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1 **Distinct T cell functional profiles in SARS-CoV-2 seropositive and seronegative children**
2 **associated with endemic human coronavirus cross-reactivity.**

3

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40 **SUMMARY** (Word count: 148/150)
41 SARS-CoV-2 infection in children typically results in asymptomatic or mild disease. There is
42 a paucity of studies on antiviral immunity in African children. We investigated SARS-CoV-
43 2-specific T cell responses in 71 unvaccinated asymptomatic South African children who
44 were seropositive or seronegative for SARS-CoV-2. SARS-CoV-2-specific CD4+ T cell
45 responses were detectable in 83% of seropositive and 60% of seronegative children.
46 Although the magnitude of the CD4+ T cell response did not differ significantly between the
47 two groups, their functional profiles were distinct, with SARS-CoV-2 seropositive children
48 exhibiting a higher proportion of polyfunctional T cells compared to their seronegative
49 counterparts. The frequency of SARS-CoV-2-specific CD4+ T cells in seronegative children
50 was associated with the endemic human coronavirus (HCoV) HKU1 IgG response. Overall,
51 the presence of SARS-CoV-2-responding T cells in seronegative children may result from
52 cross-reactivity to endemic coronaviruses and could contribute to the relative protection from
53 disease observed in SARS-CoV-2-infected children.

54

55 **Key words:** SARS-CoV-2, Children, IgG responses, T cell response, Polyfunctional profile,
56 endemic HCoV

57

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58 **INTRODUCTION:**

59 Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) infection in children
60 usually causes asymptomatic or mild illness, contrasting with the high rate of severe disease
61 reported in older adults [1,2]. As a result, global reports of coronavirus disease 2019 (COVID-
62 19) cases among children and adolescents are underreported. The United Nations
63 International Children's Emergency Fund (UNICEF) estimates that 21% of all reported
64 confirmed cases occur in individuals younger than 20 years [3].

65 In the USA, it has been documented that COVID-19-associated hospitalization rates among
66 children less than 18 years were lower compared to those in older individuals [4]. Indeed, the
67 COVID-19-Associated Hospitalization Surveillance Network (COVID-NET) reported that
68 children younger than 18 years accounted for only 4.2% of COVID-19-associated
69 hospitalization and 0.2% of COVID-19-associated in-hospital deaths [5]. In South Africa,
70 during the first 2 years of the pandemic, 12.5% of confirmed cases and 0.7% of COVID-19
71 associated in-hospital deaths were in individuals younger than 19 years [6].

72 In contrast to the low COVID-19 severity in the majority of younger individuals, it is known
73 that children are more susceptible than adults to other acute viral respiratory tract infections
74 including respiratory syncytial virus (RSV), rhinovirus (RV), influenza virus and common
75 circulating human coronaviruses (HCoV) [7]. Several age-associated factors have been
76 proposed to play a role in the reduction of severity to SARS-CoV-2 infection in children [2,8],
77 including limited comorbidities [9–11], differences in the expression of SARS-CoV-2 viral
78 entry factors [12–14], robust innate immune responses [15–18], humoral and cellular immunity [19–21]
79 and pre-existing immunity against common cold circulating endemic HCoVs [22–26]. Endemic
80 HCoVs account for 15 to 30% of respiratory infections reported annually in children [27,28].
81 These HCoVs belong to the alpha-coronavirus subfamily (HCoV-229E and HCoV-NL63)
82 and the beta-coronavirus subfamily (HCoV-OC43 and HCoV-HKU1) and have seasonal
83 infection peaks during the winter season and are responsible for high rates of infection among
84 children [29,30].

85 It is well established that protective immune responses to SARS-CoV-2 encompass both an
86 antibody as and T cell components [31]. Several studies have reported robust and durable
87 antibody and T cell responses against SARS-CoV-2, which are maintained up to 6-12 months
88 following infection [21,32–35]. However, data on T cell responses in children compared to adults
89 are conflicting, with studies reporting lower SARS-CoV-2-specific T cell responses in

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90 children [23,36,37], no differences [38] or greater T cell responses in children [22]. Many of these
91 studies make use of ELISPOT assays which preclude analysis of T cell phenotypes or
92 functional cytokine profiles.

93 In this study, we prospectively characterized specific T cell responses in SARS-CoV-2
94 seropositive and seronegative children during the COVID pandemic and determined the
95 functional profiles of SARS-CoV-2-specific T cells, in the context of their pre-existing
96 immunity against endemic beta-HCoVs. This study provides novel insights into cross-
97 reactive immunity to SARS-CoV-2 in children.

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98 **RESULTS:**

99 **Study cohort**

100 To investigate SARS-CoV-2-specific immune responses, we measured immunoglobulin (Ig)
101 G and T cell responses in 71 children recruited in Cape Town, Western Cape, South Africa.
102 The participants are described in **Figure 1A**. The median age of the children was 7 years
103 (interquartile range (IQR) 2.8-9 years) and 34% (24/71) were female. The children included
104 in this study had not received any SARS-CoV-2 vaccine prior to recruitment and no PCR-
105 confirmed infection data were available, although 58% showed SARS-CoV-2 seropositivity
106 (defined as being positive for either anti-spike or anti-nucleocapsid IgG). Samples were
107 collected between 1 February 2021 – 20 May 2021, after two infection waves in South
108 Africa, that were dominated by the ancestral D614G strain followed by the Beta variant of
109 concern [39].

110 **SARS-CoV-2-specific antibody responses in children**

111 To characterize the children serologically, we measured IgG responses against SARS-CoV-2
112 spike (S) and nucleocapsid (N) proteins using an indirect Enzyme-linked immunosorbent
113 assay (ELISA) (**Figure 1B**). A large proportion (58%, 41/71) of unvaccinated children had
114 detectable SARS-CoV-2-specific IgG against spike and/or nucleocapsid proteins and were
115 classified as seropositive. Of the seropositive children, 30% (21/71) had spike- and
116 nucleocapsid-specific IgG, 23% (16/71) had only spike-specific IgG and 5% (4/71) had only
117 nucleocapsid-specific IgG only (**Figure 1B**).-The remaining 42% (30/71) had undetectable
118 SARS-CoV-2-spike and nucleocapsid-specific IgG and were classified as seronegative
119 (**Figure 1B**). IgG responses to spike and nucleocapsid are shown for the 41 seropositive
120 children in **Figure 1C**.

121 **SARS-CoV-2-specific T cell responses in children**

122 The magnitude of SARS-CoV-2-specific T cell responses in children was quantified using a
123 whole blood assay and intracellular cytokine staining followed by flow cytometry. SARS-
124 CoV-2-specific CD4+ and CD8+ T cell responses were measured as total cytokine
125 production of interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α) or interleukin-2 (IL-2) in
126 response to a combined peptide pool covering SARS-CoV-2 spike (S), nucleocapsid (N) and
127 membrane (M) proteins (**Figure 2A**).

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128 We assessed the proportion of responders and magnitude of SARS-CoV-2-specific T cell
129 responses according to the serostatus of the children (**Figure 2B and C**). As expected, 83%
130 (34/41) of seropositive children had detectable SARS-CoV-2-specific CD4+ T cells.
131 Interestingly, 60% (18/30) of seronegative children also had detectable SARS-CoV-2-specific
132 CD4+ T cells, although this was a significantly lower proportion than observed in the
133 seropositive group (p=0.0311). The magnitude of the SARS-CoV-2-specific CD4+ T cell
134 response in seronegative responders was comparable to that observed in seropositive children
135 (median of responders: 0.04% and 0.057% respectively; p=0.437, **Figure 2B**). Conversely,
136 we did not observe significant differences in the proportion of SARS-CoV-2 CD8+ T cell
137 responders (~50%) or the frequency of SARS-CoV-2-specific CD8+ T cells between
138 seropositive and seronegative children (median of responders: 0.019% and 0.025%
139 respectively; p=0.214, **Figure 2C**).

140 Next, we evaluated the polyfunctional profile of SARS-CoV-2-responding CD4+ and CD8+
141 T cells based on the co-expression of IFN-γ, IL-2 or TNF-α (**Figure 2D, E**). The overall
142 functional profile of SARS-CoV-2-specific CD4+ T cells in seropositive children was distinct
143 from that of seronegative children (p=0.007, **Figure 2D**). SARS-CoV-2-specific CD4+ T
144 cells in seropositive children were more polyfunctional, exhibiting a higher proportion of
145 triple functional IFN-γ+IL-2+TNF-α+-producing CD4+ T cells (p=0.0044), and dual
146 expressing cells (IL-2+TNF-α+, p=0.0048; IFN-γ+IL-2+, p≤0.0001) compared to the
147 seronegative children. In contrast, seronegative children were characterized by an increased
148 proportion of IFN-γ monofunctional CD4+ T cells (p=0.0048). Considering the CD8
149 compartment, we detected no significant differences in the overall functional profile of
150 SARS-CoV-2-responding CD8+ T cells between the two groups (p=0.098), although
151 seropositive children did have a larger proportion of CD8+ T cells producing both IFN-γ and
152 IL-2 (p=0.027) and single IFN-γ (p=0.011) (**Figure 2E**).

153 Taken together, these data show that a significant proportion of seronegative children have
154 detectable SARS-CoV-2-specific CD4+ and CD8+ T cells. Notably, these CD4+ T cells
155 exhibit a distinct polyfunctional profile compared to those found in SARS-CoV-2-exposed
156 children.

157 **Pre-existing immunity to endemic common circulating human coronaviruses**

158 The presence of SARS-CoV-2-responding CD4+ T cells primarily exhibiting a
159 monofunctional profile observed in seronegative children led us to hypothesize that the T cell

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160 response could be due to cross-reactivity resulting from prior infection with common
161 circulating endemic HCoVs. Therefore, we measured endemic betacoronavirus HCoV-HKU1
162 and HCoV-OC43 spike IgG in SARS-CoV-2 seropositive and seronegative children**.** The
163 magnitude of both HCoV-HKU1 (**Figure 3A**) and HCoV-OC43 (**Supplemental Figure 1A**)
164 spike IgG was comparable in the seropositive and seronegative groups (median OD: 0.746 vs
165 0.420; p=0.153 for HCoV-HKU1 and 1.769 vs 1.281; p=0.07 for HCoV-OC43, respectively).

166 We found a significant correlation between the frequency of SARS-CoV-2-specific CD4+ T
167 cells and HCoV-HKU1-spike IgG in seronegative children (p=0.036, r=0.392, **Figure 3B**),
168 while no correlation was observed in seropositive children (p=0.936, r=-0.013, **Figure 3C**).
169 For HCoV-OC43, no correlation was detected in either the seronegative (p=0.324, r=-0.187,
170 **Supplemental Figure 1B**) or the seropositive group (p=0.171, r=-0.218, **Supplemental**
171 **Figure 1C**). Overall, these results indicate SARS-CoV-2-reactive CD4+ T cell responses in
172 seronegative children could be due in part to cross-reactive T cell immunity resulting from
173 prior infection with common cold HCoV-HKU1.

174

175 SARS-CoV-2-specific T cell responses in SARS-CoV-2 seropositive children compared 176 to COVID-19 convalescent adults

177 Blood and plasma samples were collected from 30 COVID-19 convalescent healthcare
178 workers (HCWs) participating in a longitudinal study at Groote Schuur Hospital (Cape Town,
179 Western Cape, South Africa) (**Figure 4A**). All convalescent adults had a prior PCR-
180 confirmed SARS-CoV-2 infection (median 224 days prior to sampling) and had not received
181 a COVID-19 vaccine at the time of sampling. Samples were collected between 22 January
182 2021 – 23 February 2021.

183 A trend towards a higher proportion of SARS-CoV-2 CD4 responders was observed in
184 convalescent adults (97%) compared to seropositive children (83%) (p=0.07, **Figure 4B**).
185 Moreover, in SARS-CoV-2 responders, convalescent adults did have a significantly higher
186 frequency of SARS-CoV-2-specific CD4+ T cells compared to seropositive children (median
187 of responders: 0.092% vs 0.057%; p=0.0004, respectively). Unlike CD4+ T cell responses,
188 there were no significant differences in either the proportion of CD8 responders (53% vs
189 49%, p=0.705) or the frequency of SARS-CoV-2-specific CD8+ T cells (median of
190 responders: 0.038% vs 0.019%; p=0.096) between convalescent HCWs and seropositive
191 children (**Figure 4C**). Furthermore, we observed a positive correlation between age and the

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192 frequency of SARS-CoV-2-specific CD4+ T cells (p=0.0006, r=0.396, **Figure 4D**). No
193 correlation was observed with SARS-CoV-2-specific CD8+ T cells and age (p=0.116,
194 r=0.188, **Figure 4E**). Overall, these results show that convalescent adults mount higher
195 SARS-CoV-2-specific CD4 T cell responses compared to children, which could be attributed
196 to age-related T cell development.

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197 **DISCUSSION**

198 T cells have been associated with protection from severe COVID-19 [40]. It is therefore
199 important to understand the nature of T cell responses targeting SARS-CoV-2 in children,
200 who have been largely spared from severe COVID-19. In this study, comparing SARS-CoV-
201 2-specific T cell responses in SARS-CoV-2 seropositive and seronegative children, a sizable
202 proportion of seronegative children had SARS-CoV-2-reactive CD4+ and CD8+ T cells, but
203 their CD4+ T cells exhibited a distinct functional profile compared to seropositive children.
204 Importantly, in seronegative children, the frequency of SARS-CoV-2-reactive CD4+ T cells
205 positively associated with HCoV-HKU1 spike-specific IgG antibodies. Additionally, we
206 showed that convalescent adults had a higher magnitude of CD4+ T cell responses against
207 SARS-CoV-2 compared to seropositive children, which associated positively with age.

208 Our data are in accordance with several studies showing that children develop robust humoral
209 and cellular immunity to SARS-CoV-2 [20,22,38,41,42]. In addition, SARS-CoV-2-specific T cell
210 responses have been detected in 40-60% of SARS-CoV-2 unexposed individuals including
211 children and adults [22,23,43–48] suggesting possible cross reactivity to HCoVs. One study
212 demonstrated that the proportion of T cell responders was higher in children than adults [22]. In
213 contrast, a study by Tsang et al. found that SARS-CoV-2 uninfected children failed to mount
214 detectable T cell responses [41]. A further study showed that only a small proportion of
215 seronegative children mounted a SARS-CoV-2-specific CD4+ T cell response (13%)
216 compared to 60% in seropositive siblings, despite similar exposure in shared households [42].
217 These two studies differ from our findings, where we showed that 60% of seronegative
218 children had detectable SARS-CoV-2-specific T cell responses, consistent with Dowell et al
219 [22]. The discrepancies between the studies could be due to a difference in the seasonal
220 prevalence of circulating HCoVs in each study setting [29,30,49], different T cell assays used to
221 analyze T cell responses, and/or the cohort demographics.

222 Although the source of SARS-CoV-2 reactive T cells in seronegative children remains
223 unclear, mounting evidence argues for cross-reactivity to endemic HCoVs. HCoVs (including
224 beta HCoVs-HKU1 and OC43, and alpha HCoVs-NL63 and 229E) have partial sequence
225 homology with SARS-CoV-2 [44,50]. While cross reactive humoral responses have been shown
226 not to underly this protection [51], studies have reported pre-existing T cell responses to
227 endemic HCoVs with cross-reactivity to SARS-CoV-2 in unexposed adults [47,52–57] and
228 children [22,24,58,59]. These cellular responses were targeted mainly towards the S2 subunit

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229 which is highly conserved among coronaviruses [22,25]. In our study, we showed a positive
230 correlation between the frequency of SARS-CoV-2 CD4+ T cells and the magnitude of
231 HKU1 spike-specific IgG in SARS-CoV-2 seronegative children. Our findings are supported
232 by similar findings showing that 58% (7/12) seronegative children had binding antibodies
233 against alpha and/or beta HCoV spike proteins, which they concluded were likely due to
234 recent HCoV infection [22].

235 Pre-existing immunity from endemic HCoVs infection or exposure has been shown to be
236 associated with a protective effect against COVID-19 disease in adults [53,56,57,60,61]. Sagar et al.
237 showed that individuals with a documented recent or ongoing HCoV infection and SARS-
238 CoV-2 infection had fewer COVID-19-associated complications when hospitalized,
239 compared to individuals without HCoV infection [60]. Previously, the clinical relevance of pre-
240 existing immunity and cross-reactive cellular responses in providing protection from
241 infection in children was unclear. Recently however, Dowell et al. demonstrated that as for
242 SARS-CoV-2 seropositive children, SARS-CoV-2-reactive T cell responses in SARS-CoV-2
243 seronegative children were protective against Omicron infection, suggesting a protective role
244 in SARS-CoV-2 unexposed individuals [62].

245 Polyfunctional T cells have been reported to be associated with protection against a number
246 of viral diseases [63–66]. These include protection against cytomegalovirus (CMV) infection
247 after lung transplantation [64], improved viral control of hepatitis C virus (HCV) [65] and
248 detection during human immunodeficiency virus (HIV) infection in long term non-
249 progressors [66]. We found that seropositive children had a more polyfunctional profile of
250 SARS-CoV-2-specific CD4+ T cells while CD4 responses in seronegative children exhibited
251 a predominantly monofunctional profile. The SARS-CoV-2-specific polyfunctional Th1 CD4
252 response (characterized by co-expression of IFN-γ, TNF-α and/or IL-2), as seen in
253 seropositive children, may be necessary for effective viral control and has been documented
254 in COVID-19 convalescent adults [67,68].

255 The distinct functional profiles reported in our findings could be a result of differing degrees
256 memory cell T cell differentiation or low SARS-CoV-2 peptide binding affinity with HCoV-
257 specific T cells resulting in low T cell activation. The affinity between MHC molecules
258 presenting peptide and the T cell receptor (TCR) plays a role in antigen recognition. It has
259 been proposed that T cells with high affinity TCRs have greater effector functions and
260 therefore an increased polyfunctional profile compared to low affinity TCRs [69,70]. TCR cross-

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261 recognition of peptides on MHCs that are not structurally identical results in lower binding
262 affinity which decreases T cell polyfunctionality [70,71]. It is therefore plausible that the
263 monofunctional profile of SARS-CoV-2 CD4+ T cell responses in seronegative children
264 observed in our study may be mediated by cross-recognition of pre-existing T cell immunity
265 to HCoV-HKU1, where partial sequence homology between HCoV-HKU1 and SARS-CoV-2
266 results in low peptide binding affinity and a different functional T cell profile [44,55,69,71,72]. It is
267 noteworthy to report that we did not find any association with the other beta HCoV-OC43
268 spike-specific IgG and SARS-CoV-2-specific T cells in seronegative children, which could
269 be related to differences in seasonal prevalence of types of HCoV infections.

270 Numerous studies have now compared the magnitude of the SARS-CoV-2 specific T cell
271 response in children to those detectable in adults [22,23,36–38,41]. Initially it was thought that
272 children may have a higher magnitude of T cell responses given their relative resistance to
273 severe disease and the link between T cell responses and protection from severe disease [8,73].
274 However, children were shown to have lower T cell responses than adults in most studies,
275 including the current study [23,36,37]. A plausible explanation is that adults have a mature
276 immune system with more differentiated memory T cell subsets endowed with increased
277 cytokine capacity, whereas children have an immature immune system, with many more
278 naïve T cells which have reduced cytokine producing capacity, are enriched for
279 monofunctional responses and have increased antigen dependence [74]. Despite lower
280 circulating SARS-CoV-2-specific T cells, children and adults may also have different
281 responses in the respiratory tract. There is evidence in adults demonstrating the presence of
282 SARS-CoV-2-specific T cell responses in the nasal mucosa after infection and vaccination
283 [75,76]. However comparative studies have yet to be performed in children. Additionally, several
284 studies have proposed that one potential contributing factor to age-related COVID-19 clinical
285 outcome are the robust innate immune responses observed in children, which may contribute
286 to early control of viral replication [15–17,77]. Recent studies have shown that, compared to
287 adults, SARS-CoV-2 seronegative children exhibited an increased number of innate immune
288 cells with pre-activated signatures leading to early production of interferon-mediated antiviral
289 effects in the upper respiratory tract [18,19].

290 In conclusion, our study shows that a robust SARS-CoV-2-specific T cell response is
291 observed in children, including in those with no evidence of prior SARS-CoV-2 infection.
292 We demonstrate that the magnitude of SARS-CoV-2-reactive CD4+ T cells in seronegative
293 children correlates with HCoV-HKU1 exposure. This, together with a distinct functional

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294 profile of SARS-CoV-2-specific responding CD4+ T cells observed between seropositive and
295 seronegative children provides further evidence for pre-existing T cell responses cross-
296 reactive to SARS-CoV-2.

297 **Limitations of the study**

298 This study relied on serology to determine SARS-CoV-2 exposure. With no PCR
299 confirmation of SARS-CoV-2 infection, the exact time of infection of the children could not
300 be determined. Additionally, our study investigated T cell responses against SARS-CoV-2
301 spike, nucleocapsid and membrane proteins, and not the non-structural viral proteins that can
302 also serve as targets for the T cell response, thus not capturing the full extent of T cell
303 reactivity in infected participants. Furthermore, we did not address durability of the T cell
304 responses in children due to the cross-sectional design of the study.

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318 ## METHODS:

319 **Lead contact**

320 Further information and requests for resources and reagents should be directed to and will be
321 fulfilled by the lead contact: Roanne S. Keeton (roanne.keeton@uct.ac.za).

322

323 **Materials availability**

324 Materials will be made available by request to Roanne S. Keeton (roanne.keeton@uct.ac.za).

325

326 **Data and code availability**

327 The published article includes all data generated or analyzed during this study, and
328 summarized in the accompanying tables, figures, and supplemental materials.

329

330 ## EXPERIMENTAL MODEL AND SUBJECT DETAILS

331 **Study participants**

332 Paediatric participants (n=71) were recruited from two cohorts in the Western Cape, South
333 Africa. The first cohort enrolled 50 children from the Red Cross War Memorial Children's
334 Hospital (Cape Town, Western Cape, South Africa). In this cohort 50/71 children were
335 hospitalized for non-COVID-19-related elective procedures. A further 21 children were
336 enrolled from the Drakenstein Child Health Study (DCHS) (Cape Winelands Western Cape,
337 South Africa), a birth cohort study [78]. The participants from this study were recruited between
338 1 February 2021 and 20 May 2021, after the first and the second infection waves with
339 Ancestral strain and Beta variant in South Africa [79]. Parents or legal guardians provided
340 written informed consent for all paediatric participants. For the DCHS longitudinal cohort,
341 this was renewed annually. The study was approved by the University of Cape Town Human
342 Research Ethics Committee (HREC 599/2020 for the RC cohort and HREC 401/2009 for the
343 DCHS cohort).

344 COVID-19 convalescent unvaccinated adults from a longitudinal study of healthcare workers
345 (HCW) enrolled from Groote Schuur Hospital (Cape Town, Western Cape, South Africa)

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346 were included in the study (n=30). HCW in this cohort were recruited between July 2020 and
347 January 2021 and were selected for inclusion based on a prior PCR-confirmed SARS-CoV-2
348 infection at least 3 months earlier. All participants were asymptomatic or had mild symptoms
349 and did not require hospitalization for COVID-19 and were symptom-free at the time of
350 sampling. Written informed consent was obtained from all participants and the study was
351 approved by the University of Cape Town Human Research Ethics Committee (HREC
352 190/2020 and 209/2020).

353

354 **METHODS DETAILS**

355 **SARS-CoV-2 and HCoV antigens**

356 For serology assays, recombinant SARS-CoV-2 spike S1 (Cape Bio Pharms), and SARS-
357 CoV-2 nucleocapsid (BioTech Africa) proteins were used for this study.

358 HKU1 and OC43 spike proteins were expressed in Human Embryonic Kidney (HEK) 293F
359 suspension cells by transfecting the cells with the spike plasmid. After 6 days, proteins were
360 purified using a nickel resin followed by size-exclusion chromatography. Relevant fractions
361 were collected and frozen at -80°C until use.

362 SARS-CoV-2 peptides used for T cell assays included a commercially available peptide pool
363 (15mers peptides with 11 amino acid overlap) covering the immunodominant regions of
364 SARS-CoV-2 spike protein (PepTivator SARS-CoV-2 Prot_S, Miltenyi Biotech) based on
365 the Wuhan-1 strain. The Spike peptide pool was prepared by resuspending in distilled water
366 and used at a final concentration of 1 µg/ml. SARS-CoV-2 (Wuhan-1) nucleocapsid and
367 membrane peptides (17mers with 10 amino acid overlap spanning the full proteins) were
368 obtained from BEI Resources and were prepared by resuspending in dimethyl sulfoxide
369 (DMSO, Sigma) and used at a concentration of 1 µg/ml.

370 **Enzyme-linked immunosorbent assay (ELISA)**

371 SARS-CoV-2-specific enzyme-linked immunosorbent assay (ELISA) was performed to
372 characterize the serostatus of participants, as previously described [80]. Two ug/ml of spike
373 protein was used to coat 96-well high-binding plates and incubated overnight at 4 °C. The
374 plates were incubated in a blocking buffer consisting of 1% casein, 0.05% Tween 20, 1x
375 Phosphate-Buffered Saline (PBS) for SARS-CoV-2 or 1x PBS, 5% skimmed milk powder,

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376 0.05% Tween 20 for HCoVs. Plasma samples were diluted to 1:50 for SARS-CoV-2 or 1:100
377 for HCoVs in the respective blocking buffer. For the SARS-CoV-2 ELISA, secondary
378 antibody was diluted to 1:5000 in dilution buffer and added to the plates followed by
379 SigmaFast O-phenylenediamine dihydrochloride (OPD) substrate. For the HCoV ELISAs,
380 secondary antibody was diluted to 1:3000 in blocking buffer and added to the plates followed
381 by TMB substrate (Thermofisher Scientific). Upon stopping the reaction with 1-3 M sulfuric
382 acid, absorbance was measured at a wavelength of 490 nm for SARS-CoV-2 or 450 nm for
383 HCoVs. A cut-off for positivity was set at two standard deviations (SD) above the mean
384 optical density (OD) of prepandemic samples for SARS-CoV-2 ELISAs

385 **Whole blood-based T cell assay**

386 Blood was collected in sodium heparin tubes and processed within 4-6 hours of collection.
387 The whole blood assay sample processing used for this study was adapted from a whole
388 blood intracellular cytokine detection assay designed to detect SARS-CoV-2 specific T cells
389 in adults [81,82]. Briefly, 500 µl of blood was stimulated for 24 hours at 37°C with a combined
390 pool of SARS-CoV-2 peptides including S, N and M, all at 1 µg/mL in the presence of
391 costimulatory antibodies against CD28 (clone 28.2) and CD49d (clone L25) (1 µg/mL each;
392 BD Biosciences) and Brefeldin A (10 µg/mL, Sigma-Aldrich). Unstimulated blood was
393 incubated with costimulatory antibodies, Brefeldin A and an equimolar amount of DMSO as
394 a background control. After 24 hours, blood was treated with EDTA (2 mM) for 15 minutes
395 followed by red blood cell lysis and white cell fixation using FACS lysing solution (BD
396 Biosciences) for 10 minutes. Cells were then cryopreserved in freezing media (90% fetal
397 bovine serum (FBS) and 10% DMSO) and stored at -80°C until batched analysis.

398 **Cell staining and flow cytometry**

399 Cell staining was performed on cryopreserved fixed cells that were thawed and washed with
400 1% FACS washing buffer (1% FBS in PBS). Cells were stained with the following surface
401 antibody markers: CD4 ECD (SFCI12T4D11, Beckman Coulter), CD8 BV510 (RPA-8,
402 Biolegend) and incubated at room temperature for 20 minutes. Cells were permeabilized and
403 stained with intracellular antibody markers CD3 BV650 (OKT3), IFN-γ AlexaFluor® 700
404 (B27), TNF-α BV786 (Mab11) and IL-2 APC (MQ1-17H12) (all from Biolegend). Finally,
405 cells were washed and fixed with Cellfix (BD Biosciences). Samples were acquired on a
406 multiparameter BD Fortessa flow cytometer using Diva software version 8 and analyzed
407 using FlowJo v10. Results are expressed as the frequency of CD4+ or CD8+ T cells

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408 expressing IFN-γ, TNF-α or IL-2. Cytokine responses presented are background subtracted
409 values (from the frequency of cytokine produced by unstimulated cells).

410

411 QUANTIFICATION AND STATISTICAL ANALYSIS

412 Statistical analyses were performed in Prism (v9.4.1; GraphPad Software Inc, San Diego,
413 CA, USA). Non-parametric tests were used for all comparisons. The Mann-Whitney and
414 Wilcoxon signed rank tests were used for unmatched and paired samples, respectively. Chi-
415 square tests were used for comparisons between the proportion of responders represented as
416 pie charts. All correlations reported are non-parametric Spearman's correlations. Analysis of
417 cytokine co-expressing populations was performed using SPICE version 5.1. *P* values less
418 than 0.05 were considered statistically significant. Details of analyses performed for each
419 experiment are described in the figure legends.

420

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454

455 **AUTHOR CONTRIBUTIONS**

456 R.S.K., K.W. and W.A.B. conceived the study and designed the experiments. N.S.B.B., A.N.
457 and R.B. performed T cell assays. M.B.T., V.S. and A.W. performed SARS-CoV-2 ELISAs.
458 S.V.G. and S.R.B. performed HCoV ELISAs. N.S.B.B., T.M.G., P.L.M., C.R., W.A.B. and
459 R.S.K. analyzed the data. K.W., L.Z., and C.B. established and led the Red Cross childrens
460 cohort. H.J.Z. established and led the Drakenstein Child Health cohort including a COVID
461 sub-study. M.B and L.W. managed the Drakenstein Child Health cohort and repository.
462 N.A.B.N. established and led the adult HCW cohort and M.M., S.S. and M.A. recruited
463 participants and managed the cohort. N.S.B.B., C.R., W.A.B. and R.S.K. wrote the paper. All
464 authors reviewed and edited the manuscript.

465

466 **DECLARATION OF INTERESTS**

467 The authors have no competing interests.

468

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469 **FIGURE LEGENDS**

470 **Figure 1: SARS-CoV-2-specific antibody responses in children**

471 (A) The demographic characteristics of 71 unvaccinated children included in this study. Age,
472 gender, SARS-CoV-2 vaccination status, SARS-CoV-2 serology and collection date are
473 shown.

474 (B) Proportion of children exhibiting antibody responses to SARS-CoV-2 spike (S) and
475 nucleocapsid (N) proteins.

476 (C) The magnitude of SARS-CoV-2 S and N IgG antibodies (OD_{490nm}) measured by ELISA
477 in seropositive children (n=41). The dotted lines indicate the cut-off for positivity which was
478 calculated as the mean optical density of COVID-19 prepandemic control samples. Statistical
479 analysis was performed using the Wilcoxon signed rank test; p values <0.05 were considered
480 statistically significant and are bolded.

481

482 **Figure 2: SARS-CoV-2-specific T cell responses in children**

483 (A) Representative flow cytometry plots of SARS-CoV-2-specific interferon-γ (IFN-γ), tumor
484 necrosis factor-α (TNF-α) and interleukin-2 (IL-2) cytokine production from CD4+ (left) and
485 CD8+ (right) T cells in response to SARS-CoV-2 peptide stimulation. NS: no stimulation,
486 SNM: combined peptide pool of SARS-CoV-2-spike, nucleocapsid and membrane proteins.

487 (B) Frequency of SARS-CoV-2-specific CD4+ T cells producing any of the measured
488 cytokines (IFN-γ, IL-2 or TNF-α). Children were grouped according to SARS-CoV-2
489 serostatus (light blue: n=41 SARS-CoV-2 seropositive; dark blue: n=30 SARS-CoV-2
490 seronegative). Bars represent the median of the responders, and median values are indicated.
491 The pie charts represent the proportion of responders with detectable T cell response to
492 SARS-CoV-2 SNM peptides.

493 (C) Frequency of SARS-CoV-2-specific CD8+ T cells producing any of the measured
494 cytokines (IFN-γ, IL-2 or TNF-α). Children were grouped according to SARS-CoV-2
495 serostatus (light red: n=41 SARS-CoV-2 seropositive children; dark red: n=30 SARS-CoV-2
496 seronegative children). Statistical comparisons in (B) and (C) were performed using the
497 Mann-Whitney test between seropositive and seronegative children and the Chi-square test to

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498 compare the percentage of responders; p values <0.05 were considered statistically significant
499 and are bolded.

500 (D) Polyfunctional profile of SARS-CoV-2-specific CD4+ and (E) CD8+ T cells in
501 seropositive and seronegative unvaccinated children. The x-axis illustrates each combination
502 which is indicated with a black circle for the presence of IFN-γ, IL-2 and TNF-α. The
503 medians and interquartile range are shown. Each response pattern (any possible combination
504 of IFN-γ, IL-2 and TNF-α production) is color coded and summarized in the pie charts, with
505 each pie slice representing the median contribution of each combination to the total SARS-
506 CoV-2 responses. The permutation test was used to compare the statistical differences
507 between the pie charts and the Mann Whitney Sum Test to compare response patterns
508 between seropositive and seronegative children; p values <0.05 were considered statistically
509 significant and are bolded.

510 **Figure 3: SARS-CoV-2 cross-reactivity to endemic beta-HCoV in children**

511 (A) The magnitude of HCoV-HKU-1 spike IgG levels were measured by ELISA in SARS-
512 CoV-2 seropositive (light blue; n=41) and seronegative (dark blue; n=29) children. Plasma
513 sample was insufficient for one seronegative child therefore OD for HCoV-HKU1 was not
514 measured for this participant. The bars represent the median values. A statistical comparison
515 was performed using the Mann-Whitney test between seropositive and seronegative children;
516 a p value <0.05 was considered statistically significant.

517 (B) Correlation between the frequency of SARS-CoV-2-specific CD4+ T cells and HCoV-
518 HKU-1-spike IgG levels in SARS-CoV-2 seronegative children (n=29). One participant had
519 insufficient sample available to be included in this assay.

520 (C) Correlation between the frequency of SARS-CoV-2-specific CD4+ T cells and HCoV-
521 HKU-1-spike IgG levels in SARS-CoV-2 seropositive children (n=41). Statistical
522 comparisons for (B) and (C) were performed using a two-tailed non-parametric Spearman
523 rank tests; p values <0.05 were considered statistically significant and are bolded and
524 correlation coefficients values are shown.

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527 **Figure 4: SARS-CoV-2-specific T cell responses in children compared to convalescent**
528 **adults**

529 (A) The demographic characteristics of 30 unvaccinated convalescent adults included in this
530 study. Age, sex, SARS-CoV-2 PCR-positivity, days since PCR test and collection date are
531 shown.

532 (B) Frequency of SARS-CoV-2-specific CD4+ T cells producing any of the measured
533 cytokines (IFN-γ, TNF-α, or IL-2) in SARS-CoV-2 convalescent HCW (purple; n=30) and
534 seropositive children (light blue; n=41). The pie charts represent the proportion of responders
535 with a detectable T cell response to SARS-CoV-2 SNM combined peptide pools. Bars
536 represent median of the responders.

537 (C) Frequency of SARS-CoV-2-specific CD8+ T cells producing any of the measured
538 cytokines (IFN-γ, TNF-α, or IL-2) in SARS-CoV-2 convalescent HCW (dark red; n=30) and
539 seropositive children (light red; n=41). The bars represent the median of the responders and
540 median values are indicated. Statistical comparisons were performed using the Mann-
541 Whitney test between seropositive children and adults and the Chi-square test was used to
542 compare the percentage of responders; p values <0.05 were considered statistically significant
543 and are bolded.

544 (D) Correlations between SARS-CoV-2-specific CD4+ or CD8+ T cells and age in
545 convalescent HCW (purple and dark red; n=30) and seropositive (light blue and light red;
546 n=41) children. Statistical comparisons were performed using a two-tailed non-parametric
547 Spearman rank tests; p values <0.05 were considered statistically significant and are bolded
548 and correlation coefficients are shown.

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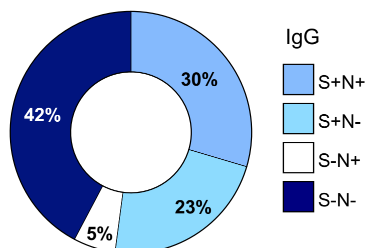
Figure 1

A

	Children
N	71
Age (median, IQR)	7 (2.8 - 9)
Gender (n, % female)	24 (34%)
SARS-CoV-2 vaccination (n, %)	0 (0%)
SARS-CoV-2 seropositive (n, %)	41 (58%)
Sample collection date	1 February 2021 – 20 May 2021

B All children (n=71)



C SARS-CoV-2 seropositive children (n=41)

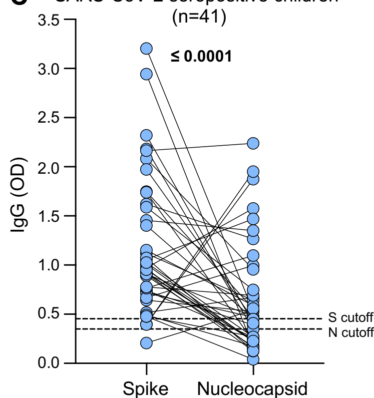


Figure 2

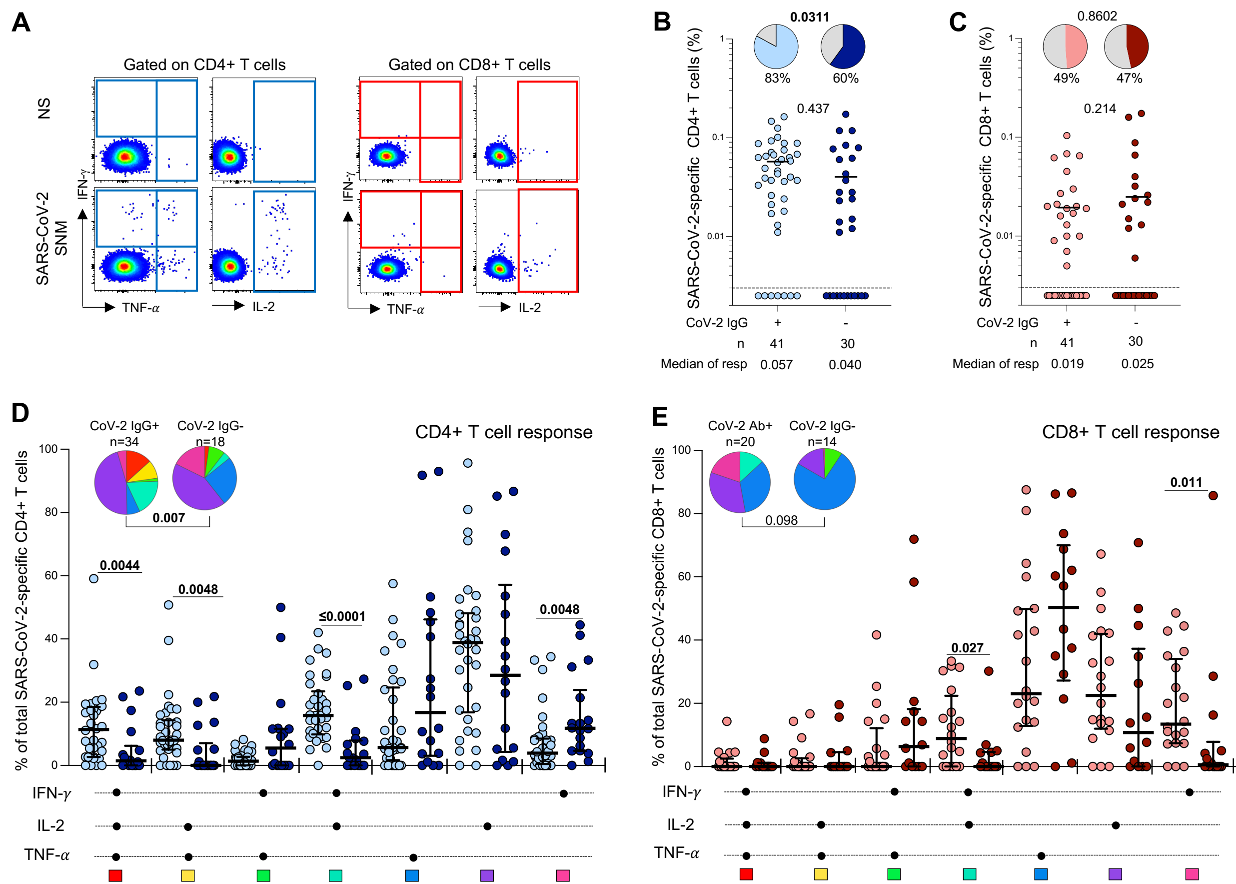


Figure 3

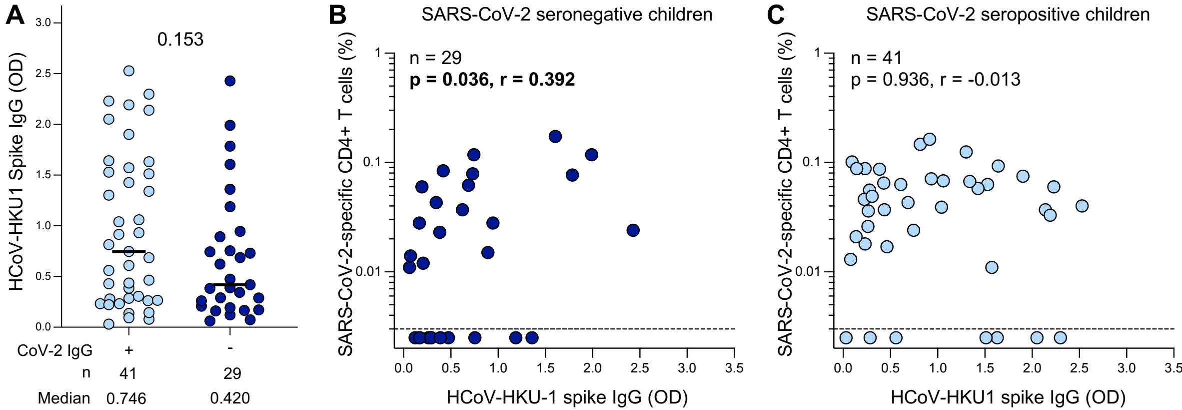


Figure 4

A

	Convalescent adults
N	30
Age (median, IQR)	37.5 (32.3 - 45.3)
Gender (n, % female)	22 (73%)
SARS-CoV-2 PCR positivity (n, %)	30 (100%)
Days since PCR+ (median, IQR)	224 (189 - 239)
Sample collection date	22 January 2021 - 23 February 2021