi}CD16^+$ (Figure 3D), but not $CD14^{lo}CD16^+$ monocytes (Figure 3E), between the two time points compared to non-SCs. The number of mDCs ($HLA\text{-}DR^+CD14^-CD11c^+$), which are important antigen-presenting cells, were on average lower in SCs than in non-SCs in the acute samples, although this difference did not meet the threshold of statistical significance ($p = 0.1$; Figures 3F and 3G).

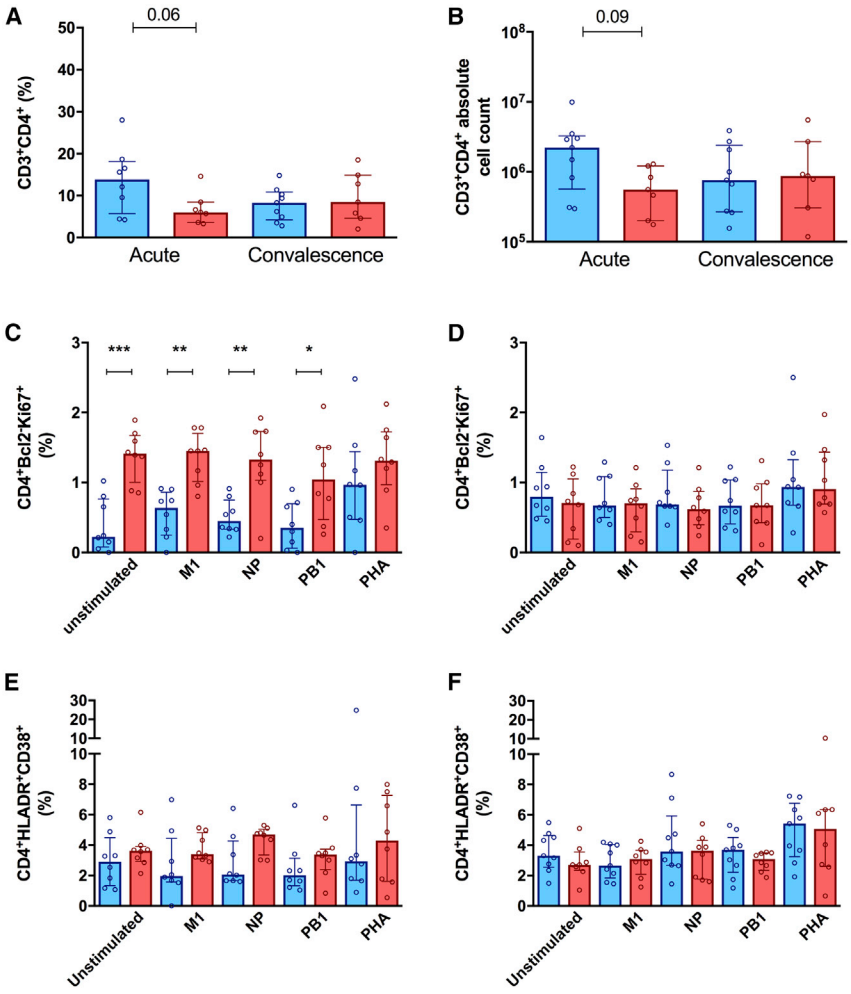

Figure 2. Peripheral CD4⁺ T cell profile in the SCs (red, n = 7) and non-SCs (blue, n = 9) during the acute and convalescence phases

(A and B) Frequency (A) and (B) number of total $CD3^+CD4^+$ T cells during the acute and convalescence phases.

(C–F) Frequency of proliferating ($Bcl2^-$ and $Ki67^+$) and activated ($HLA\text{-}DR^+CD38^+$) $CD4^+$ T cells, during the (C and E) acute phase and (D and F) the convalescence phase, respectively. Cells were stimulated overnight with either media (unstimulated), pooled influenza peptides corresponding to the matrix (M1), nucleoprotein (NP), and PB1 protein, or the non-specific mitogen, phytohemagglutinin (PHA).

*$p < 0.05$ and **$p < 0.01$, ***$p < 0.001$, by 1-way analysis of variance, with Bonferroni corrections applied for multiple comparisons. Lines and error bars indicate the median and interquartile range.

$CD14^{hi}CD16^+$ monocytes were also important in the mucosal antibody response

Having determined the correlations between $CD16^+$ monocytes and serum antibody responses, we next asked whether similar patterns were seen in the mucosal compartment. To address this, we measured the influenza-specific IgA and IgG titers in the nasal washes of influenza-confirmed individuals from a cohort in which we had previously characterized their monocyte subpopulations.[31] As there is no equivalent seroconversion standard for mucosal antibody titers, we identified 10 individuals with influenza-specific IgA titer >40 in their nasal wash samples within 2 weeks of symptom onset as SCs (GMT, 95% CI: 186.6, 98.4–354.1) (Figure 4A) and another 9 individuals, with no detectable IgA titers during the course of study, as non-SCs (Table S2). To ensure the validity of our classification of SCs and non-SCs, we also evaluated the influenza-specific IgG responses. Nine of the 10 SCs had influenza-specific IgG titers >40 (GMT, 95% CI: 28.9, 2.8–292), while none of the non-SCs had detectable titers at any of the time points tested (Figure 4B). While classical monocytes were predominant in the peripheral blood, it was $CD14^{hi}CD16^+$ monocytes that were mostly recovered in the nasal washes (Figure 4C). Within 1 week of enrollment, greater numbers of $CD14^{hi}CD16^-$ and fewer numbers of $CD14^{hi}CD16^+$ monocytes were found in SCs compared to the non-SCs (Figure 4C). Thus, the early dynamics of the monocyte subpopulations in the nasal mucosa were also important in regulating downstream mucosal antibody response.

DISCUSSION

Seroconversion in influenza is typically defined by measuring the strain-specific HAI antibody response and is generally thought to follow infection. Somewhat unexpectedly, we found that only 32% of individuals who were PCR confirmed to be infected with influenza virus met this standard definition of seroconversion in SHIVERS. While technical reasons (e.g., late sampling of sera, mismatched antigen) may have accounted for some of this seroconversion failure, we were able to identify cellular signatures associated with the ability, or lack thereof, to seroconvert in a proportion of infected individuals. When we more closely examined those SCs who had low HAI antibody titers in their acute samples, the magnitude of their increase in HA-specific IgG and NAI antibody titers were only modest compared to HAI antibody responses, suggesting that the distinct cellular immune profile that we observed here is important for the induction of conformationally specific HAI antibodies and not necessarily the magnitude of antibody response overall. On average, the $CD8^+$ T cell responses were not significantly different between SCs and non-SCs, suggesting that the $CD4^+$ and $CD8^+$ T cell responses are regulated independently, which is consistent with findings previously observed in mouse studies.[32]

The distinct roles of the monocyte subpopulations were of particular interest. The $CD14^{lo}CD16^{hi}$ population, which has been reported to have potent antiviral function,[33] was reported to be associated with increased disease severity after influenza, Ebola, and Zika virus infection.[34–36] However, much less is known

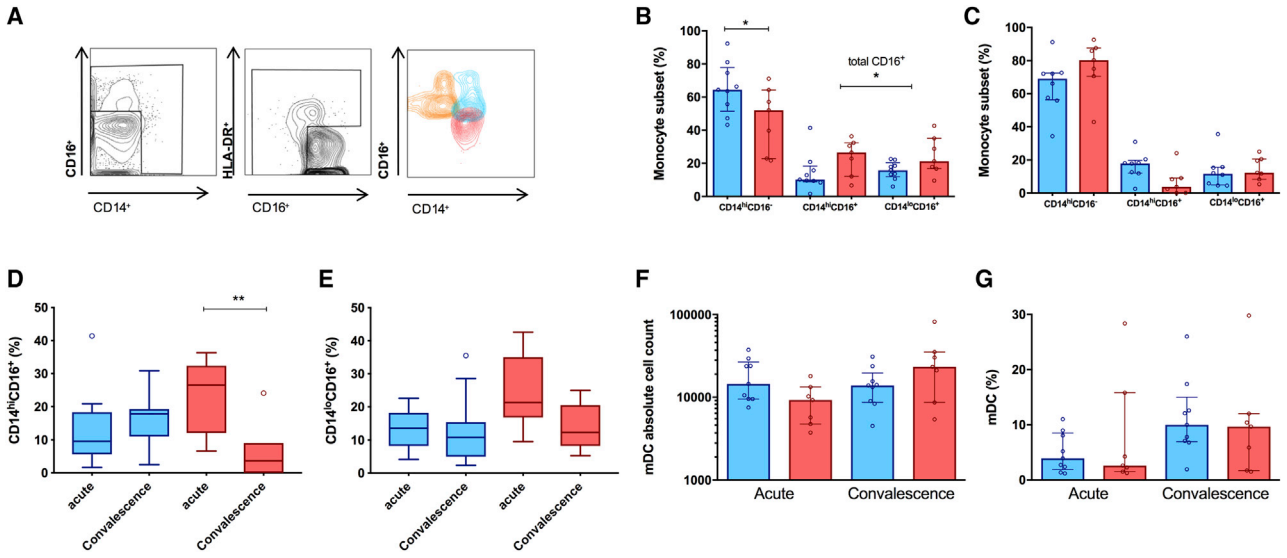

Figure 3. Profile of the monocyte subpopulations and myeloid dendritic cells (mDCs) in the peripheral blood of the SCs (red, n = 7) and non-SCs (blue, n = 9) during the acute and convalescence phases

(A) Gating strategy to identify the monocyte subpopulations based on the relative expression levels of the markers CD14 and CD16: "classical" monocytes are $CD14^{hi}/CD16^{-}$ (red), "inflammatory" monocytes are $CD14^{hi}/CD16^{+}$ (blue), and "patrolling" monocytes are $CD14^{lo}/CD16^{+}$ (orange).

(B and C) Percentages of each monocyte subpopulations (per total monocytes) during (B) acute phase and (C) convalescence phase in the SC and non-SC groups.

(D and E) Percentage of (D) $CD14^{hi}/CD16^{+}$ and (E) $CD14^{lo}/CD16^{+}$ cells in the acute and convalescence time points samples.

(F and G) Number (F) and (G) percentage of $CD14^{-}CD11^{+}$ mDCs in the SCs and non-SCs during the acute and convalescence phases.

*$p < 0.05$ and **$p < 0.01$, by 1-way analysis of variance, with Bonferroni corrections applied for multiple comparisons. Lines and error bars indicate the median and interquartile range.

about the function of the $CD14^{hi}CD16^{+}$ monocytes. The finding that mDCs and $CD14^{hi}CD16^{+}$ monocytes were associated with antibody responsiveness have been reported for bloodborne viral infections such as dengue and Zika,[34,37–39] which suggests that these are broadly responsive markers for viral infections. $CD14^{hi}CD16^{+}$ monocytes appear to be primed for efficient antigen presentation. In macaques that were vaccinated intramuscularly with mRNA encapsulated within lipid nanoparticles, $CD14^{hi}CD16^{+}$ monocytes were found to be the most efficient in engulfing and translating the foreign mRNA, particularly at the draining lymph nodes.[40] Similarly, in dengue virus-infected non-human primates, $CD14^{hi}CD16^{+}$ monocytes trafficked to draining lymph nodes to promote the differentiation of resting B cells into plasmablasts.[34] The depletion of the $CD14^{hi}CD16^{+}$ monocytes in blood and within the airways of SCs observed in our study potentially reflect the active migration of these cells to the lymph nodes.

The higher incidence of seroconversion in hospitalized individuals supports a correlation between the severity of infection and antibody responsiveness in the periphery and nasal mucosa.[5,41] Although our data cannot preclude the possibility of antibody-dependent enhancement (ADE) of influenza disease, we think it is unlikely within the context of our study design (antibody increases were detected post-hospitalization) and other available evidence. ADE of influenza disease has mostly been described during the 2009 H1N1 pandemic[42,43] and within critically ill or fatal cases.[44,45] In our cohort, although they were hospitalized, most of the patients were on the "milder" end of the severity spectrum.[36] Furthermore, in the present and previous SHIVERS studies on the immunological correlates of disease severity, we found no significant differences in the acute-phase antibody titers between mild (outpatient) and severe (hospitalized) patients. Thus, it appears that, paradoxically, a strong proinflammatory response that is associated with symptomatic infections[31,36] could also help induce a good antibody response, while a weak signal may not provide adequate stimulation.[46] This is a particularly important consideration for the ongoing coronavirus disease 2019 (COVID-19) pandemic, in which a proportion of mild or asymptomatic COVID-19 cases fail to mount detectable antibody responses despite evidence of active infection, while severely ill patients had comparably stronger antibody responses.[8,9,47] Incidentally, the lack of association between age and seroconversion indicates that disease severity may be a stronger factor driving post-infection antibody response than age, suggesting a strong proinflammatory response may compensate for the decline in immune function in the elderly. However, it should be noted that the association between antibody response and severity of infection should not be broadly interpreted, as it had also been shown that fatal H7N9 and COVID-19 patients failed to mount robust antibody responses in some cases.[47,48]

In summary, our data provide immunological evidence that respiratory viral infections do not always lead to successful seroconversions. These data have implications for our understanding of post-infection immunity and on studies that rely on such metrics. Identification of key cellular elements associated with robust antibody response will also help in our understanding of vaccine failure and contribute to the development of more efficacious vaccines.

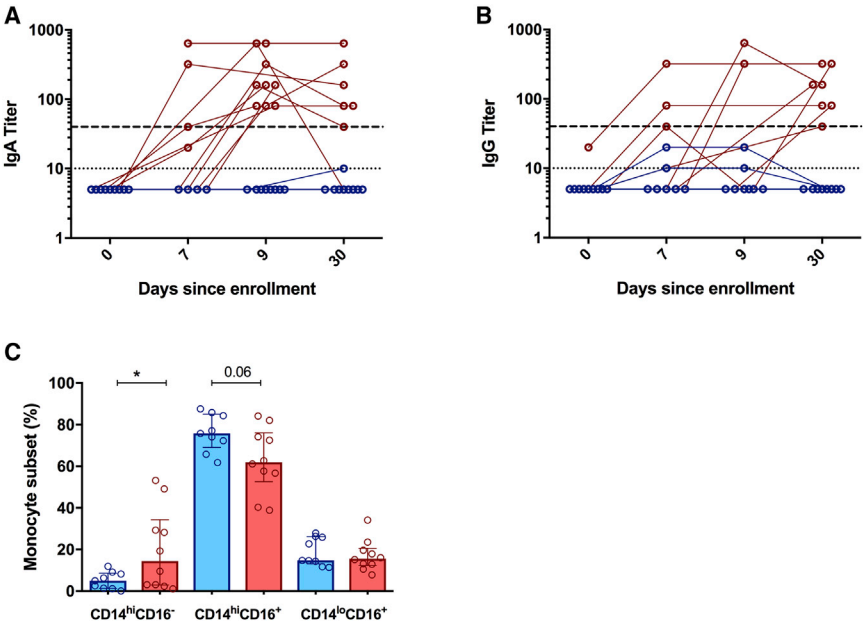

Figure 4. Immune responses in the nasal airways of participants in the FLU09 cohort

(A and B) Influenza-specific (A) IgA and (B) IgG titers in the nasal washes collected on days 0, 7, 9, and 28 post-enrollment of SCs (red, n = 10) and non-SCs (blue, n = 9). SCs were defined as individuals with IgA titers >40 at any time points during the study, whereas non-SCs were individuals who did not.

(C) Percentages of each monocyte subpopulation (per total monocytes) in the nasal airways of SCs and non-SCs collected within 1 week of enrollment. The monocyte populations are identified based on the relative expression levels of markers CD14 and CD16 as $CD14^{hi}/CD16^{-}$ (classical), $CD14^{hi}/CD16^{+}$ (inflammatory), and $CD14^{lo}/CD16^{+}$ (patrolling) monocytes.

*$p < 0.05$, by 1-way analysis of variance, with Bonferroni corrections applied for multiple comparisons. Samples were run in duplicate wells in the ELISA assays. Lines and error bars indicate the median and interquartile range.

Limitations of study

Our study has several limitations. First, the subcohorts used for cellular immune profile analyses were small, largely because we restricted analyses to individuals from whom first samples were collected within 14 days of symptom onset. Obtaining samples early in the disease course is often a challenge in clinical studies of respiratory infections, particularly those that target patients with milder symptoms such as ours. Second, we were not able to provide any indication on the functionality or subset of the reactive $CD4^+$ T cells. Third, although the internal viral proteins were reportedly the predominant targets of T cell responses,[49] we did not explore $CD4^+$ T cell reactivity to the surface proteins HA and NA. Such studies in the future could be useful to further identify which subset of these $CD4^+$ T cells are primarily responsible for driving the antibody response. Finally, it is unknown whether the present failure to mount an antibody response would have any impact on the memory B cell responses and their capacity to mount a recall response. Addressing these issues will greatly advance our understanding of the cellular correlates for antibody responsiveness.

STAR★METHODS

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

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xcrm.2021.100237.

CONSORTIA

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AUTHOR CONTRIBUTIONS

Q.S.H. was the lead investigator for the overall SHIVERS study. P.G.T. and R.J.W. conceived and designed the immunological experiments. S.-S.W., C.M.O., M.Z., X.-Z.J.G, L.J., T.J., and J.R. performed the laboratory assays and the analysis for the serology and cellular data. B.W. and T.W. were responsible for data management and statistical analyses. M.-A.W. oversaw the overall SHIVERS program and provided critical input for the manuscript. R.S. was the project coordinator. G.E.R. led the SHIVERS patient enrollment and sample collection process. S.-S.W., P.G.T., and R.J.W. wrote the manuscript. All of the authors reviewed and approved the final version of the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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REFERENCES

1. Chen, M.I., Barr, I.G., Koh, G.C., Lee, V.J., Lee, C.P., Shaw, R., Lin, C., Yap, J., Cook, A.R., Tan, B.H., et al. (2010). Serological response in RT-PCR confirmed H1N1-2009 influenza a by hemagglutination inhibition and virus neutralization assays: an observational study. PLoS ONE *5*, e12474.
2. Chen, M.I., Lee, V.J., Lim, W.Y., Barr, I.G., Lin, R.T., Koh, G.C., Yap, J., Cui, L., Cook, A.R., Laurie, K., et al. (2010). 2009 influenza A(H1N1) seroconversion rates and risk factors among distinct adult cohorts in Singapore. JAMA *303*, 1383–1391.
3. Huang, K.Y., Li, C.K., Clutterbuck, E., Chui, C., Wilkinson, T., Gilbert, A., Oxford, J., Lambkin-Williams, R., Lin, T.Y., McMichael, A.J., and Xu, X.N. (2014). Virus-specific antibody secreting cell, memory B-cell, and sero-antibody responses in the human influenza challenge model. J. Infect. Dis. *209*, 1354–1361.
4. Memoli, M.J., Shaw, P.A., Han, A., Czajkowski, L., Reed, S., Athota, R., Bristol, T., Fargis, S., Risos, K., Powers, J.H., et al. (2016). Evaluation of Antihemagglutinin and Antineuraminidase Antibodies as Correlates of Protection in an Influenza A/H1N1 Virus Healthy Human Challenge Model. MBio *7*, e00417-16.
5. Murphy, B.R., Rennels, M.B., Douglas, R.G., Jr., Betts, R.F., Couch, R.B., Cate, T.R., Jr., Chanock, R.M., Kendal, A.P., Maassab, H.F., Suwanagool, S., et al. (1980). Evaluation of influenza A/Hong Kong/123/77 (H1N1) ts-1A2 and cold-adapted recombinant viruses in seronegative adult volunteers. Infect. Immun. *29*, 348–355.
6. Gorse, G.J., Donovan, M.M., and Patel, G.B. (2020). Antibodies to coronaviruses are higher in older compared with younger adults and binding antibodies are more sensitive than neutralizing antibodies in identifying coronavirus-associated illnesses. J. Med. Virol. *92*, 512–517.
7. Ko, J.H., Müller, M.A., Seok, H., Park, G.E., Lee, J.Y., Cho, S.Y., Ha, Y.E., Baek, J.Y., Kim, S.H., Kang, J.M., et al. (2017). Serologic responses of 42 MERS-coronavirus-infected patients according to the disease severity. Diagn. Microbiol. Infect. Dis. *89*, 106–111.
8. Lee, Y.L., Liao, C.H., Liu, P.Y., Cheng, C.Y., Chung, M.Y., Liu, C.E., Chang, S.Y., and Hsueh, P.R. (2020). Dynamics of anti-SARS-Cov-2 IgM and IgG antibodies among COVID-19 patients. J. Infect. *81*, e55–e58.
9. Zhang, G., Nie, S., Zhang, Z., and Zhang, Z. (2020). Longitudinal Change of Severe Acute Respiratory Syndrome Coronavirus 2 Antibodies in Patients with Coronavirus Disease 2019. J. Infect. Dis. *222*, 183–188.
10. Goodnow, C.C., Vinuesa, C.G., Randall, K.L., Mackay, F., and Brink, R. (2010). Control systems and decision making for antibody production. Nat. Immunol. *11*, 681–688.
11. Havenar-Daughton, C., Carnathan, D.G., Torrents de la Peña, A., Pauthner, M., Briney, B., Reiss, S.M., Wood, J.S., Kaushik, K., van Gils, M.J., Rosales, S.L., et al. (2016). Direct Probing of Germinal Center Responses Reveals Immunological Features and Bottlenecks for Neutralizing Antibody Responses to HIV Env Trimer. Cell Rep. *17*, 2195–2209.
12. Pulendran, B., and Ahmed, R. (2011). Immunological mechanisms of vaccination. Nat. Immunol. *12*, 509–517.
13. Garner-Spitzer, E., Kundi, M., Rendi-Wagner, P., Winkler, B., Wiedermann, G., Holzmann, H., Herzog, C., Kollaritsch, H., and Wiedermann, U. (2009). Correlation between humoral and cellular immune responses and the expression of the hepatitis A receptor HAVcr-1 on T cells after hepatitis A re-vaccination in high and low-responder vaccinees. Vaccine *27*, 197–204.
14. Garner-Spitzer, E., Wagner, A., Paulke-Korinek, M., Kollaritsch, H., Heinz, F.X., Redlberger-Fritz, M., Stiasny, K., Fischer, G.F., Kundi, M., and Wiedermann, U. (2013). Tick-borne encephalitis (TBE) and hepatitis B nonresponders feature different immunologic mechanisms in response to TBE and influenza vaccination with involvement of regulatory T and B cells and IL-10. J. Immunol. *191*, 2426–2436.
15. Li, S., Nakaya, H.I., Kazmin, D.A., Oh, J.Z., and Pulendran, B. (2013). Systems biological approaches to measure and understand vaccine immunity in humans. Semin. Immunol. *25*, 209–218.
16. Li, S., Rouphael, N., Duraisingham, S., Romero-Steiner, S., Presnell, S., Davis, C., Schmidt, D.S., Johnson, S.E., Milton, A., Rajam, G., et al. (2014). Molecular signatures of antibody responses derived from a systems biology study of five human vaccines. Nat. Immunol. *15*, 195–204.
17. Tsang, J.S., Schwartzberg, P.L., Kotliarov, Y., Biancotto, A., Xie, Z., Germain, R.N., Wang, E., Olnes, M.J., Narayanan, M., Golding, H., et al.; Baylor HIPC Center; CHI Consortium (2014). Global analyses of human immune variation reveal baseline predictors of postvaccination responses. Cell *157*, 499–513.
18. Wrammert, J., Koutsonanos, D., Li, G.M., Edupuganti, S., Sui, J., Morrissey, M., McCausland, M., Skountzou, I., Hornig, M., Lipkin, W.I., et al. (2011). Broadly cross-reactive antibodies dominate the human B cell response against 2009 pandemic H1N1 influenza virus infection. J. Exp. Med. *208*, 181–193.
19. Wrammert, J., Onlamoon, N., Akondy, R.S., Perng, G.C., Polsrila, K., Chandele, A., Kwissa, M., Pulendran, B., Wilson, P.C., Wittawatmongkol, O., et al. (2012). Rapid and massive virus-specific plasmablast responses during acute dengue virus infection in humans. J. Virol. *86*, 2911–2918.
20. Wrammert, J., Smith, K., Miller, J., Langley, W.A., Kokko, K., Larsen, C., Zheng, N.Y., Mays, I., Garman, L., Helms, C., et al. (2008). Rapid cloning of high-affinity human monoclonal antibodies against influenza virus. Nature *453*, 667–671.
21. Hobson, D., Curry, R.L., Beare, A.S., and Ward-Gardner, A. (1972). The role of serum haemagglutination-inhibiting antibody in protection against challenge infection with influenza A2 and B viruses. J. Hyg. (Lond.) *70*, 767–777.
22. Wong, S.S., Duan, S., DeBeauchamp, J., Zanin, M., Kercher, L., Sonnberg, S., Fabrizio, T., Jeevan, T., Crumpton, J.C., Oshansky, C., et al. (2017). The immune correlates of protection for an avian influenza H5N1 vaccine in the ferret model using oil-in-water adjuvants. Sci. Rep. *7*, 44727.
23. Truelove, S., Zhu, H., Lessler, J., Riley, S., Read, J.M., Wang, S., Kwok, K.O., Guan, Y., Jiang, C.Q., and Cummings, D.A. (2016). A comparison of hemagglutination inhibition and neutralization assays for characterizing immunity to seasonal influenza A. Influenza Other Respir. Viruses *10*, 518–524.
24. Food and Drug Administration Division of Vaccines and Related Product Applications, Center for Biologics Evaluation and Research (2007). Guidance for industry: clinical data needed to support the licensure of seasonal

inactivated influenza vaccines. https://www.fda.gov/media/73706/download.

25. Memoli, M.J., Czajkowski, L., Reed, S., Athota, R., Bristol, T., Proudfoot, K., Fargis, S., Stein, M., Dunfee, R.L., Shaw, P.A., et al. (2015). Validation of the wild-type influenza A human challenge model H1N1pdMIST: an A(H1N1)pdm09 dose-finding investigational new drug study. Clin. Infect. Dis. *60*, 693–702.
26. Monto, A.S., Petrie, J.G., Cross, R.T., Johnson, E., Liu, M., Zhong, W., Levine, M., Katz, J.M., and Ohmit, S.E. (2015). Antibody to Influenza Virus Neuraminidase: An Independent Correlate of Protection. J. Infect. Dis. *212*, 1191–1199.
27. Thompson, M.G., Gaglani, M.J., Naleway, A.L., Dowell, S.H., Spencer, S., Ball, S., Levine, M., and Fry, A. (2016). Reduced serologic sensitivity to influenza A virus illness among inactivated influenza vaccinees. Vaccine *34*, 3443–3446.
28. Han, A., Czajkowski, L.M., Donaldson, A., Baus, H.A., Reed, S.M., Athota, R.S., Bristol, T., Rosas, L.A., Cervantes-Medina, A., Taubenberger, J.K., and Memoli, M.J. (2019). A Dose-finding Study of a Wild-type Influenza A(H3N2) Virus in a Healthy Volunteer Human Challenge Model. Clin. Infect. Dis. *69*, 2082–2090.
29. Huang, Q.S., Bandaranayake, D., Wood, T., Newbern, E.C., Seeds, R., Ralston, J., Waite, B., Bissielo, A., Prasad, N., Todd, A., et al.; Southern Hemisphere Influenza and Vaccine Effectiveness Research and Surveillance (SHIVERS) Investigation Team (2019). Risk Factors and Attack Rates of Seasonal Influenza Infection: Results of the Southern Hemisphere Influenza and Vaccine Effectiveness Research and Surveillance (SHIVERS) Seroepidemiologic Cohort Study. J. Infect. Dis. *219*, 347–357.
30. Wong, K.L., Yeap, W.H., Tai, J.J., Ong, S.M., Dang, T.M., and Wong, S.C. (2012). The three human monocyte subsets: implications for health and disease. Immunol. Res. *53*, 41–57.
31. Oshansky, C.M., Gartland, A.J., Wong, S.S., Jeevan, T., Wang, D., Roddam, P.L., Caniza, M.A., Hertz, T., Devincenzo, J.P., Webby, R.J., et al. (2014). Mucosal immune responses predict clinical outcomes during influenza infection independently of age and viral load. Am. J. Respir. Crit. Care Med. *189*, 449–462.
32. Koyama, S., Ishii, K.J., Kumar, H., Tanimoto, T., Coban, C., Uematsu, S., Kawai, T., and Akira, S. (2007). Differential role of TLR- and RLR-signaling in the immune responses to influenza A virus infection and vaccination. J. Immunol. *179*, 4711–4720.
33. Shi, C., and Pamer, E.G. (2011). Monocyte recruitment during infection and inflammation. Nat. Rev. Immunol. *11*, 762–774.
34. Kwissa, M., Nakaya, H.I., Onlamoon, N., Wrammert, J., Villinger, F., Perng, G.C., Yoksan, S., Pattanapanyasat, K., Chokephaibulkit, K., Ahmed, R., and Pulendran, B. (2014). Dengue virus infection induces expansion of a CD14(+)CD16(+) monocyte population that stimulates plasmablast differentiation. Cell Host Microbe *16*, 115–127.
35. Lüdtke, A., Ruibal, P., Becker-Ziaja, B., Rottstegge, M., Wozniak, D.M., Cabeza-Cabrerizo, M., Thorenz, A., Weller, R., Kerber, R., Idoyaga, J., et al. (2016). Ebola Virus Disease Is Characterized by Poor Activation and Reduced Levels of Circulating CD16+ Monocytes. J. Infect. Dis. *214* (*Suppl 3*), S275–S280.
36. Wong, S.S., Oshansky, C.M., Guo, X.J., Ralston, J., Wood, T., Seeds, R., Newbern, C., Waite, B., Reynolds, G., Widdowson, M.A., et al. (2018). Severe Influenza Is Characterized by Prolonged Immune Activation: Results From the SHIVERS Cohort Study. J. Infect. Dis. *217*, 245–256.
37. Cros, J., Cagnard, N., Woollard, K., Patey, N., Zhang, S.Y., Senechal, B., Puel, A., Biswas, S.K., Moshous, D., Picard, C., et al. (2010). Human CD14dim monocytes patrol and sense nucleic acids and viruses via TLR7 and TLR8 receptors. Immunity *33*, 375–386.
38. Michlmayr, D., Andrade, P., Gonzalez, K., Balmaseda, A., and Harris, E. (2017). $CD14^{+}CD16^{+}$ monocytes are the main target of Zika virus infection in peripheral blood mononuclear cells in a paediatric study in Nicaragua. Nat. Microbiol. *2*, 1462–1470.
39. O'Connor, M.A., Tisoncik-Go, J., Lewis, T.B., Miller, C.J., Bratt, D., Moats, C.R., Edlefsen, P.T., Smedley, J., Klatt, N.R., Gale, M., Jr., and Fuller, D.H. (2018). Early cellular innate immune responses drive Zika viral persistence and tissue tropism in pigtail macaques. Nat. Commun. *9*, 3371.
40. Liang, F., Lindgren, G., Lin, A., Thompson, E.A., Ols, S., Röhss, J., John, S., Hassett, K., Yuzhakov, O., Bahl, K., et al. (2017). Efficient Targeting and Activation of Antigen-Presenting Cells In Vivo after Modified mRNA Vaccine Administration in Rhesus Macaques. Mol. Ther. *25*, 2635–2647.
41. He, Y., Abid, A., Fisher, R., Eller, N., Mikolajczyk, M., Welliver, R.C., Sr., Bonner, A.B., Scott, D.E., and Reed, J.L. (2013). Mucosal antibody responses are directed by viral burden in children with acute influenza infection. Influenza Other Respir. Viruses *7*, 46–54.
42. Co, M.D., Terajima, M., Thomas, S.J., Jarman, R.G., Rungrojcharoenkit, K., Fernandez, S., Yoon, I.K., Buddhari, D., Cruz, J., and Ennis, F.A. (2014). Relationship of preexisting influenza hemagglutination inhibition, complement-dependent lytic, and antibody-dependent cellular cytotoxicity antibodies to the development of clinical illness in a prospective study of A(H1N1)pdm09 Influenza in children. Viral Immunol. *27*, 375–382.
43. Tsuchihashi, Y., Sunagawa, T., Yahata, Y., Takahashi, H., Toyokawa, T., Odaira, F., Ohyama, T., Taniguchi, K., and Okabe, N. (2012). Association between seasonal influenza vaccination in 2008-2009 and pandemic influenza A (H1N1) 2009 infection among school students from Kobe, Japan, April-June 2009. Clin. Infect. Dis. *54*, 381–383.
44. Monsalvo, A.C., Batalle, J.P., Lopez, M.F., Krause, J.C., Klemenc, J., Hernandez, J.Z., Maskin, B., Bugna, J., Rubinstein, C., Aguilar, L., et al. (2011). Severe pandemic 2009 H1N1 influenza disease due to pathogenic immune complexes. Nat. Med. *17*, 195–199.
45. To, K.K., Zhang, A.J., Hung, I.F., Xu, T., Ip, W.C., Wong, R.T., Ng, J.C., Chan, J.F., Chan, K.H., and Yuen, K.Y. (2012). High titer and avidity of nonneutralizing antibodies against influenza vaccine antigen are associated with severe influenza. Clin. Vaccine Immunol. *19*, 1012–1018.
46. Banchereau, R., Baldwin, N., Cepika, A.M., Athale, S., Xue, Y., Yu, C.I., Metang, P., Cheruku, A., Berthier, I., Gayet, I., et al. (2014). Transcriptional specialization of human dendritic cell subsets in response to microbial vaccines. Nat. Commun. *5*, 5283.
47. Wang, Y., Zhang, L., Sang, L., Ye, F., Ruan, S., Zhong, B., Song, T., Alshukairi, A.N., Chen, R., Zhang, Z., et al. (2020). Kinetics of viral load and antibody response in relation to COVID-19 severity. J. Clin. Invest. *130*, 5235–5244.
48. Wang, Z., Wan, Y., Qiu, C., Quiñones-Parra, S., Zhu, Z., Loh, L., Tian, D., Ren, Y., Hu, Y., Zhang, X., et al. (2015). Recovery from severe H7N9 disease is associated with diverse response mechanisms dominated by $CD8^{+}$ T cells. Nat. Commun. *6*, 6833.
49. Sant, A.J., DiPiazza, A.T., Nayak, J.L., Rattan, A., and Richards, K.A. (2018). CD4 T cells in protection from influenza virus: viral antigen specificity and functional potential. Immunol. Rev. *284*, 91–105.
50. Shu, B., Wu, K.H., Emery, S., Villanueva, J., Johnson, R., Guthrie, E., Berman, L., Warnes, C., Barnes, N., Klimov, A., and Lindstrom, S. (2011). Design and performance of the CDC real-time reverse transcriptase PCR swine flu panel for detection of 2009 A (H1N1) pandemic influenza virus. J. Clin. Microbiol. *49*, 2614–2619.
51. Harris, P.A., Taylor, R., Thielke, R., Payne, J., Gonzalez, N., and Conde, J.G. (2009). Research electronic data capture (REDCap)–a metadata-driven methodology and workflow process for providing translational research informatics support. J. Biomed. Inform. *42*, 377–381.
52. Couzens, L., Gao, J., Westgeest, K., Sandbulte, M., Lugovtsev, V., Fouchier, R., and Eichelberger, M. (2014). An optimized enzyme-linked lectin assay to measure influenza A virus neuraminidase inhibition antibody titers in human sera. J. Virol. Methods *210*, 7–14.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-human CD16-FITC (clone: 3G8)	Becton Dickinson	Cat# 555406; RRID: AB_395806
Anti-human CD11b-PE (clone: ICRF44)	eBioscience	Cat# 12-0118-42; RRID: AB_2043799
Anti-human CD192-AF647 (clone: 48607)	Becton Dickinson	Cat# 558406; RRID: AB_647150
Anti-human CD14-PECy7 (clone: M5E2)	Becton Dickinson	Cat# 557742; RRID: AB_396848
Anti-human CD11c-PECy5 (clone: B-ly6)	Becton Dickinson	Cat# 551077; RRID: AB_394034
Anti-human HLADR-APCeF780 (clone: LN3)	eBioscience	Cat# 14-9956-82; RRID: AB_468639
Anti-human IFNg-PECy7 (clone: 4S.B3)	Becton Dickinson	Cat# 561036; RRID: AB_2033977
Anti-human TNFa-APC (clone: MAb11)	Becton Dickinson	Cat# 554514; RRID: AB_398566
Anti-human CD8-PE (clone: RPA-T8)	Becton Dickinson	Cat# 555367; RRID: AB_395770
Anti-human CD3-PerCPC5.5 (clone: SK7)	Becton Dickinson	Cat# 340949; RRID: AB_400190
Anti-human CD4-FITC (clone: RPA-T4)	Becton Dickinson	Cat# 561842; RRID: AB_10892821
Anti-human CD3-APC (clone: SK7)	Becton Dickinson	Cat# 340440; RRID: AB_400513
Anti-human CD8-PerCPCy5.5 (clone: SK1)	Biolegend	Cat# 344710; RRID: AB_2044010
Anti-human CD38-PE (clone: HB-7)	Biolegend	Cat# 356604; RRID: AB_2561900
Anti-human DR-APCeF (clone: 780)	eBioscience	Cat# 50-162-21; RRID: AB_2890179
Anti-human Ki67-PECy7 (clone: Ki-67)	Biolegend	Cat# 350526; RRID: AB_2562872
Anti-human Bcl2-AF488 (clone: 100)	Biolegend	Cat# 633506; RRID: AB_2028390
Anti-human CD16-FITC (clone: 3G8)	Becton Dickinson	Cat# 555406; RRID: AB_395806
Anti-human IgA-Alkaline Phosphatase	MabTech	Cat# 3310-3; RRID: AB_2890180
Anti-human IgG- Alkaline Phosphatase	Jackson Immunoresearch	Cat# 109-055-003; RRID: AB_2337599
Bacterial and virus strains		
Beta-propiolactone-inactivated virus A/California/7/09 (H1N1)	World Health Organization (WHO) Collaborating Center (Melbourne)	N/A
Beta-propiolactone-inactivated virus A/Victoria/361/2011	World Health Organization (WHO) Collaborating Center (Melbourne)	N/A
Beta-propiolactone-inactivated virus A/Switzerland/9715293/2013	World Health Organization (WHO) Collaborating Center (Melbourne)	N/A
Beta-propiolactone-inactivated virus B/Brisbane/60/2008	World Health Organization (WHO) Collaborating Center (Melbourne)	N/A
Beta-propiolactone-inactivated virus B/Massachusetts/02/2012	World Health Organization (WHO) Collaborating Center (Melbourne)	N/A
Beta-propiolactone-inactivated virus B/Phuket/3073/2013	World Health Organization (WHO) Collaborating Center (Melbourne)	N/A
Reverse-genetics (6+2) H6N1_[A/California/7/09(H1N1)]	St. Jude's Children's Research Hospital (SJCRH)	N/A
Reverse-genetics (6+2) H6N2_[A/Victoria/361/2011(H3N2)]	St. Jude's Children's Research Hospital (SJCRH)	N/A
Reverse-genetics (6+2) H6N2_[A/Switzerland/9715293/2013(H3N2)]	St. Jude's Children's Research Hospital (SJCRH)	N/A
Biological samples		
Sera and peripheral blood mononuclear cells isolated from participants enrolled in SHIVERS	Auckland, New Zealand	N/A

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Nasal wash supernatant and peripheral blood mononuclear cells isolated from participants enrolled in FLU09	Memphis, Tennessee	N/A
Chemicals, peptides, and recombinant proteins		
Purified Influenza H1 from A/California/04/09	BEI Resources	Cat# NR13691
Purified Influenza H3 from A/Perth/16/2009	BEI Resources	Cat# NR42974
Purified Concentrated A/CA/04/2009 (H1N1)	St. Jude's Children's Research Hospital (SJCRH)	N/A
Purified Concentrated A/Victoria/361/2011	St. Jude's Children's Research Hospital (SJCRH)	N/A
Purified Concentrated A/Switzerland/9715293/2013 (H3N2)	St. Jude's Children's Research Hospital (SJCRH)	N/A
Purified Concentrated B/Massachusetts/2/2012	St. Jude's Children's Research Hospital (SJCRH)	N/A
Purified Concentrated B/Phuket/3073/2013	St. Jude's Children's Research Hospital (SJCRH)	N/A
Peptides for Expected Conserved MHC Class I Epitopes of Influenza Virus A Proteins	BEI Resources	Cat# NR-2667
Fetuin	Sigma	Cat# F3385
Lectin from *Arachis hypogaea* (peanut)-Peroxidase	Sigma	Cat# L7759-1MG
10 x Coating Buffer	KPL	Cat# 50-84-01
3,3,5,5-Tetramethylbenzidine (TMB) Liquid Substrate System	Sigma	Cat# T0440-1L
p -Nitrophenyl Phosphate (PNPP) Microwell Substrate System	KPL	Cat# 50-80-00
Benzonase	Sigma	Cat# B7651
Bovine Serum Albumin	Sigma	Cat# A8327
Stop Solution (1N Sulfuric Acid)	Fisher Scientific	Cat# SA212-1
Phytohemagglutinin, PHA-P	Sigma	Cat# L1668-5MG
Brefeldin A Solution (1,000X)	Biolegend	Cat# 420601
Deposited data		
Raw and analyzed data	This paper	Mendeley Data: https://doi.org/10.17632/rwxp2nfvys.1
Experimental models: Cell lines		
Human embryo kidney (HEK) 293T cells	ATCC	CRL-3216
Embryonated chicken eggs	St. Jude's Children's Research Hospital (SJCRH)	N/A
Recombinant DNA		
Plasmid expressing HA from A/Teal/Hong Kong/W312/1997(H6N1)	St. Jude's Children's Research Hospital (SJCRH)	N/A
Plasmid expressing NA from A/Victoria/361/2011(H3N2)	St. Jude's Children's Research Hospital (SJCRH)	N/A
Plasmid expressing NA from A/Switzerland/9715293/2013(H3N2)	St. Jude's Children's Research Hospital (SJCRH)	N/A
Plasmid expressing PB2 from A/Puerto Rico/8/1934 (H1N1)	St. Jude's Children's Research Hospital (SJCRH)	N/A
Plasmid expressing PB1 from A/Puerto Rico/8/1934 (H1N1)	St. Jude's Children's Research Hospital (SJCRH)	N/A

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Plasmid expressing PA from A/Puerto Rico/8/1934 (H1N1)	St. Jude's Children's Research Hospital (SJCRH)	N/A
Plasmid expressing NP from A/Puerto Rico/8/1934 (H1N1)	St. Jude's Children's Research Hospital (SJCRH)	N/A
Plasmid expressing M from A/Puerto Rico/8/1934 (H1N1)	St. Jude's Children's Research Hospital (SJCRH)	N/A
Plasmid expressing NS from A/Puerto Rico/8/1934 (H1N1)	St. Jude's Children's Research Hospital (SJCRH)	N/A
Software and algorithms		
FlowJo version 10	FlowJo, LLC	https://www.flowjo.com/solutions/flowjo
Prism (version 8)	Graphpad Software	https://www.graphpad.com:443/scientific-software/prism/
Research Electronic Data Capture (REDCap)	The REDCap Consortium	https://www.project-redcap.org/
Other		
96-well High-binding ELISA plates	Corning	Cat# 3590
Dulbecco's 1X PBS with 0.9 mM $CaCl_2$ and 0.5 mM $MgCl_2$	Life Technologies	Cat# 14040-182

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Richard J. Webby (Richard.webby@stjude.org)

Materials availability

This study did not generate new unique reagents.

Data and code availability

Original data have been deposited to Mendeley Data: [https://doi.org/10.17632/rwxp2nfvys.1].

EXPERIMENTAL MODEL AND SUBJECT DETAILS

SHIVERS cohort

For the Southern Hemisphere Influenza and Vaccine Effectiveness, Research and Surveillance (SHIVERS) cohort, participants (N = 66) were enrolled according to the World Health Organization (WHO)'s case definition for influenza-like-illness (ILI) or severe acute respiratory illness (SARI) from general practices or hospitals in Auckland between August and October of 2013, which coincided with the peak influenza season in New Zealand.[29] To detect influenza and other respiratory viruses, the US Centre for Disease Control's real-time reverse transcriptase (rRT) PCR protocols were used.[50] Participants whose samples tested positive by PCR for influenza were then contacted by the study nurse for the first sampling of whole blood and sera (considered to be an acute sample in the current study). A second blood sample was collected two weeks later (considered to be a convalescent sample in this study). Samples were processed at a central laboratory to isolate the peripheral blood mononuclear cells (PBMCs) using Ficoll-Paque (GE Lifesciences). Cells were stored in liquid nitrogen while sera were stored at −80°C until use. Study data were collected and managed using Research Electronic Data Capture (REDCap) tools hosted at the Institute of Environmental Science and Research.[51]

For the cellular immunology subcohort, we selected individuals with paired blood samples that had an acute sample collected within 14 days of symptom onset and an hemagglutination-inhibition (HAI) titer < 40 (N = 16). Seroconverters (SC) were selected based if they had at least a 4-fold increase in HAI-antibody titer to the infecting subtype in the convalescent sera (N = 7) while non-seroconverters (SC) had no such increase (N = 7).

FLU09 cohort

To validate the findings in the nasal mucosa, we examined the nasal wash supernatant and cells collected from influenza virus infected participants recruited through the FLU09 study. FLU09 was a longitudinal cohort study based in Memphis, TN.[31] Individuals in this study were recruited if they had onset of symptoms within the last 96 hours and were confirmed to be influenza positive during

the 2009/2010 and 2010/2011 influenza seasons. Influenza diagnosis was based US Centre for Disease Control's real-time reverse transcriptase (rRT) PCR protocol.[50] Nasal washes were collected to approximate these intervals: day 0, 3, 7, 10 and 28 after enrolment. Based on the mucosal IgA response, we defined individuals with robust antibody productions (converters) as those with an influenza-IgA titer of > 40 detected at any of the study days whereas non-converters are those with IgA titer < 40. From a cohort of N = 58 influenza confirmed cases and for which we have associated flow cytometry data, we identified N = 10 converters and N = 9 non-converters. These monocyte populations were characterized in nasal wash samples collected within a week after enrolment. In individuals where multiple samples were available, the earliest available time-points were used.

Ethics approval and study design considerations

The SHIVERS received ethics approval from the New Zealand Northern (A) Health & Disability Ethics Committee under references NTX/11/11/102/AM02, AM05, AM06, AM13, and AM14. The FLU09 study received ethics approval from the Institutional Review Boards of St. Jude Children's Research Hospital and the University of Tennessee Health Science Center / Le Bonheur Children's Hospital. Written, informed consent was obtained from all participants or if under the consenting age, consent was obtained from parents or legal guardians. Both studies were performed in accordance with the Declaration of Helsinki. Sample sizes were determined based on the availability of samples. Age and gender of subjects included in the study are available at Mendeley Data [https://doi.org/10.17632/rwxp2nfvys.1]. No randomization was performed and sample size were determined based on availability of samples. All laboratory experiments were conducted in a blinded manner. Serostatus of individuals were identified only during the analysis step.

METHOD DETAILS

Serology

Serologic testing by hemagglutination-inhibition assay (HAI) and neuraminidase-inhibition assay (NAI) for the SHIVERS cohort was performed at the National Influenza Center at the Institute of Environmental Science and Research (ESR) as previously described.[29] Sera pre-treated with receptor-destroying enzyme (RDE) to remove non-specific inhibitors of agglutinations, were tested against the infecting subtypes using the 2013 Southern Hemisphere vaccine strains [(A/Victoria/361/2011 (H3N2), or the two B-lineages; B/Brisbane/60/2008 (Victoria-lineage) or B/Phuket/3073/2013 (Yamagata-lineage)]. For influenza B, the antigen that induced a greater fold change was selected for analysis. Reference antigens which were propagated in embryonated chicken eggs were provided by World Health Organization Collaborating Centres (WHOCC)-Atlanta and WHOCC-Melbourne. These antigens were standardized by the haemagglutination (HA) assay to 4 HA units per 25 μL using 1.0% guinea pig erythrocytes. Serum samples were titrated in a serial two-fold dilutions with a starting dilution of 1:10 and ending at 1:640. Paired sera were tested together in the same run. HAI-antibody titer was measured as the reciprocal of the highest dilution causing complete haemagglutination inhibition of erythrocytes by the influenza virus. For those sera with HAI titer of > 1:640, further serial two-fold dilution were performed in order to detect the highest dilution titer that fully prevented haemagglutination. Results were accepted if sera and guinea-pig erythrocyte cell controls provided the correct non-agglutinated pattern and the positive controls were within two-fold of the mean titer.

The enzyme-linked lectin assay (ELLA) was used to test for NAI-specific antibodies using recombinant reverse-genetic viruses expressing the NA of the target strains with a mismatched HA as previously described.[52] Briefly, plasmids comprising of HA from an H6 avian virus A/Teal/Hong Kong/W312/1997(H6N1), target NA, and the six internal genes from the A/Puerto Rico/8/1934 (H1N1) strains were co-transfected into 293T cells at 1 μg each using Lipofectamine 2000. After 48 hours, culture supernatants were harvested and injected into embryonated chicken eggs to amplify the recovered viruses. After 48 hours, egg-allantoic fluid were harvested and stored as virus stocks once the viral sequence was confirmed by Sanger sequencing. Virus stocks were inactivated using beta-propiolactone (BPL) at 1:1000 w/v for 72 hours at 4°C prior to use in ELLA assay. To determine the working concentration, virus stocks were titrated to determine the dilution that gave 90% of the maximum optical density readout in the ELLA assay done without the addition of serum samples.

For the ELLA assay, 96-well microtiter plates were coated overnight with 25 μg/ml of fetuin, diluted in bicarbonate coating buffer (KPL). Sera were tested in serial 2-fold dilutions from 1:10 to 1:5120. Serial dilutions of heat-inactivated sera were transferred to fetuin-coated plates, reference antigens were added at the predetermined dilution, and then plates incubated for 16–18 hours at 37°C in dry heat, followed by the addition of peroxidase-labeled peanut lectin for 2 hours at room temperature. Bound lectin was detected with 3,3,5,5-Tetramethylbenzidine (TMB) Liquid Substrate System (Sigma-Aldrich). Paired sera were tested together in the same run. The NAI-titer was measured as the reciprocal of the highest dilution that resulted in more than 50% signal inhibition compared to virus-only wells. Sera with a NAI titer of > 1:5120 were assigned a titer of 1:10240.

All sera samples were tested in single wells in at least two separate experiments. Samples that show greater than two-fold inter-assay variation were retested. Individual assay titers that were within two-fold of each other were averaged to obtain the final titers used for analysis. Samples with a HAI or NAI titer of < 1:10 were assigned a titer of 1:5 for the purposes of computing seroconversion. A fourfold or greater rise in HAI antibody titers in paired sera, with the second titer at least 1:40 was considered as HAI or NAI seroconversion.

Enzyme-linked immunosorbent assay

Sera from the SHIVERS subcohort were tested for influenza-specific IgG using purified, concentrated virus stocks while nasal wash samples in the FLU09 study were tested for the presence of influenza HA-specific IgA or IgG using purified protein. All antigens are representative of the infecting subtypes in circulation when samples were collected. For SHIVERS samples, plates were coated overnight at 4°C with purified, concentrated virus stocks of A/California/04/2009 (H1N1) or A/Victoria/361/2011 (H3N2), while plates for FLU09 samples were coated with either purified H1 from A/California/04/09 (NR13691, BEI Resources) or H3 from A/Perth/16/2009 (NR42974, BEI Resources) at 1 μg/ml. Negative wells were coated with buffer only. Plates were washed six times with phosphate-buffered saline (PBS) supplemented with 0.05% Tween-20 (PBS-T) and blocked for 2 hours in PBS supplemented with 5% fetal bovine serum (FBS). Serially diluted sera (at a starting dilution of 1:100) or nasal wash supernatants (at a starting dilution of 1:10) were added to the plate in duplicates. Serum samples were incubated for 2 hours while nasal wash samples were incubated overnight at 4°C. After incubation, plates were washed as before and incubated with alkaline-phosphatase conjugated anti-human IgA (MabTech) or IgG (Jackson) for 2 or 1 hour, respectively. After a final wash step, plates were developed with the p-nitrophenyl phosphate (pNPP) substrate system (KPL). Absorbance was read at 450 nm and the last dilution that gave a positive/negative optical density readout ratio of > 2 was determined as the end-point titer.

Flow cytometry

Cryopreserved cells were thawed gently in warm, complete RPMI (supplemented with 10% FBS, 10 U/ml Penicillin/Streptomycin and 2 mM glutamine) media in the presence of 25 U/ml Benzonase. Cells were centrifuged at 450 x g for 5 minutes at room temperature and cell pellet were resuspended to the concentration of 1×10^7 cells/ml in pre-warmed, benzonase-free media. For each sample, 100 μl of cells (1×10^6 cells) were aliquoted into one well for the proliferation and monocytes and myeloid dendritic cell (mDC) panel and into five-wells for the intracellular cytokine staining (ICS) panel, in 96-well plates. Three staining panels were used: a T cell proliferation panel consisting of antibodies for CD3, CD8, CD38, DR, Ki67 and Bcl2, a monocyte/mDC panel consisting of antibodies for CD16, CD11b, CD192, CD14, CD11c and HLA-DR and an ICS panel consisting of antibodies for IFN-γ, TNF-α, CD8, CD3 and CD4.

For staining of the monocyte/mDC surface markers, cells were pelleted at 450 x g for 8 mins and resuspended in 25 μl of staining cocktail containing the pre-determined concentration of antibodies. Cells were incubated for 30 minutes on ice. Cells were washed in FACS buffer (PBS with 2% FBS and 0.1% sodium azide) and fixed in 1% formaldehyde diluted in PBS for 10 min on ice, in the dark. Cells were pelleted, supernatant discarded and subsequently washed twice in FACS buffer prior to acquisition.

ICS staining was performed on *in vitro* stimulated cells using pooled peptides derived from the influenza virus Matrix-1 (M1), nucleoprotein (NP), and polymerase basic 1 (PB1) proteins (NR-2667, BEI Resources). Pooled peptides were added at a working concentration of 1 μg/ml per peptide into the protein specific pools. Phytohemagglutinin (PHA) at 1 μg/ml or media alone were used as controls. Cells were stimulated for 2 hours at 37°C in 5% CO_2. After 2 hours, 5 μg/ml of Brefeldin A were added to the wells and incubated for another 4 hours. Cells were then pelleted and the supernatant discarded. Cells were washed and resuspended in surface antibody markers in 25 μl volume and incubated for 30 minutes on ice. Cells were washed and fixed as described above prior to permeabilization. To permeabilize the cells for intracellular staining, pelleted cells were resuspended in 100 μl of 0.5% Saponin in FACS buffer and incubated for 15 min on ice in the dark. After incubation, cells were washed once and resuspended in 25 μl of intracellular staining cocktail containing IFN-γ and TNF-α or Ki67 and Bcl2 and incubated for 30 minutes on ice. Cells were washed twice prior to acquisition. All staining was performed in the dark. Single-color controls, unstimulated cells and unstained cells were included for compensation and control.

Samples were acquired on a LSRII flow cytometer (Becton Dickinson) and analyzed using FlowJo v10 (FlowJo, LLC). For some analyses, not all samples were analyzed due to sample availability (e.g poor PBMC viability). Data were expressed as absolute cell numbers, derived by back-calculation of the percentage of recovered live cells or as percentages of parent population. FlowJo data were exported as CSV files to Graphpad Prism for statistical analyses. Medians were used to describe the average responses and interquartile range were used to describe the measures of variability. No technical replicates were performed due to the lack of samples.

QUANTIFICATION AND STATISTICAL ANALYSIS

All categorical data were analyzed by Fischer's or Chi-square Test. Antibody data and data that failed to meet assumptions of normality were log-transformed and tested using unpaired Student's t test. Differences between paired samples were analyzed by paired t test. Flow cytometric data were analyzed by one-way analysis of variance with Bonferroni corrections applied for multiple comparisons, where applicable. All analyses were done using GraphPad Prism (v8). P value of < 0.05 is considered statistically significant, unless otherwise indicated.