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ORIGINAL ARTICLE

Humoral and cellular immune responses in vaccinated and unvaccinated children following SARS-CoV-2 Omicron infection

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Abstract

Objectives. The immune response in children elicited by SARS-CoV-2 Omicron infection alone or in combination with COVID-19 vaccination (hybrid immunity) is poorly understood. We examined the humoral and cellular immune response following SARS-CoV-2 Omicron infection in unvaccinated children and children who were previously vaccinated with COVID-19 mRNA vaccine. **Methods.** Participants were recruited as part of a household cohort study conducted during the Omicron predominant wave (Jan to July 2022) in Victoria, Australia. Blood samples were collected at 1, 3, 6 and 12 months following COVID-19 diagnosis. Humoral immune responses to SARS-CoV-2 Spike proteins from Wuhan, Omicron BA.1, BA.4/5 and JN.1, as well as cellular immune responses to Wuhan and BA.1 were assessed. **Results.** A total of 43 children and 113 samples were included in the analysis. Following Omicron infection, unvaccinated children generated low antibody responses but elicited Spike-specific CD4 and CD8 T-cell responses. In contrast, vaccinated children infected with the Omicron variant mounted robust humoral and cellular immune responses to both ancestral strain and Omicron subvariants. Hybrid immunity persisted for at least 6 months post infection, with cellular immune memory characterised by the generation of Spike-specific polyfunctional CD8 T-cell responses. **Conclusion.** SARS-CoV-2 hybrid immunity in children is characterised by persisting SARS-CoV-2 antibodies and robust CD4 and CD8 T-cell activation and polyfunctional responses. Our findings contribute to understanding hybrid immunity in

children and may have implications regarding COVID-19 vaccination and SARS-CoV-2 re-infections.

Keywords: cellular immune responses, children, hybrid immunity, SARS-CoV-2

INTRODUCTION

Coronavirus disease 2019 (COVID-19) in children is generally mild or asymptomatic.[1,2] The lower clinical severity in children compared with adults is thought to be because of a faster and stronger innate immune response to SARS-CoV-2.[3] However, a small proportion of children develop severe acute infection or multisystem inflammatory syndrome in children (MIS-C), which requires hospitalisation.[4–6] Furthermore, the risk of post-acute sequelae of COVID-19 (PASC) following SARS-CoV-2 infection,[7] as well as the indirect impact of COVID-19 in children and parents/guardians (i.e. sick days, absence from school, loss wages), can significantly impact their overall quality of life.[8] Importantly, children remain a source of transmission within the household.[9]

The increased transmissibility of the Omicron variant, and the overall low vaccination rates in children at the start of the Omicron wave, resulted in an absolute and relative increase in COVID-19 cases among children from late 2021.[1] In adults, COVID-19 booster vaccination schedules are associated with a significant reduction in SARS-CoV-2 Omicron-related hospitalisation and death.[10,11] In children aged 5–17 years, a two-dose primary COVID-19 vaccination schedule was recommended mostly in middle- and high-income countries,[2] with booster doses generally recommended only for immunocompromised children.[12] In Australia, the current COVID-19 vaccine programme focusses on adults aged $\geq$ 18 years, and COVID-19 vaccination is not recommended for children under 5 years unless they are immunocompromised or have a disability or complex health condition.[13] Reports suggest COVID-19 vaccination is moderately effective against Omicron infection and hospitalisation in children.[14,15]

Given the low vaccine uptake among children and the increasing reports of breakthrough infections, the importance of understanding the nature and persistence of immune responses in children will help inform effective disease prevention strategies. Hybrid immunity is defined as the immunity that develops following the combination of SARS-CoV-2 infection and COVID-19 vaccination, irrespective of the order.[16] To date, most studies of COVID-19 hybrid immunity have been in adults[17–19] and have reported significantly more robust humoral and cellular immune responses than those arising from vaccination or infection alone.[17,20] This heightened immune response is likely to translate into more durable protection over a longer period.[21,22] However, it is uncertain whether similar robust hybrid immunity occurs in children as there are marked differences in the anti-SARS-CoV-2 immune response to infection between children and adults.[3,23]

To address these knowledge gaps, we aimed to assess humoral and cellular immune responses in unvaccinated and vaccinated children who were infected during the Omicron wave in Victoria, Australia, which started in November 2021.

RESULTS

COVID-19 Omicron cohort

A total of 43 children were recruited at the Royal Children's Hospital, Victoria, Australia, between Jan 2022 and July 2022, a period when the SARS-CoV-2 Omicron variant was predominant. Children aged between 3 months and 17 years with COVID-19 confirmed by SARS-CoV-2 PCR or rapid antigen test (RAT) on nasopharyngeal swab were included in this analysis (Table 1). Children infected with earlier SARS-CoV-2 variants were excluded. Our hybrid group was defined as children who were vaccinated with the ancestral COVID-19 vaccine prior to SARS-CoV-2 infection. As vaccination was not available for healthy children under 5 years in Australia, children in the hybrid group of our study were older (11 years, 5–17 years) than children with SARS-CoV-2 infection only (median, range: 1 year, 0.4–7 years). This age difference limited our ability to undertake comparisons between these groups. Therefore, we focussed on separate analyses of

Table 1. COVID-19 Omicron cohort

		Hybrid		
	Infection only (N = 17)	Total (N = 26)	[a]1 dose (N = 8)	[b]2 or 3 doses (N = 18)
Age, median (range)	1 (0.4, 7)	11 (5, 17)	8 (5, 12)	13 (6, 17)
Sex, female (%)	7 (41%)	13 (50%)	3 (37.5%)	10 (56%)
Time since last infection, median days (range)	34 (13, 78)	31 (13, 81)	31 (28, 60)	31 (13, 81)
Time since last vaccine dose, median days (range)	–	73 (26, 113)	20 (11, 47)	109 (60, 135)
Up to May 2022 (BA1 wave), *N*	12	18	8	10
From June 2022 (BA4/5 wave), *N*	5	8	0	8

[a]Two participants received their 2nd dose ≤ 5 days prior to infection.
[b]Two participants received three doses.

the immune response in unvaccinated and vaccinated children with Omicron infection. The time since last infection was similar for both the infection and hybrid groups (31 vs 34 days, respectively, Table 1), with the majority of infections occurring when the SARS-CoV-2 BA.1 variant was circulating according to Nextstrain.org.[24]

Omicron infection elicits Spike-specific CD4 and CD8 T-cell responses among children aged under 5 years

We measured both humoral (IgG and surrogate neutralising antibodies) and cellular immune responses [intracellular cytokine secretion and activation-induced marker (AIM) expression] against the SARS-CoV-2 Spike protein [ancestral strain (WA.1), BA.1, BA.4/5 and JN.1 (IgG only)] in children 1 month following Omicron infection (Figure 1a). Children infected with Omicron mounted low IgG concentrations and surrogate neutralising antibody titres to all tested strains (Figure 1b and c). IgG-binding antibody units (BAU mL^{-1}) against WA.1, BA.1 and BA.4/5 are shown in Supplementary figure 1. As expected, Omicron-infected children had significantly higher IgG and surrogate neutralising antibodies against BA.1 and BA.4/5 variants than WA.1 (Figure 1b and c). Most children (13/17, 76.5%) had no surrogate neutralising antibodies against WA.1 (Figure 1c).

While the antibody responses were generally weak, Omicron infection generated Spike-specific CD4 and CD8 T-cell responses by AIM expression following WA.1 or BA.1 stimulation (Figure 1d and e). The expression of individual AIMs (CD69, CD137 and OX-40) on CD4 and CD8 T cells following WA.1 and BA.1 stimulation are shown in Figure 1f. However, only CD4 T cells but not CD8 T cells exhibited polyfunctional responses against WA.1 and BA.1 in terms of IFNγ, TNFα and IL-2 expression compared with unstimulated cells (Figure 1d and e). Individual CD4 and CD8 T-cell cytokine expression profiles are shown in Supplementary figure 2.

Hybrid immunity induces robust humoral and cellular immunity

We used the same experimental approach to assess humoral (IgG and surrogate neutralising antibodies) and cellular immune responses (intracellular cytokine and AIM expression) in children with hybrid immunity (Figure 2a). One month following Omicron BA.1 or BA.4/5 infection, robust IgG antibody responses were observed across all tested SARS-CoV-2 variants among previously vaccinated children (Figure 2b). All children with hybrid immunity had strong surrogate neutralising antibody titres against WA.1, BA.1 and BA.4/5. Surrogate neutralising antibody titres against BA.1 and BA.4/5 were lower than WA.1 (Figure 2c).

Children with hybrid immunity exhibited both CD4 and CD8 T-cell activation, as well as polyfunctional responses against WA.1 and BA.1 compared with unstimulated cells (Figure 2d and e). Individual CD4 and CD8 T-cell cytokine expression profiles are shown in Supplementary figure 3. The expression of individual AIMs (CD69, CD137 and OX-40) on both CD4 and CD8 T cells following WA.1 and BA.1 stimulation are shown Figure 2f.

When we stratified the hybrid group based on the number of vaccine doses received before infection, we found that children who received two/three doses have significantly higher IgG concentrations against BA.1 and JN.1 than

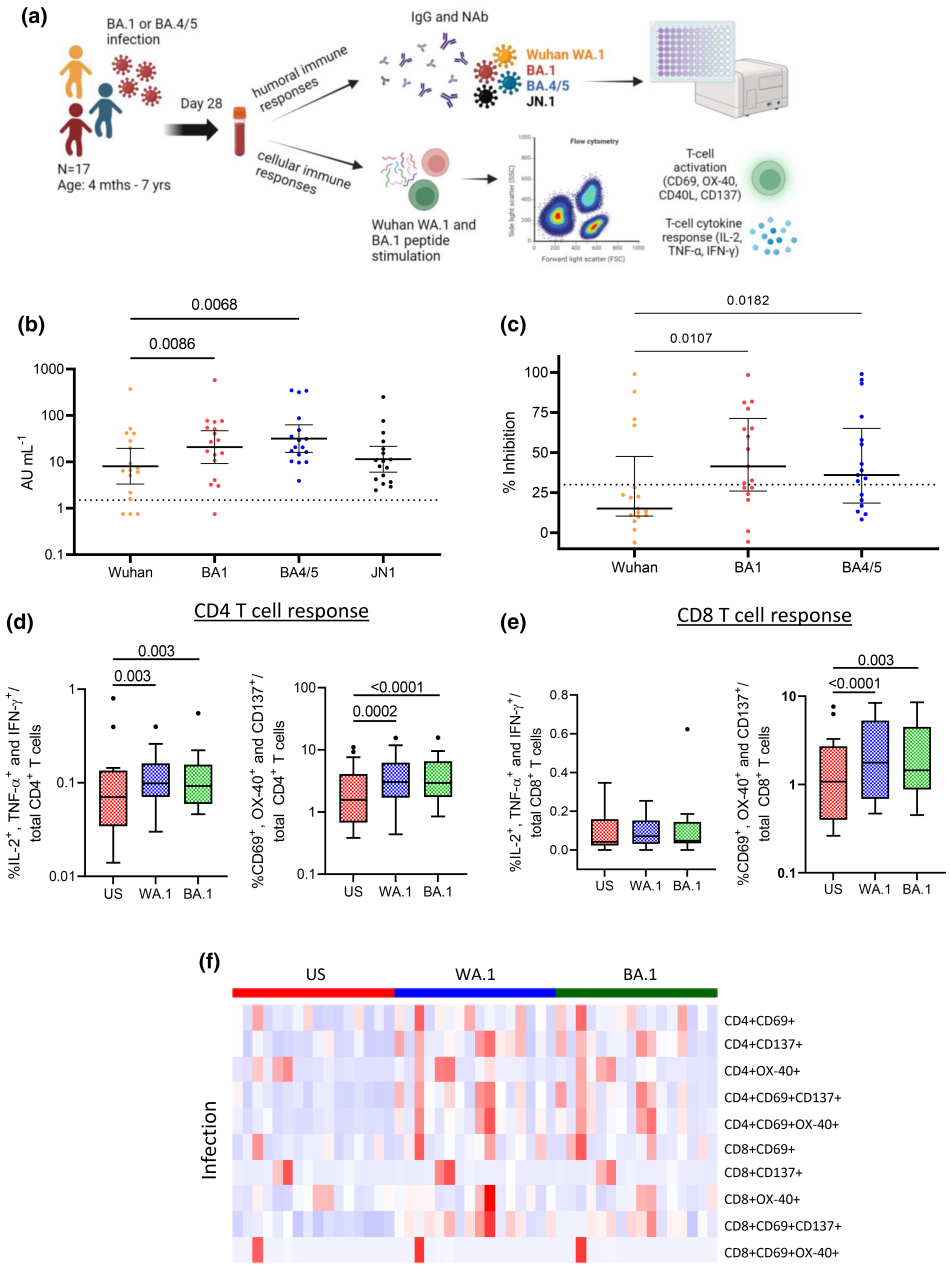

Figure 1. SARS-CoV-2 humoral and cellular immune responses in unvaccinated children at 1 month post Omicron infection. **(a)** Blood samples collected 28 days post infection were used to measure SARS-CoV-2 humoral (IgG and surrogate neutralising antibodies; NAb) (N = 17) and cellular immune response (via AIM and ICS expression following stimulation with the overlapping peptide pools of the Wuhan (WA.1) or omicron BA.1 full-length spike) (N = 16). **(b)** SARS-CoV-2 IgG concentration against WA.1 (orange), omicron BA.1 (red), BA.4/5 (blue) and JN.1 (black) measured by ELISA. **(c)** SARS-CoV-2 neutralising antibody response (% inhibition) against WA.1, omicron BA.1 and BA.4/5 measured by surrogate virus neutralisation test. **(d)** $CD4^-$ and **(e)** $CD8^-$ Spike-specific T cell response characterised by polyfunctionality (%$IL\text{-}2^+$, TNF-α^+ and $IFN\gamma^+$) or expression of activation-induced markers (%$CD69^+$, $OX\text{-}40^+$ and $CD137^+$) following WA.1 or BA.1 stimulation. **(f)** Heatmap of each activation marker and in combination following WA.1 or BA.1 stimulation. IgG data are presented as assay units (AU mL^{-1}) and surrogate neutralising antibody data are presented as percent inhibition (%inhibition). IgG binding antibody units against WA.1, BA.1 and BA.4/5 are available in Supplementary figure 1. Error bars represent geometric mean concentrations ± 95% CI and median with interquartile range for IgG and surrogate neutralising antibody data, respectively. A Friedman's test was used to compare the different variants. T-cell data are presented as a boxplot (median, min–max and outliers shown as dots). A Friedman's test with a Benjamini–Hochberg post-hoc adjustment was used to compare between unstimulated and stimulated conditions. An adjusted P-value < 0.05 was considered significant.

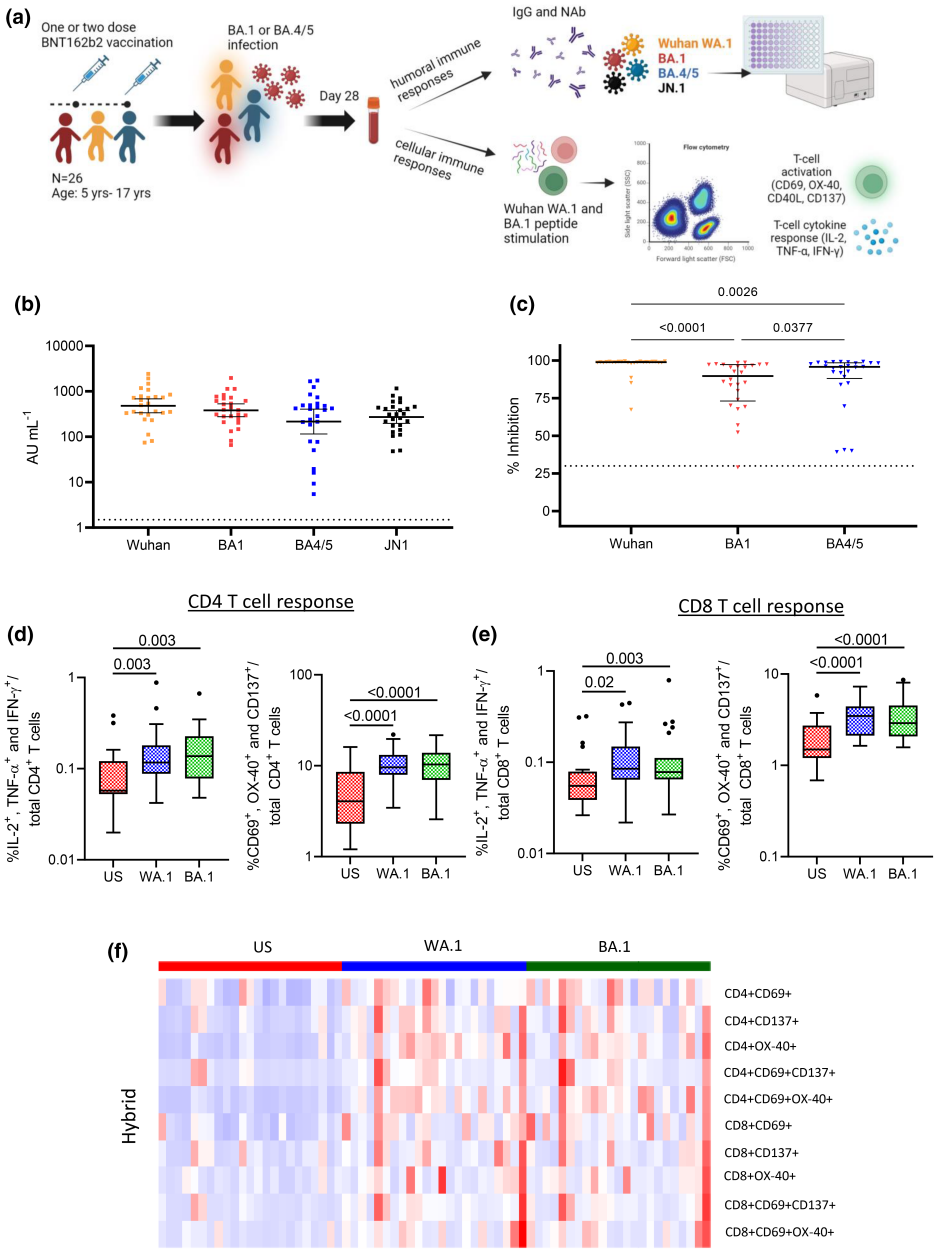

Figure 2. SARS-CoV-2 humoral and cellular immune responses in previously vaccinated children at 1 month post Omicron infection. **(a)** Blood samples collected 28 days post infection were used to measure SARS-CoV-2 humoral (IgG and surrogate neutralising antibodies; NAb) (N = 26) and cellular immune response (via AIM and ICS expression following stimulation with the overlapping peptide pools of the Wuhan (WA.1) or omicron BA.1 full-length spike) (N = 23). **(b)** SARS-CoV-2 IgG concentration against WA.1 (orange), omicron BA.1 (red), BA.4/5 (blue) and JN.1 (black) measured by ELISA. **(c)** SARS-CoV-2 neutralising antibody response (% inhibition) against WA.1, omicron BA.1 and BA.4/5 measured by surrogate virus neutralisation test. **(d)** $CD4^-$ and **(e)** $CD8^-$ Spike-specific T cell response characterised by polyfunctionality (%$IL\text{-}2^+$, $TNF\text{-}\alpha^+$ and $IFN\gamma^+$) or expression of activation-induced markers (%$CD69^+$, $OX\text{-}40^+$ and $CD137^+$) following WA.1 or BA.1 stimulation. **(f)** Heatmap of each activation marker and in combination following WA.1 or BA.1 stimulation. IgG data are presented as assay units (AU mL^{-1}) and surrogate neutralising antibody data are presented as percent inhibition (%inhibition). IgG binding antibody units against WA.1, BA.1 and BA.4/5 are available in Supplementary figure 1. Error bars represent geometric mean ± 95% CI and median with interquartile range for IgG and surrogate neutralising antibody data, respectively. A Friedman's test was used to compare the antibody response across different variants. T-cell data are presented as a boxplot (median, min–max and outliers shown as dots). A Friedman's test with a Benjamini–Hochberg post hoc adjustment was used to compare between unstimulated and stimulated conditions. An adjusted P-value < 0.05 was considered significant.

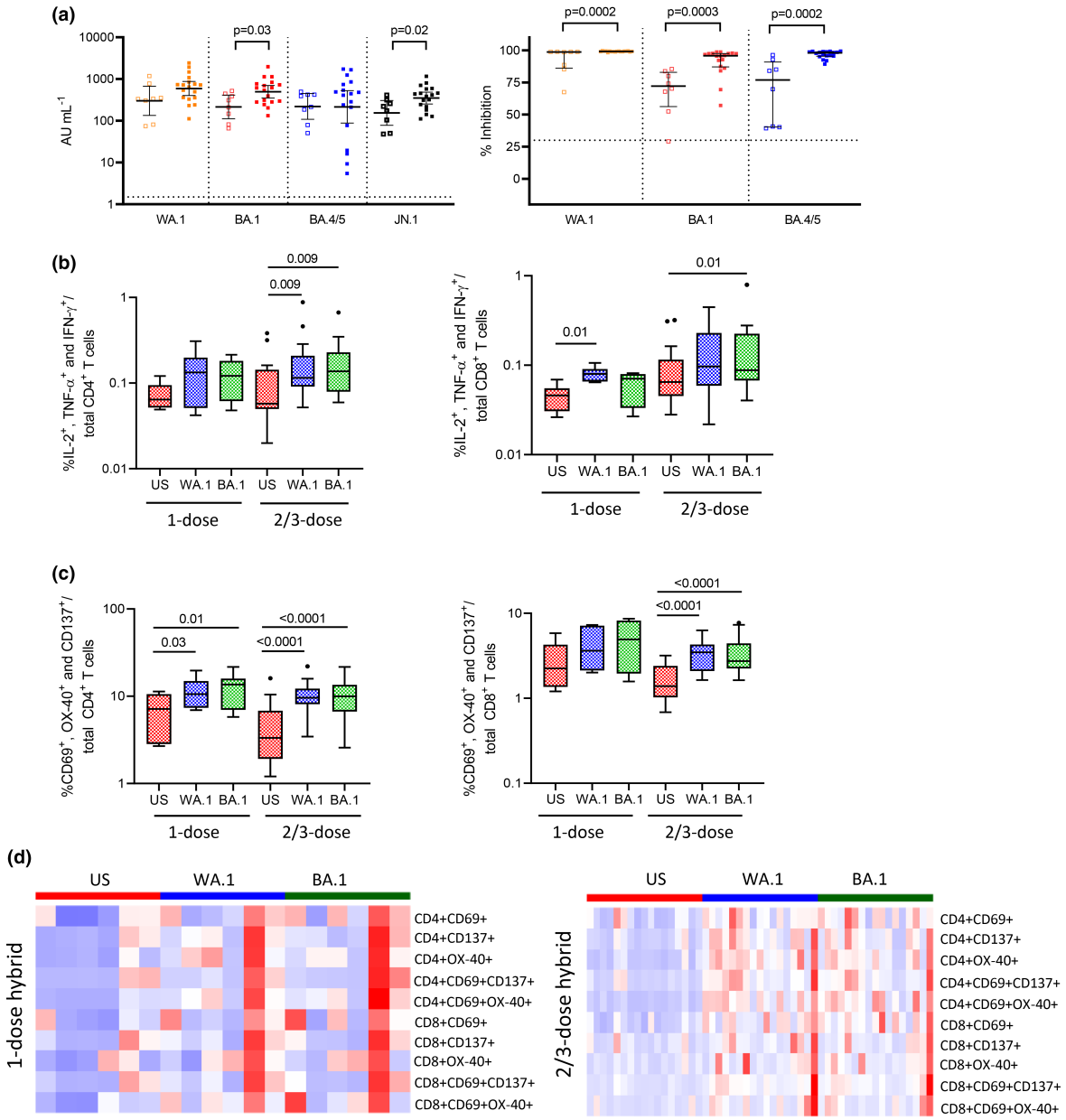

Figure 3. SARS-CoV-2 humoral and cellular immune responses in previously vaccinated children at 1 month post Omicron infection stratified by vaccine doses. Blood samples collected 28 days post infection were used to measure SARS-CoV-2 humoral (IgG and surrogate neutralising antibodies; NAb) (N = 26; N = 18 vaccinated with two/three dose and N = 8 vaccinated with one dose) and cellular immune response (via AIM and ICS expression following stimulation with the overlapping peptide pools of the Wuhan (WA.1) or omicron BA.1 full-length spike) (N = 23; N = 17 vaccinated with two/three dose and N = 6 vaccinated with one dose). **(a)** SARS-CoV-2 IgG concentration and surrogate neutralising antibody response (% inhibition) against WA.1 (orange), omicron BA.1 (red), BA.4/5 (blue) and JN.1 (black) by vaccine doses (N = 18 vaccinated with two/three dose; closed symbols and N = 8 vaccinated with one dose; open symbols) **(b)** $CD4^-$ and **(c)** $CD8^-$ Spike-specific T-cell response by vaccine dose, characterised by polyfunctionality (%$IL\text{-}2^+$, $TNF\text{-}\alpha^+$ and $IFN\gamma^+$) or expression of activation-induced markers (%$CD69^+$, $OX\text{-}40^+$ and $CD137^+$) following WA.1 or BA.1 stimulation. **(d)** Heatmap of each activation marker and in combination following WA.1 or BA.1 stimulation by vaccine dose. IgG data are presented as assay units (AU mL^{-1}) and surrogate neutralising antibody data are presented as percent inhibition (% inhibition). Error bars represent geometric mean ± 95% CI and median with interquartile range for IgG and neutralising antibody data, respectively. A Mann–Whitney U-test was used to compare antibody response between one-dose and two-/three-dose group. T-cell data are presented as a boxplot (median, min–max and outliers shown as dots). A Friedman's test with a Benjamini–Hochberg post hoc adjustment was used to compare between unstimulated and stimulated conditions. An adjusted P-value < 0.05 was considered significant.

children who received only one dose (Figure 3a). Consistent with the IgG responses, surrogate neutralising antibodies against WA.1, BA.1 and BA.4/5 were also significantly higher in children who received two/three doses compared with children who received one dose (Figure 3a). In contrast, there was no Spike-specific CD4, or CD8 T-cell responses observed irrespective of the number of vaccine doses received. Both one- and two/three-dose groups induced higher polyfunctional and activation marker expression on CD4 and CD8 T cells following WA.1 or BA.1 stimulation compared with unstimulated cells. However, the small sample size in the one-dose group may have precluded statistical significance for some populations (i.e. $IL2^{+}TNF^{+}IFNg^{+}$ CD4 and $CD69^{+}OX40^{+}CD137^{+}$ CD8) (Figure 3b and c). The expression of individual AIMs (CD69, CD137 and OX-40) on CD4 and CD8 T cells following WA.1 and BA.1 stimulation for both one-dose and two/three-dose groups are shown in Figure 3d.

Persistence and recall of SARS-CoV-2 immune memory following vaccination and/or infection

A subset of children in both the infection only group and hybrid group were followed up at 3, 6 and 12 months post infection to assess humoral and cellular immune responses (Figure 4a). For this analysis, we also included a group of vaccinated but not infected children (verified by negative N protein serostatus throughout follow-up) (Supplementary table 1). Samples from the vaccinated only group were collected at a median duration of 4 months post vaccination with a 3- and 6-month follow up after the 4-month time point.

Figure 4b shows the SARS-CoV-2 Spike IgG and surrogate neutralising antibody kinetics for the ancestral strain, BA.1 and BA.4/5, as well as IgG against SARS-CoV-2 JN.1 Spike protein across the hybrid, vaccine-only and infection-only groups. Participants in the one-dose hybrid group were excluded for this analysis as they received their second dose of vaccine within 3 months post infection, which is reflected in the elevated Spike-specific IgG responses at this time point (Supplementary figure 4). For each group, IgG antibody responses across each variant were similar over the 12-month follow-up period (Figure 4b). Importantly, the IgG concentration and surrogate neutralising antibody titres in the hybrid group remained high over the 12-month follow-up, although the number of participants at the 12-month time point were small ($n = 3$) (Figure 4b). In the infection-only group, surrogate neutralising antibody titres against BA.1 and BA.4/5 rose at 3 months, remained stable at 6 months post infection and then declined by 12 months (Figure 4b). As expected, surrogate neutralising antibody titres against the ancestral strain remained low or undetectable during the follow-up period in the infection only group but was high for the hybrid and vaccine-only group (Figure 4b). Interestingly, in the vaccine-only group, the median surrogate neutralising antibody titres against BA.4/5 across all time points were higher than BA.1. The reason for this is unclear although previous studies have detected and documented higher BA4/5 than BA.1 surrogate neutralising antibodies among vaccinated individuals.[25–27] In addition, the semi-quantitative nature of SARS-CoV-2 surrogate virus neutralisation test (sVNT) may have contributed to the observed differences between BA.1 and BA.4/5 surrogate neutralising titres in the vaccine-only group.

We also investigated T-cell memory in peripheral blood mononuclear cells (PBMC) samples collected at 6 months post infection among the hybrid and infection only group, and at 4 months post vaccination among the vaccine-only group. For the infection only group, Spike-specific CD4 T cell but not CD8 T-cell responses persisted to 6 months post infection as shown by the increased AIM expression following WA.1 or BA.1 stimulation compared with unstimulated cells (Figure 4c). In line with their SARS-CoV-2 infection history, a higher BA.1 but not WA.1 polyfunctional CD4 T-cell response was also observed, although this was not statistically significant (Figure 4c). Individual CD4 and CD8 T-cell cytokine expression profiles for the infection-only group are shown in Supplementary figure 5. For the vaccine-only group, both CD4 and CD8 T-cell AIM expression and polyfunctionality were higher following WA.1 or BA.1 stimulation compared with unstimulated control (Figure 4d).

For the hybrid group, a strong polyfunctional CD8 T-cell response to BA.1 at 6 months post infection was observed, a feature that was not seen in the infection-only or vaccine-only group (Figure 4e). Individual CD4 and CD8 T-cell cytokine expression profiles for the hybrid group

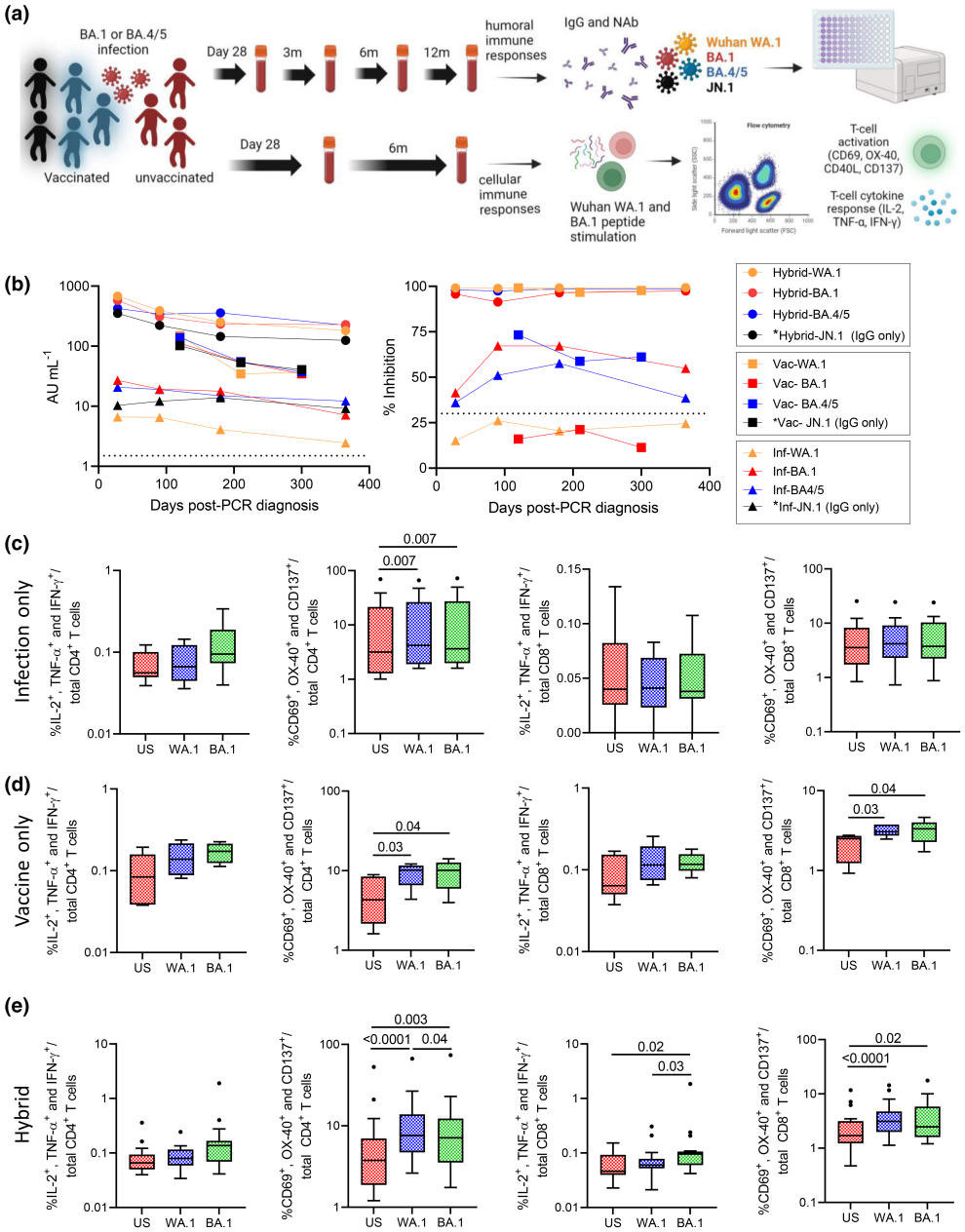

Figure 4. Persistence of immunity in vaccinated children with or without Omicron infection, and unvaccinated children with Omicron infection. **(a)** Children were followed up for 12 months with humoral responses measured at 1 month (Hybrid: $N = 18$; Infection only: $N = 17$), 3 months (Hybrid: $N = 12$; Infection only: $N = 12$), 6 months (Hybrid: $N = 13$; Infection only: $N = 9$) and 12 months (Hybrid: $N = 3$; Infection only: $N = 3$) post-infection, while cellular immune response was measured at 1 month (Hybrid: $N = 17$; Infection only: $N = 16$) and 6 months post infection (Hybrid: $N = 17$; Infection only: $N = 9$) where samples were available. Vaccine-only children had samples taken at 4 months post vaccination ($N = 7$ and $N = 5$ for humoral and cellular immune response, respectively) and was followed up for a further 6 months ($N = 4$). **(b)** SARS-CoV-2 IgG kinetics and surrogate neutralising antibody kinetics measured against ancestral (WA.1) (closed circle), omicron BA.1 (open triangle), BA.4/5 (half-closed square) and JN.1 (open diamond; *IgG only); Blue symbols and lines: children vaccinated with two/three doses with omicron infection; red symbols and lines: unvaccinated children with Omicron infection; black symbols and lines: vaccinated children. Data points are presented as medians. (%IL-2$^+$, TNF-α^+ and IFNγ^+) or expression of activation-induced markers (%CD69$^+$, OX-40$^+$ and CD137$^+$) following WA.1 or BA.1 stimulation among **(c)** infection only group, **(d)** Vaccine-only group and **(e)** Hybrid group. T cell data are presented as a boxplot (median, min–max and outliers shown as dots). A Friedman's test with a Benjamini–Hochberg post hoc adjustment was used to compare between unstimulated and stimulated conditions. An adjusted P-value < 0.05 was considered significant.

are shown in Supplementary figure 6. Consistent with the polyfunctional response, higher CD4 and CD8 T-cell activation were observed in the hybrid group following WA.1 or BA.1 stimulation compared with the unstimulated control. The CD4 activation response and CD8 polyfunctional response were higher for BA.1 than WA.1 in the hybrid group, reflecting their SARS-CoV-2 Omicron exposure (Figure 4e). The individual AIM markers (CD69, CD137 and OX-40) for both CD4 and CD8 T cells following WA.1 and BA.1 stimulation for both infection-only and hybrid groups are shown in Supplementary figure 7.

DISCUSSION

In this study, we found that unvaccinated children infected with Omicron induced weak antibody responses, but generated Spike-specific CD4 and CD8 T-cell responses, of which the CD8 T-cell response waned by 6 months. In contrast, children previously vaccinated with two/three doses of monovalent mRNA COVID-19 vaccine developed robust humoral and cellular immune responses that were sustained for at least 6 months following SARS-CoV-2 Omicron infection. Because of the age difference in vaccinated and unvaccinated cohorts, we are unable to directly compare their immune responses. Nevertheless, the difference in immunity and persistence observed among unvaccinated children and vaccinated children following Omicron infection may have implications on SARS-CoV-2 reinfection rates as seen with adults.[21]

Most of the evidence on hybrid immunity and protection are derived from studies in adults.[17–21] We found that children with hybrid immunity generated robust antibody responses to both the ancestral strain contained in the vaccine and to Omicron BA.1, BA.4/5 and JN.1 subvariants. Furthermore, Spike-specific IgG responses had a dose–response relationship with the number of vaccine doses, consistent with hybrid immunity data from children following Omicron infection,[27] and analogous data in adults.[28,29] It is important to note that the SARS-CoV-2 JN.1 variant was not circulating at the time when the samples were collected. The similarly high IgG antibody response against JN.1 as with BA.1 or BA.4/5 variant suggests that there may be some protection against this variant among children with hybrid immunity, although this needs to be interpreted with caution as the IgG response was based on S1 and S2 trimer protein (while the other variants were based on S1 protein only). A recent study found children with hybrid immunity generate cross-neutralising antibodies to Omicron sub variants (i.e. XBB), consistent with our findings.[30] Unvaccinated children infected with Omicron had much weaker antibody responses. As neutralising antibody titres correlate with protection against symptomatic COVID-19 infection,[31–33] our results indicate that unvaccinated children may remain unprotected against SARS-CoV-2 reinfection, particularly against emerging variants. Indeed, effectiveness against reinfection among unvaccinated children dropped to 54% within 3 months of Omicron infection compared with 70% among vaccinated children.[34] However, it is worth noting that secondary Omicron infection of previously Omicron-infected children induced stronger antibody responses than primary Omicron infection (but still lower than hybrid immunity).[35] This suggests a limited humoral immune memory response following primary Omicron infection.

T-cell immunity is thought to play an important role in limiting COVID-19 severity.[36] Studies investigating the T-cell response in children with hybrid immunity following Omicron infection is limited. A recent study in children found that breakthrough infection boosts ancestral Spike-specific T-cell responses, as well as non-Spike T-cell responses (i.e. nucleocapsid, membrane, ORF3a- and ORF7/8).[30] Our findings suggest hybrid immunity boosts Spike-specific Omicron T-cell responses. While both infection only and hybrid groups in our study generated Spike-specific CD4 and CD8 T-cell responses, a polyfunctional CD8 T-cell response was only seen in the hybrid group. This finding is consistent with adult studies of hybrid immunity that found preferential expansion of CD8 T cells specific for the SARS-CoV-2 Spike protein[37,38] and a recent study in children.[30] Our data add support to previous studies in adults and children showing how hybrid immunity might facilitate faster viral clearance and resolution of symptoms,[39–44] although we were not able to address this directly as clinical and virological data were not available. In addition, immune imprinting, characterised by reduced T-cell responses against Omicron compared with responses against Wuhan or Delta variants, has been reported in adults with Omicron breakthrough infection.[28] However, this phenomenon was not observed in our hybrid group or in another study in children.[35] Further

investigations are needed to confirm this difference in immune imprinting phenomenon between children and adults.

An important finding in our study is the persistence of humoral and cellular immunity in children with hybrid immunity to both BA.1 and ancestral strain. Prior studies in adults have documented persistent T- and B-cell responses up to 8 months after SARS-CoV-2 infection, but these were conducted during the pre-Omicron period.[45] One study in children found persistent hybrid immunity for at least 12 months, although that was to the ancestral strain.[30] We observed a higher CD8 polyfunctional response against BA.1, but the small numbers in the group at the 6-month time point may have increased the possibility of a type II error. Hybrid immunity in adults was shown to provide the highest level of protection 6–8 months after last immune exposure.[46] Our data, therefore, suggest that hybrid immunity may reduce the risk of COVID-19 reinfection in children for the same duration, although this may vary with emerging variants. Our study examined hybrid immunity in the context of vaccination preceding breakthrough infection. Whether hybrid immunity in children varies depending on the sequence of exposures is unknown, although evidence to date in adults and children does not support such a difference in immune response or protection.[20,21,27]

Higher antibody responses but not CD4 or CD8 T-cell responses were found in children vaccinated with two/three doses compared with one dose following Omicron infection. A retrospective study that included partially vaccinated children reported lower vaccine effectiveness against infection, but similar effectiveness against severe COVID-19, compared with fully vaccinated children.[47] A recent retrospective cohort study in Israeli children and adolescent children found a single dose of mRNA vaccine confers substantial protection against SARS-CoV-2 Delta and Omicron BA.1/BA.2 reinfection,[22] but not against BA.4/5 reinfection. Our antibody data suggest two/three doses compared with one dose may offer better protection against Omicron subvariant infection, although the level of antibody correlates have not been identified.

Limitations of the study include the small sample size, and not being able to directly compare immune responses in unvaccinated and vaccinated children with Omicron infection due their age differences. However, we and others have previously found no difference in antibody responses between children aged < 5 years and children aged 5–12 years who recovered from mild COVID.[48,49] In addition, we did not have samples from the vaccine only group at a similar 1-month time point post-vaccination for comparison, as participants were recruited into this cohort based on detection of SARS-CoV-2 infection within households, and most vaccine-eligible children had been infected prior to recruitment. We also did not measure the measure T-cell response to non-Spike proteins such as N protein or ORF proteins, which may also be of interest in the response to infection. Lastly, our data on hybrid immunity may not be directly generalisable to other circulating or future SARS-CoV-2 variants (i.e. XBB variants, BA.2.86) as they have different mutations. We were also not able to measure neutralising antibodies and T-cell responses to JN.1 variant as these reagents were not available at the time of this study.

In conclusion, our findings indicate that there are added immune benefits of COVID-19 vaccination shown through robust CD4 and CD8 T-cell polyfunctional and activation responses in children with hybrid immunity. Our findings have important implications regarding vaccination and SARS-CoV-2 re-infections, providing evidence that could help to inform global COVID-19 vaccination strategies in children, particularly for those at highest risk of severe disease.

METHODS

Study design

In this case-ascertained study based in Victoria, Australia, suspected SARS-CoV-2 cases and household members of suspected cases were tested by RT-PCR on nasopharyngeal swabs at the Royal Children's Hospital (outpatient testing) or by nasal RAT at home during the Omicron predominant wave (January to July 2022).[24] Confirmed SARS-CoV-2 cases and household members were invited to participate and provide blood samples approximately 1 month following PCR/RAT diagnosis and over the subsequent 12 months. Written informed consent and assent were obtained from adults/parents and children, respectively, after the nature and possible consequences of the studies were explained. The study was conducted with the approval of the Royal Children's Hospital Human Research Ethics Committee (HREC): HREC/63666/RCHM-2019.

SARS-CoV-2 ELISA

A SARS-CoV-2 in-house ELISA was used to measure Spike-specific IgG antibody responses. Details of the ELISA method have been previously published.[50] The ELISA method

was previously validated against two commercial assays (Diasorin LIAISON® and Wantai) and a SARS-CoV-2 microneutralisation assay.[50] The Wuhan, Omicron BA.1 and BA.4/5 Spike proteins (S1 antigen) and JN.1 Spike protein (S1 + S2 antigen) were sourced from Sino Biological (Sino Biological Inc., China). Seropositive samples were titrated and calculated based on the World Health Organization SARS-CoV-2 pooled serum standard (NIBSC code: 20/130, National Institute of Biological Standards and Controls, UK). Spike-specific IgG data are presented as assay units per mL (AU mL^{-1}). The cut-off for seropositivity was 1.5 AU mL^{-1} based on pre-pandemic samples, whereas seronegative samples were given half of the seropositive cutoff value. Results for WA.1, BA.1 and BA.4/5 are available as binding antibody units per mL (BAU mL^{-1}) (Supplementary figure 1).

Surrogate virus neutralisation test

SARS-CoV-2 surrogate virus neutralisation test (sVNT) was used to measure the surrogate neutralising antibody responses according to the manufacturer's instructions (GenScript, New Jersey, USA). All serum samples were measured at a 1:10 dilution. For Omicron sVNT, an Omicron-specific horse-radish peroxidase (HRP)-conjugated receptor-binding domain (RBD) was used instead of the Wuhan HRP-RBD, and the Omicron-specific surrogate neutralising antibody standard was used as the positive control. The results were reported as a percentage (%) of inhibition calculated from the formula; % inhibition = [1−(optical density value of sample/optical density value of the background)] × 100%. Results < 30% inhibition are considered negative, while ≥ 30% inhibition are considered positive for surrogate neutralising antibodies.

Peripheral blood mononuclear cells (PBMCs) stimulation

Cryopreserved PBMCs were thawed at 37°C and diluted to 1×10^6 cells in RPMI1640 media supplemented with 10% fetal bovine serum (FBS), 1000 IU penicillin–streptomycin and 200 nM L-glutamine (R10). PBMCs were stimulated with 1 μg/mL of SARS-CoV-2 Wuhan- or Omicron BA.1-specific full-length Spike peptides (PepTivator®, Miltenyi Biotec, Bergisch Gladbach, Germany) for 24 h at 37°C, 5% CO_2. For intracellular cytokine analysis, PBMCs were blocked with monensin and brefeldin A (1:1000) at 2 h post stimulation, while PBMCs used for cellular activation assays were left unblocked.

Cellular activation and intracellular cytokine response

Peripheral blood mononuclear cells were stained with fluorescently labelled monoclonal antibodies for flow cytometry analysis. Following 24-h stimulation, PBMCs were washed in 1 mL of flow buffer (2% FBS in PBS). For the activation assay, PBMCs were stained with 50 μL of activation antibody cocktail (Supplementary table 2) for 20 min on ice. After incubation, these cells were washed with 1 mL of flow buffer and then resuspended in 100 μL of flow buffer for acquisition. For intracellular cytokine analysis, PBMCs were stained with 50 μL of surface antibody cocktail 1 (containing CD3, CD4, CD8, CD161, Vα7.2, γδTCR and Vδ2) for 20 min on ice (Supplementary table 3). After incubation, the cells were washed with 1 mL of flow buffer and fixed using the Cytofix/Cytoperm Fixation and Permeabilization kit (BD Biosciences, San Diego, USA) for 20 min on ice. The cells were then washed twice with 1 mL of 1× permeabilisation buffer and then stained with 50 μL antibody cocktail 2 (containing IFN-γ, TNF-α and IL-2) for 20 min on ice (Supplementary table 3). The cells were then washed once with permeabilisation buffer followed by once with flow buffer. The cells were then resuspended in 100 μL flow buffer the acquisition on the Cytek Aurora. Compensation was performed at the time of acquisition using compensation beads. Data were analysed using the Flowjo v10.7.1 software. Gating strategies to identify ICS^+ and AIM^+ cells are shown in Supplementary figure 8.

Statistical analysis

Antibody concentrations and surrogate neutralising antibodies were reported as geometric mean concentration (GMC) and percent (%) inhibition, respectively. Comparisons within groups for the Spike-specific IgG antibody concentrations (AU mL^{-1}) and surrogate neutralising antibodies (% inhibition) across variants were compared using a Friedman test. A Mann–Whitney *U*-test was used to compare antibody responses between one-dose and two-/three-dose group. All cellular data are plotted as boxplots (median, min–max whiskers and outliers shown as dots). Comparisons within groups for intracellular cytokine and activation data were performed using a Friedman test with a Benjamini–Hochberg post hoc adjustment. A *P*-value < 0.05 was considered statistically significant. All analyses were performed with GraphPad Prism version 9.0 (GraphPad Software, https://www.graphpad.com). Heatmaps were generated with the *ggplot2* package using R v4.1.2.

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AUTHOR CONTRIBUTIONS

Jeremy Anderson: Conceptualization; data curation; formal analysis; investigation; methodology; writing – original draft; writing – review and editing. **David P Burgner:** Investigation; writing – review and editing. **Nigel W Crawford:** Funding acquisition; investigation; writing – review and editing. **Nigel Curtis:** Investigation; writing – review and editing. **Lien Anh Ha Do:** Investigation; writing – review and editing. **Rachel A Higgins:** Methodology. **Sedi Jalali:** Methodology; writing – review and editing. **Paul V Licciardi:** Conceptualization; funding acquisition; investigation; methodology; supervision; writing – original draft; writing – review and editing. **Adrian Marcato:** Funding acquisition; writing – review and editing. **Nadia Mazarakis:** Conceptualization; data curation; formal analysis; investigation; methodology; writing – review and editing. **Alissa McMinn:** Project administration. **Sarah McNab:** Investigation; writing – review and editing. **Jodie McVernon:** Funding acquisition; writing – review and editing. **Kim Mulholland:** Investigation; writing – review and editing. **Melanie R Neeland:** Investigation; writing – review and editing. **Yan Yung Ng:** Methodology. **Jill Nguyen:** Project administration. **Daniel G Pellicci:** Methodology; writing – review and editing. **Leanne Quah:** Methodology. **Richard Saffery:** Investigation; writing – review and editing. **Andrew C Steer:** Funding acquisition; investigation; writing – review and editing. **Zheng Quan Toh:** Conceptualization; data curation; formal analysis; investigation; methodology; writing – original draft; writing – review and editing. **Shidan Tosif:** Funding acquisition; investigation; methodology; writing – review and editing.

CONFLICT OF INTEREST

NWC received funding from the National Institute of Health for influenza and COVID-19 research. All other authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.