1   Persistent SARS-CoV-2 infection and increasing viral variants in children and young

2   adults with impaired humoral immunity

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31

32 **Summary**

33 *Background*

34 There is increasing concern that persistent infection of SARS-CoV-2 within

35 immunocompromised hosts could serve as a reservoir for mutation accumulation and

36 subsequent emergence of novel strains with the potential to evade immune responses.

37 *Methods*

38 We describe three patients with acute lymphoblastic leukemia who were persistently

39 positive for SARS-CoV-2 by real-time polymerase chain reaction. Viral viability from

40 longitudinally-collected specimens was assessed. Whole-genome sequencing and

41 serological studies were performed to measure viral evolution and evidence of immune

42 escape.

43 *Findings*

44 We found compelling evidence of ongoing replication and infectivity for up to 162 days

45 from initial positive by subgenomic RNA, single-stranded RNA, and viral culture

46   analysis. Our results reveal a broad spectrum of infectivity, host immune responses,

47   and accumulation of mutations, some with the potential for immune escape.

48   *Interpretation*

49   Our results highlight the need to reassess infection control precautions in the

50   management and care of immunocompromised patients. Routine surveillance of

51   mutations and evaluation of their potential impact on viral transmission and immune

52   escape should be considered.

53   *Funding*

63

64   **Introduction**

65   Infection with severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), the

66   cause of coronavirus disease 2019 (COVID-19) is most commonly detected using real-

67   time polymerase chain reaction (RT-PCR). Viral load typically peaks with onset of

68   symptoms and wanes to undetectable levels by week three, when patients generally

69   begin to develop antibodies.[1] While the Centers for Disease Control and Prevention

70   (CDC) recommends quarantining for 14 days following exposure to COVID-19, the time

71   course of infection may vary due to factors including age, immune status, and disease

72   severity.[2] In most patients, culture, contact tracing, and subgenomic RNA detection

73   studies have not demonstrated infectivity beyond 10 days of symptom onset.[3,4] In rare

74   cases prolonged shedding of SARS-CoV-2 has been observed in immunocompromised

75   adults, making study of this population critically important.[5–8] However, the temporal

76   dynamics of SARS-CoV-2 infectivity and the evolution of SARS-CoV-2 mutational

77   profiles over prolonged periods of infection in immunocompromised patients, particularly

78   in children, have not been described. Beyond the implications for individual patients, the

79   emergence of B.1.1.7 SARS-CoV-2 and other lineages with potential for immune

80   evasion has led to greater focus on the importance of genomic surveillance.[9,10] Here,

81   we describe prolonged SARS-CoV-2 RT-PCR positivity in two children and one young

82   adult undergoing therapy for B-cell acute lymphoblastic leukemia (ALL). In addition to

83   evidence of ongoing replication, two of these cases demonstrated significant intra-host

84   SARS-CoV-2 mutational accumulation and host immune responses that may have

85   contributed to their disease course.

86

87   **Methods**

88   **Case histories**

89   Patient 1 is a previously healthy female under 5 years of age with no significant past

90   medical history prior to several admissions to the emergency department (ED) for

91   worsening pancytopenia, decreased appetite, and abdominal pain. Her bone marrow

92   biopsy revealed 58% blasts consistent with B-cell ALL and a chemotherapy regimen

93   was initiated (Figure 1A, Table 1). Asymptomatic screening for SARS-CoV-2 by RT-

94   PCR at time of discharge revealed a positive result (day 0). She was re-admitted to the

95   hospital on day 3 for fatigue and vomiting as well as cough, malaise, and

96   gastrointestinal symptoms and discharged on day 6. She consistently tested positive for

97   SARS-CoV-2 during follow-up screens for her chemotherapy until she finally tested

98   negative on day 91 without any notable respiratory symptoms (Figure 1A, Figure 2A).

99

100   Patient 2 is a male in the 20-25 year age range who was previously diagnosed with B-

101   cell ALL six months prior to his first SARS-CoV-2 positive RT-PCR. He received

102   combination chemotherapy, however continued to have detectable residual disease at

103   the end of the consolidation phase, prompting initation of CD19-directed chimeric

104   antigen receptor T cell therapy (CAR-T, Tisagenlecleucel, Cassiopeia protocol). Upon a

105   follow up visit to the transplant and cell therapy clinic 3 weeks later, the patient was

106   tested for SARS-CoV-2 due to concerns for possible contact exposure and was positive

107   (day 0). On day 6 after the initial positive test he presented to the ED with decreased

108   appetite, fever, and two days of dry cough. His chest x-ray showed a diffuse interstitial

109   prominence and a small bilateral pleural effusion. He developed intermittent fevers with

110   increased oxygen requirement over the next several days, including an episode of acute

111   hypoxemic respiratory failure necessitating 10 liters of oxygen via face mask on day 18.

112   He received a 5-day course of Remdesivir. From then on his oxygen requirement was

113   weaned down to 2-3 liters via nasal cannula (NC), however he continued to have

114   intermittent fevers and bilateral patchy ground glass opacities on chest CT. He

115   improved after a second 5-day course of Remdesivir, weaned down off supplemental

116   O2 to his nighttime baseline of 0.5L/NC while asleep (used to treat his mild obstructive

117   sleep apnea) and was discharged on day 45. He was hospitalized four additional times

118   for persistent fevers with negative infectious work-up except for a persistently positive

119   SARS-CoV-2 RT-PCR with consistently high (Ct range: 17.2 – 28.1) viral loads. Weekly

120   convalescent plasma therapy was initiated on day 103 and changed to biweekly

121   beginning day 144. Unfortunately on day 156, he developed worsening cough, new

122   fevers, and was found to be hypoxemic down to 80% needing supplemental oxygen

123   with 4L/NC. Full infectious disease workup failed to demonstrate any additional infection

124   besides persistently positive SARS-CoV-2 RT-PCR. He was admitted to the hospital

125   and received convalescent plasma. Thereafter, he resumed weekly convalescent

126   plasma infusions. His anti-SARS-CoV-2 IgG was finally positive on day 103 which was

127   likely due to the plasma infusion. He has remained SARS-CoV-2 RT-PCR positive

128   through his most recent test on day 250 at the time of this publication.

129

130   Patient 3 is a male under 5 years of age who was diagnosed with high-risk B-cell ALL 7

131   months prior to presentation to the ED with fever and confirmed positive for SARS-CoV-

132   2 upon admission (day 0). At admission he demonstated tachycardia and pancytopenia

133   that were attributed to his chemotherapy. After clinical improvement, he was discharged

134   on day 6 but re-admitted on day 14 with recurrent fever and tachycardia. He continued

135   to be febrile, had worsening pancytopenia, pulmonary infiltrates, and increasing oxygen

136   requirements to 5L via face mask by day 32, at which time he received a 5-day regimen

137   of Remdesivir. His fevers and respiratory distress improved and he was discharged on

138   day 40, only to be readmitted for fever on day 43 and discharged on day 51. The patient

139   then started maintenance chemotherapy. Anti-SARS-CoV-2 IgG was repeatedly

140   negative or borderline and finally became positive on day 83. He was re-admitted on

141   day 162 for possible urinary tract infection and subsequently developed tachypnea,

142   fever, and cytopenia. Chest CT scans on days 47, 96, and 166 showed persistent

143   multifocal pneumonia and scattered areas of ground-glass and consolidative opacities,

144   with shifting opacities that increased in some areas but improved in others. He improved

145   on antibiotics and was discharged on resolution of his fevers on day 173. He was last

146   RT-PCR positive on day 162 and finally negative on day 196. A surveillance chest CT

147   scan on day 230 showed near complete resolution of his pulmonary disease.

148

149   **Study cohort**

150   Specimens from all three patients were obtained at Children's Hospital Los Angeles

151   (CHLA) between May 7 to November 21, 2020 for clinical testing for various indications

152   including hospital admissions both related and unrelated to COVID-19, asymptomatic

153   pre-procedural screening, evaluation of COVID-19 related and unrelated symptoms,

154   and after a high-risk exposure to someone with confirmed SARS-CoV-2 infection. Data

155   for demographics, clinical course and management were obtained from the electronic

156   medical record (EMR). Disease severity was categorized using Centers for Disease

157   Control and Prevention's classification as previously described.[11] The study was

158   approved by the CHLA Institutional Review Board (CHLA-16-00429).

159

160   **Detection of SARS-CoV-2 RNA**

161 Nasopharyngeal swabs or combined oropharyngeal and nares swabs were sent to the

162 Clinical Virology Laboratory at CHLA for testing. The molecular assays used were the

163 CDC 2019-Novel Coronavirus Real-Time RT-PCR assay, the Taqpath COVID-19 RT-

164 PCR assay (Thermo Fisher, Walham, MA), the Xpert Xpress SARS-CoV-2 assay

165 (Cepheid, Sunnyvale, CA), and the Simplexa COVID-19 assay (Diasorin Molecular,

166 Cypress, CA). Total nucleic acid was extracted according to manufacturer's instructions

167 as outlined in the respective Emergency Use Authorization.[11–14] Viral loads (copies/mL)

168 were calculated based on a standard curve generated by testing samples with known

169 viral copy numbers for each assay as previously described.[11]

170

171 **Viral culture**

172 Viral culture was performed using SARS-CoV-2-susceptible VeroE6TMPRSS2 cells[15,16]

173 adapted from the VeroE6 cell line (ATCC CRL-1586) to express the TMPRSS2

174 protease at levels approximately 10-fold higher than that found in the human lung. 150

175 µL of clinical specimen was used to inoculate one well of a 24 well plate of

176 VeroE6TMPRSS2 cells as previously described.[15,16] After a 2-hour incubation at 37ºC,

177 the inoculum was removed and replaced with 0.5 ml infection media and the plates

178 incubated at 37ºC for 5 days. The plates were scored daily for cytopathic effect and

179 supernatants harvested when >75% of the cells had detached. The presence of SARS-

180 CoV-2 viruses in the harvested supernatants was then verified by quantitative RT-PCR

181 using the CDC N1 and RNase P primer sets on RNA extracted with the viral RNA

182 isolation kit (Qiagen, Germantown, MD).

183

Quantitative Two-step strand-specific SARS-CoV-2 RT-PCR

184

185   A two-step strand-specific SARS-CoV-2 RT-PCR assay targeting the *envelope* (E) gene

186   was performed as previously described by Hogan et al. as a biomarker to predict

187   presence of actively replicating virus.[17] In the first set of reactions, reverse transcription

188   with strand-specific primers converts SARS-CoV-2 RNA to complementary DNA

189   (cDNA). In the second step, the cDNA is amplified by real-time PCR in using the Rotor-

190   Gene Q instrument (QIAGEN). A standard-curve to convert minus-strand Ct values to

191   copies/µL was generated using *in vitro* transcribed minus-strand *E* gene RNA.

192

Subgenomic SARS-CoV-2 RT-PCR

193

194   One-step subgenomic (sg) SARS-CoV-2 RT-PCR utilizing a forward primer targeting

195   the 5' leader sequence and reverse primer and probe complementary to *E* gene

196   sequences was adapted from Wolfel et al. and combined in multiplex with the Hong

197   Kong Orf1ab and Centers for Disease Control and Prevention RNAse P primer/probe

198   sets[18,19] as an additional biomarker for actively replicating virus. Real-time RT-PCR was

199   performed using the Rotor-Gene Q instrument. A standard-curve to convert sgRNA Ct

200   values to copies/µL was generated using *in vitro* transcribed plus-strand RNA encoding

201   the leader and *E* gene sequences (Log_{10} copies/µL = -0.288*Ct + 11.351).

202

Measurement of anti-SARS-CoV-2 antibodies in serum

203

204   IgG to the S1 domain of SARS-CoV-2 spike was measured from serum within 24 hours

205   of collection with a commercial enzyme-linked immunosorbent assay (ELISA) kit

206   (EUROIMMUN, Lubeck, Germany).[20] A ratio of sample to calibrator optical density of <

207 0.8 is considered negative, ≥ 0.8 to <1.1 is considered borderline, and ≥1.1 is

208 considered positive.

209

210 In addition, IgA, IgG and IgM to SARS-CoV-2 nucleocapsid (N) protein, spike S1, and

211 receptor binding domain (RBD) and the competition ELISA procedure were measured

212 as recently described by Röltgen et al.[21]

213

214 **Viral genome library construction and sequencing**

215 Whole-genome sequencing (WGS) libraries of viral cDNA were prepared as previously

216 described using the CleanPlex SARS-CoV-2 Research and Surveillance NGS Panel

217 (Paragon Genomics, Hayward, CA).[11] Libraries were then quantified and normalized to

218 approximately 7nM and pooled to a final concentration of 4nM; libraries were denatured

219 and diluted according to Illumina protocols and loaded on the MiSeq at 10pM. Paired-

220 end and dual-indexed 2x150bp sequencing was done using Micro Kit v2 (300 Cycles).

221 Libraries yielding average depth ≥ 1000x were considered high quality and used for

222 downstream analysis, and variant calling was performed for nucleotide positions with

223 ≥100x coverage.

224

225 **Genome assembly and variant calling**

226 Coverage profiles, variant calls and consensus genomes were generated using the in-

227 house software system LUBA (Lightweight Utilities for Bioinformatics Analysis).[11] Ion

228 Torrent aligner TMAP (Thermo Fisher) was used to generate AmpliSeq BAMs.

229 Sequencing of majority of clinical specimens by two alternate methods were also

230   pursued to validate Paragon variant calls (appendix p 1-3). Paragon and Twist

231   nucleotide sequences were aligned with NovoAlign (Novocraft Technologies, Selengor,

232   Malaysia) aligner. Ion Torrent aligner TMAP (Thermo Fisher) was used to generate

233   AmpliSeq BAMs.

234

235   **Role of the funding source**

236   The funder had no role in study design, data collection, data analysis, data

237   interpretation, or writing of the report. The corresponding author had full access to all

238   the data in the study and had final responsibility for the decision to submit for

239   publication.

240

241   **Results**

242   <u>Viral detection by RT-PCR and culture</u>

243   Specimens from all three patients were collected and used to detect SARS-CoV-2 RNA

244   over the course of 6 months. In addition to viral culture, we performed strand-specific

245   and subgenomic RT-PCR to detect ongoing replication.[4,17,18] The viral load in patient 1

246   was highest at day 0 and then gradually declined and was last reliably detected on day

247   46 (Figure 2A). Culturable virus was only detected in the day 0 sample and subsequent

248   cultures were negative. The SARS-CoV-2 minus strand and subgenomic RNA was only

249   detected from the first sample taken on day 0 and not in any subsequent samples

250   (Figure 2A).

251

252 The viral load in patient 2 remained consistent through day 172 despite two courses of

253 Remdesivir and weekly administration of convalescent plasma. Consistent with these

254 results, SARS-CoV-2 was recovered by viral culture for up to day 144. Viral cultures

255 were not explored past 144 days from initial positive. Strikingly, the minus strand and

256 subgenomic RNA was detected in nearly all of the samples from patient 2 and the Ct

257 values trended with the corresponding viral load (Figure 2B).

258

259 Patient 3 also maintained consistent viral loads through day 162 despite weak

260 seroconversion on day 83, and finally tested negative on day 196 (Figure 2C). He

261 demonstrated variable viral recovery with positive cultures from days 0, 22, and 139,

262 and negative cultures from days 15, 67, and 162 which we believe reflects poor sample

263 integrity or a transient reduction in infectious virus production given that subgenomic

264 and minus strand RNA was readily detectable up through day 162.

265

266 <u>Antibody response</u>

267 We assessed each patient's serological response profile using ELISAs to measure IgA,

268 IgG, and IgM antibodies specific to the SARS-CoV-2 receptor binding domain (RBD),

269 S1, and N proteins. Patient 1 had a strong IgA, IgG, and IgM response to all tested

270 antigens at days 46 and 117, consistent with their more typical clinical course of

271 infection and recovery, with viral RNA not reliably detected after day 46 (Figure 3A). In

272 contrast, patient 2, who had culturable virus up to day 144 and positive viral RNA up to

273 day 172, was more weakly IgG positive and was negative for IgA and IgM to all tested

274 SARS-CoV-2 antigens from days 110-144 (Figure 3B). Importantly, paired sera

275   obtained from patient 2 before and after convalescent plasma infusion showed

276   increased IgG levels after infusion which were rapidly waning. As this patient had B-cell

277   aplasia following CAR T-cell therapy, his ability to mount an immune response was

278   impaired. Patient 3 had detectable viral RNA at least up to day 162, and showed

279   serological results spanning days 83-176 with a weakly positive IgG and unusually high

280   levels of IgM for the RBD, S1, and Spike proteins, but negative N antibodies (Figure

281   3C). Antibody levels were compared to four non-immunocompromised COVID-19

282   patients, who showed the typical increase of anti-SARS-CoV-2 IgG, IgM, and IgA

283   antibodies between one to two weeks after symptom onset. IgA and IgM antibody levels

284   began to wane about 3 to 4 weeks after symptom onset, while IgG levels were more

285   stable (Figure 3D).

286

287   We also measured the ability of the patients' sera to interfere with the interaction

288   between the RBD of the SARS-CoV-2 spike protein and the angiotensin-converting

289   enzyme 2 (ACE2) receptor *in vitro* (Figure 3E). Sera from patient 1 from days 46 and

290   117 achieved 100% blocking, whereas patient 2's sera only weakly blocked the ACE2-

291   RBD interaction on day 123 post-infusion and the other timepoints failed to block the

292   interaction. Samples from patient 3 from days 83, 141, and 176 also showed poor

293   blocking activity (Figure 3E). Plasma samples from the four control patients showed

294   varying degrees of RBD-ACE2 blocking activity. In a previous study higher RBD-ACE2

295   blocking percentages were correlated with higher anti-RBD antibody levels[21] The

296   antibody results were overall consistent with what we observed from the blocking assay,

297   with the strongest immune response in patient 1, least effective response in patient 2,

298   and a weak response in patient 3.

299

300   Genomic sequencing analysis

301   Specimens from patient 1 showed only a single major allele variant difference between

302   days 0 and 27. Decreasing viral load precluded sequencing of specimens from later

303   timepoints. Patients 2 and 3 demonstrated an increase in intra-host viral diversity over

304   time in variants of both high (major) and low (minor) allele frequencies across the viral

305   genome (Figure 4). In patient 2, 3 major allele variants emerged between days 0 and 40

306   with an additional 4 major and 7 minor allele variants by day 144. In patient 3, 13 major

307   and 8 minor allele variants emerged by day 162. Virus clade predictions did not change

308   across the specimens from each patient (patients 1 and 3: clade 20C and patient 2:

309   clade 20A), providing further support that these patients were continuously infected

310   rather than reinfected. Of particular note was the emergence of several spike gene

311   mutations including 3 inframe deletions at residues S:Δ141-143, S:Δ145, and S:Δ141-

312   144, a deletion-insertion at S:Δ211-212, and several nonsynonymous mutations

313   including N440K, V483A, and E484Q at residues that have independently mutated in

314   other lineages (Figure 4, appendix p 6-12). Sequencing of additional library

315   preparations by two alternate methods confirmed the variant calls (appendix p 1-3).

316

317   **Discussion**

318   We report two pediatric and one young adult patient with B-cell ALL with remarkably

319   different courses of SARS-CoV-2 infection. Patient 1 had milder symptoms with rapidly

320   declining and nonviable virus and serological evidence of a robust immune response

321   that is representative of a typical COVID-19 clinical course.[1] By contrast, patients 2 and

322   3 experienced more severe COVID-19 characterized by serological evidence of a

323   weaker immune response with evidence of prolonged infectious virus shedding and

324   mutation accumulation demonstrating emergence of potential escape mutations.

325

326   The infection control implications make it critically important to closely monitor

327   immunocompromised patients who are persistently positive by SARS-CoV-2 RT-PCR

328   and to determine whether or not the virus is actively replicating for a prolonged period of

329   time. Using RT-PCR to detect subgenomic and negative-strand replication

330   intermediates, we were able to identify ongoing replication throughout the samples from

331   patient 2 (up to 144 days) and patient 3 (up to 162 days). It is notable that subgenomic

332   RNA was readily detected for this length of time given that it is rarely detected past 8

333   days.[4,5,18] Overall the detection of viral intermediates correlated very well with the viral

334   culture data, suggesting these may serve an expeditious molecular surrogate for

335   infectivity. Viral culture was intermittently positive up to day 139 in patient 3. As culturing

336   methods are limited by sample quality and lower sensitivity compared to molecular

337   methods, it is more likely that the consistently positive results by subgenomic and

338   negative-strand RT-PCR accurately represented continued replication within patient 3.

339

340   There has been considerable interest in the rapidly spreading SARS-CoV-2 B.1.1.7

341   variant due to its potential increased transmissibility. Relative to the SARS-CoV-2

342   reference sequence, B.1.1.7 has acquired 17 mutations, 8 of which are in the spike

343   gene. There is concern that such a divergent variant could have emerged from long-

344   term replication within an immunocompromised host, especially with the lack of closely

345   related viral isolates.[9] Although we did not find these mutations in our patients, we did

346   observe mutations in several regions within the spike gene. A V70P mutation appeared

347   at day 162 in patient 3, overlapping the region where a S:Δ69-70del in the B.1.1.7

348   variant may enhance infectivity.[22] Several other observed mutations mapped to

349   important residues in the spike gene. We observed emergence of a S:Δ141-144 and

350   S:Δ145 deletion in patient 2 and a S:Δ141-143 deletion in patient 3. Similar mutations in

351   this region of the spike gene have been found to abolish the binding of the anti-spike

352   protein 4a8 blocking/neutralizing monoclonal antibody (Figure 3, Tables S3-4).[7,23]

353   Additionally, in patient 3, N440K within the spike RBD emerged with an allele frequency

354   of 0.51 on day 162 (Figure 3, Table S4). This variant was also found by another group

355   to confer antibody escape.[24] This mutation is adjacent to the position of a N439K variant

356   of interest currently circulating in several lineages in Europe, which may enhance affinity

357   of the binding of the spike protein to the ACE2 receptor and influences binding of

358   monoclonal antibodies.[25] At day 139 the sequenced specimen from patient 3 also had

359   mutations at V483A and E484Q within the RBD. These residues were similarly

360   associated with *in vitro* antibody binding and escape, and there has been interest in

361   another mutation at the same position, E484K, which has emerged in the S.501Y.V2

362   lineage in South Africa (Figure 3, Table S4).[24,26–28] Similar mutations at these positions

363   (N440D, E484A, and E484K) have independently arisen within other persistently

364   infected, immunocompromised patients.[5,7]

365

366   Analysis of patient antibody responses was performed to aid in understanding the

367   persistent detection of SARS-CoV-2. Patient 1 was associated with very limited

368   detection of active replication and had high levels of ACE2-RBD blocking antibodies, a

369   surrogate measure of potential viral neutralizing antibodies *in vivo*. Patients 2 and 3 by

370   contrast both had poor antibody responses, associated with evidence of prolonged

371   infectivity. In immunocompetent patients, IgM, IgG, and IgA antibodies are generally

372   detectable by day 20 post symptom onset.[21] Patient 3 had an atypical persistence of

373   high IgM levels on days 83, 141, and 176 without evidence of evolution to IgG or IgA,

374   suggestive of an impaired immune response perhaps related to his partially

375   immunocompromised state. It is unlikely that the detection of IgM in this patient is

376   related to infection with a new viral strain as the majority of the mutations that emerged

377   in patient 3 are rarely found in the general population (Table S4). In general, all three

378   patients had higher levels of antibodies targeting RBD and S1 compared to N, and this

379   pattern has been previously observed in patients with milder illness compared to

380   patients who died or were admitted to intensive care units.[21] However, compared to

381   patients 1 and 2, patient 3 completely lacked antibodies to the N protein (Figure 3C). It

382   is possible that the spike-focused immune response, which weakly blocked ACE2, led

383   to the emergence of escape mutants. There were more mutations in the spike gene,

384   particularly in the RBD region, in patient 3 compared to patient 2 and many of these

385   emerged on day 139 or 162, a time period where his antibody levels were rising. Patient

386   2 was treated with two courses of remdesivir and several infusions of convalescent

387   plasma, however antibody levels waned rapidly after each infusion, possibly due to

388   redistribution in the body or partial consumption and clearance. These treatments failed

389   to control viral replication and did not apparently lead to as many mutations with the

390   potential for immune escape as observed in patient 3. We note that anti-SARS-CoV-2

391   antibody levels start to decrease as soon as one month after symptom onset in some

392   previously healthy COVID-19 patients, therefore it is possible that our measurements at

393   later time points may underestimate the peak antibody levels acheived by patients 2

394   and 3 throughout their clinical course.[21]

395

396   Our work demonstrates that immunocompromised pediatric and young adult patients

397   are susceptible to prolonged viral infections with prolonged infectious virus shedding

398   and mutation accumulation. The patients we report are immunocompromised to varying

399   degrees, and support a potential correlation between host immune response and the

400   emergence of viral variants, some of which may have the potential to escape antibody

401   neutralization. One of the limitations of a small case series is the generalizability of

402   findings and more work is needed to better understand the many host factors that can

403   influence viral clearance and mutation within these patients. However, we conclude that

404   the observed expanded intra-host heterogeneity of viral isolates from pediatric and

405   young adult patients with suppressed immunity and correspondingly prolonged infection

406   raises the possibility of emergence of variants with the potential to evade immune

407   responses elicited during the course of infection or induced by vaccine. This possibility

408   warrants further study and, in the meantime, serious consideration of extended infection

409   control precautions and genomic monitoring in this patient population.

410

411   **Acknowledgments**

412   The authors thank the ATUM Bio team for optimization, production, and sharing of

413   antigens used in this study. We would like to acknowledge NCBI, GISAID, and

414   Nextstrain for providing valuable resources for SARS-CoV-2 genomics. We thank the

415   National Institute of Infectious Diseases, Japan, for providing VeroE6TMPRSS2 cells.

416

417   **Conflict of Interest**

418   S.D.B. has consulted for Regeneron, Sanofi, and Novartis on topics unrelated to this

419   study. S.D.B., and K.R. have filed provisional patent applications related to serological

420   tests for SARS-CoV-2 antibodies. All other authors have no competing interests.

421

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508 **Research in context**

509 *Evidence before this study*

510 There has been substantial interest in the phenomenon of prolonged shedding of

511 SARS-CoV-2. Although an increasing number of case reports have described the

512 course of disease in immunocompromised individuals, definitive evidence for prolonged

513 active replication with infectious virus and corresponding immunological data has been

514 scarce. We searched PubMed, bioRxiv, and medrxiv for these reports using the terms

515 "immunocompromised," "immunosuppressed," "SARS-CoV-2," "replication," "shedding,"

516 and "infectivity" for relevant articles published up until December 26, 2020. We found

517 four articles that have utilized longitudinal sequencing, detection of subgenomic RNA,

518 and/or viral culture to demonstrate ongoing infectivity in immunocompromised adults.

519

520 *Added value of this study*

521 Our study presents a more complete picture of the spectrum of prolonged infection for

522 up to 162 days, which is the longest reported to-date, in three immunocompromised

523 patients using viral culture, detection of both subgenomic and single-stranded RNA,

524 whole-genome sequencing, and characterization of host serological responses.

525 Secondly, this is the first report, to our knowledge, characterizing prolonged infection in

526 pediatric and young adult patients. Thirdly, we demonstrated the clear presence of intra-

527 host viral heterogeneity as evidence of ongoing viral evolution within the hosts. We note

528 two of our patients acquired several mutations in the gene encoding the spike protein at

529 specific residues that may be important for antibody binding. Finally, we present a

530   potential correlation between the host immune response and the emergence of

531   mutations in the spike gene.

532

533   *Implications of all the available evidence*

534   Given recent interest in the emergence of a potentially more transmissible variant of

535   SARS-CoV-2, there is a need to better understand the dynamics of within-host

536   accumulation of mutations in immunocompromised hosts, whether or not they remain

537   infectious, if these mutations could mediate immune escape, and what kind of

538   immunological selection pressures may drive such mutations. Altogether, these findings

539   have important public health implications for infection control precautions and

540   management in this patient population.

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553   **Figure Legends**

554   **Figure 1. Clinical timeline of symptoms, hospital admissions, and treatment**.

555   Timelines for patient 1 (A), patient 2 (B), and patient 3 (C) are labelled by date from

556   initial positive RT-PCR (day 0). Colored bars indicate time periods where patients were

557   symptomatic, required supplementary oxygen, or received treatment (Remdesivir or

558   convalescent plasma). The phases of chemotherapy are also shown.

559

560   **Figure 2. Viral load by routine, negative-strand, and subgenomic RT-PCR.** Time

561   course of viral load from nasopharyngeal or combined nares/oropharyngeal swabs

562   collected from each patient. Viral culture results are indicated in pink. Corresponding

563   serum anti-SARS-CoV-2 IgG values are plotted in blue. For patient 2, IgG was

564   measured before and after administration of convalescent plasma at the indicated

565   timepoints.

566

567   **Figure 3. Anti-SARS-CoV-2 antibody responses in three immunocompromised**

568   **pediatric and young adult patients.**

569   Serum samples from two immunocompromised children (A, C) and one young adult (B)

570   were collected up to 176 days after initial positive SARS-CoV-2 PCR test. Plasma

571   samples from four non-immunocompromised COVID-19 patients were analyzed for

572   comparison (D). Antibodies specific for SARS-CoV-2 Spike RBD (top row), S1 subunit

573   (second row), and N protein (third row) were measured using ELISA at 1:100

574   serum/plasma dilution. RBD-ACE2 blocking activity was assessed in a competition

575   ELISA and is shwon as percentage of blocking (E). The cutoff for seropositivity was

576   defined as the mean absorbance + 3 SD of 94 pre-pandemic plasma samples in each

577   assay (A, B, C, D). Color coding for isotypes: IgG (blue), IgM (green), IgA (red). Dotted

578   lines depict the cutoff for seroconversion for the different isotypes in each assay.

579   Individual donors were shown with different symbols (D). * indicates timepoints of

580   convalescent plasma infusion in patient 2.

581

582   **Figure 4. Accumulation of SARS-CoV-2 variants in three persistently positive**

583   **pediatric and young adult patients with hematologic malignancies.** Each row

584   represents viral culture, strand-specific RT-PCR (ssRT-PCR), subgenomic RT-PCR

585   (sgRT-PCR) and sequencing from longitudinally derived specimens numbered by days

586   from initial positive test (day 0). Boxes represent distinct variants, and shading reflects

587   variant allele frequency (cutoff = 0.25). Corresponding genes are labeled in top row and

588   colored to represent variant annotation (see legend). Specimens from two patients

589   demonstrate progressive accumulation of multiple variants and variants of lower allele

590   frequencies (Patient 2, difference of 7 major allele variants between days 0 and 144;

591   Patient 3, difference of 13 major allele variants between days 0 and 162). In addition to

592   point mutations, four different inframe deletions were observed to develop in the S gene

593   in Patient 2 and Patient 3. Several variants (both major and minor alleles) reverted at
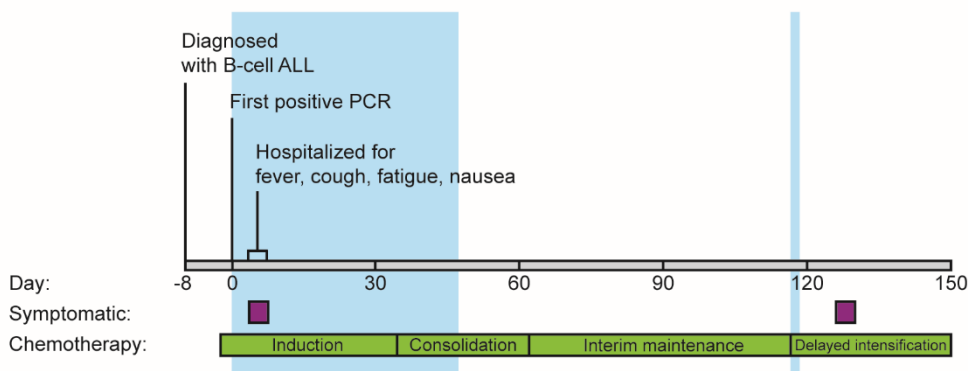
594   later timepoints.

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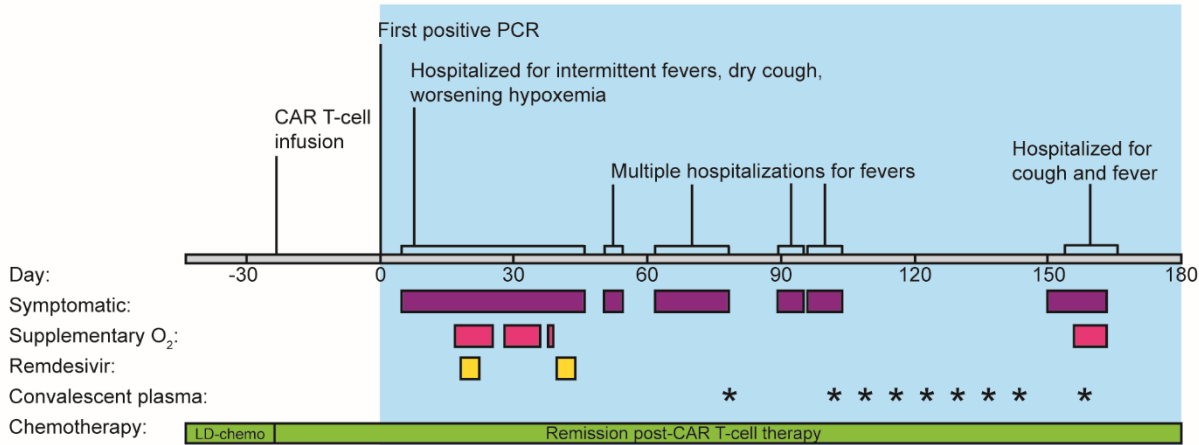596   UTR, untranslated region; S, spike; E, envelope; M, matrix; N, nucleocapsid
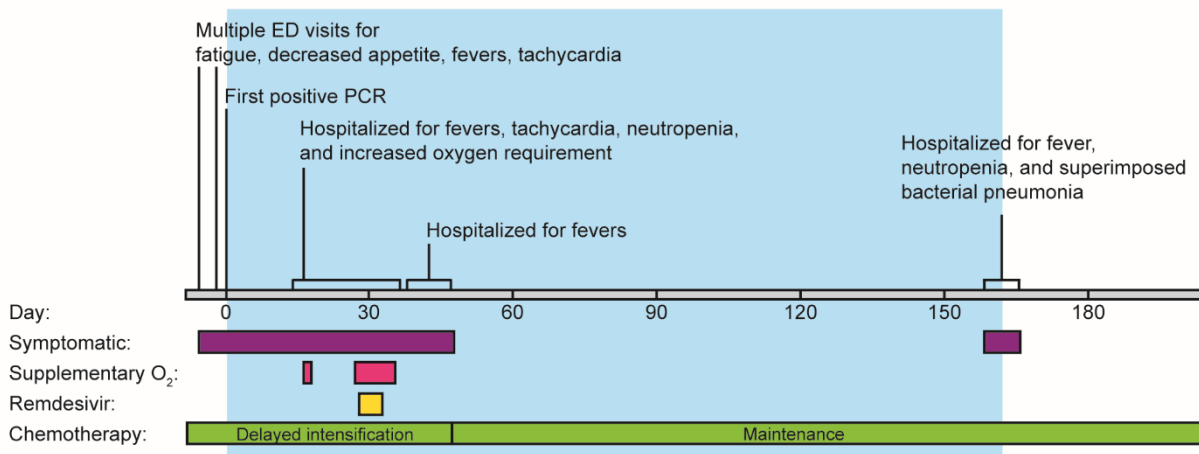
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599 **Figure 1.**

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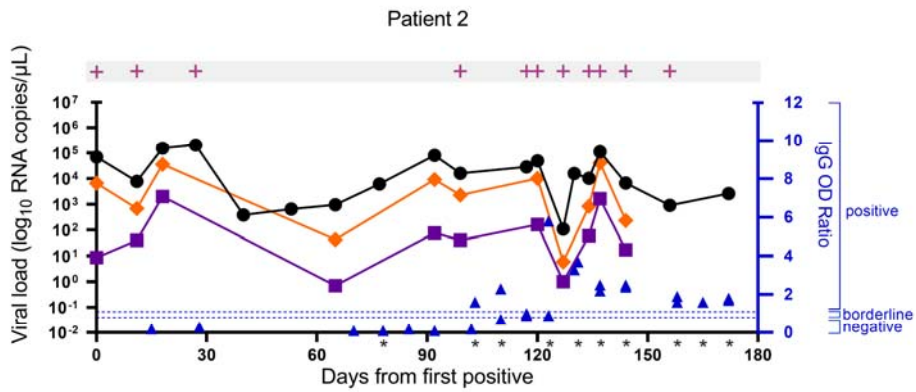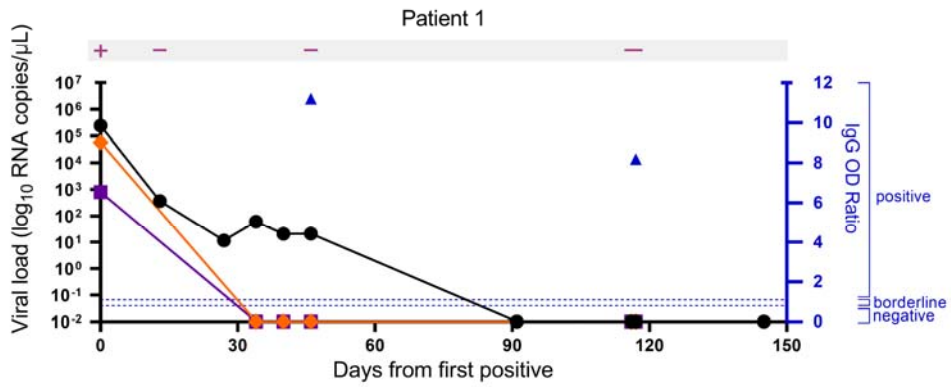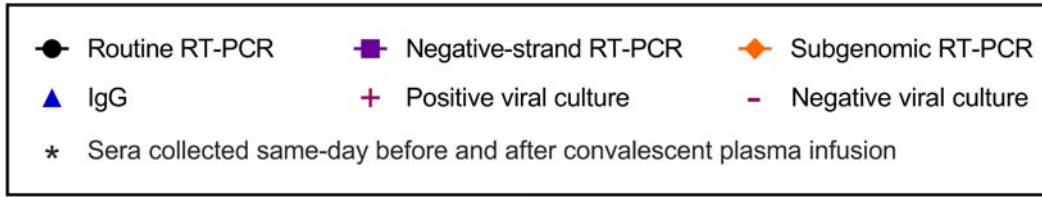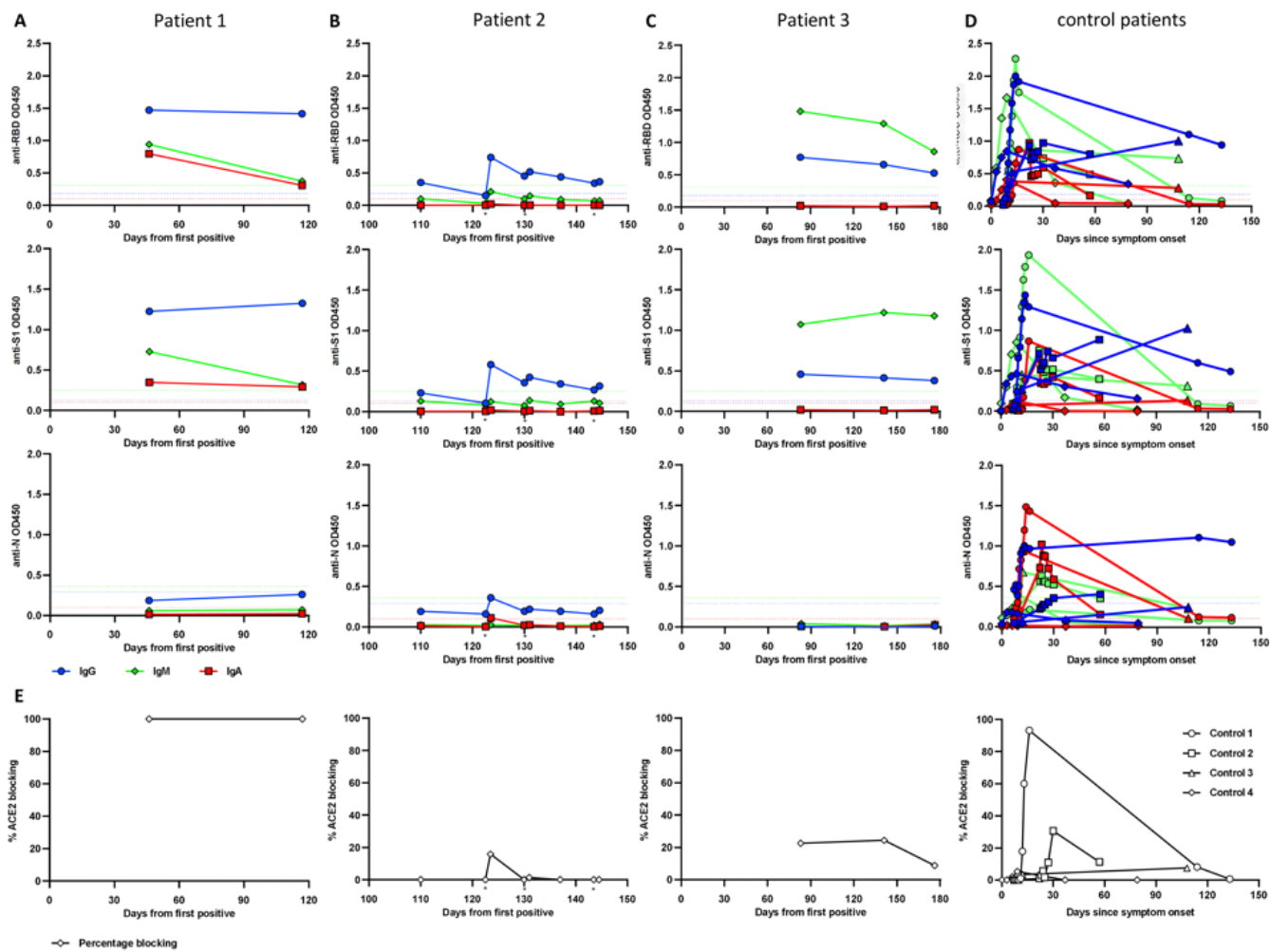

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603 **Figure 2.**
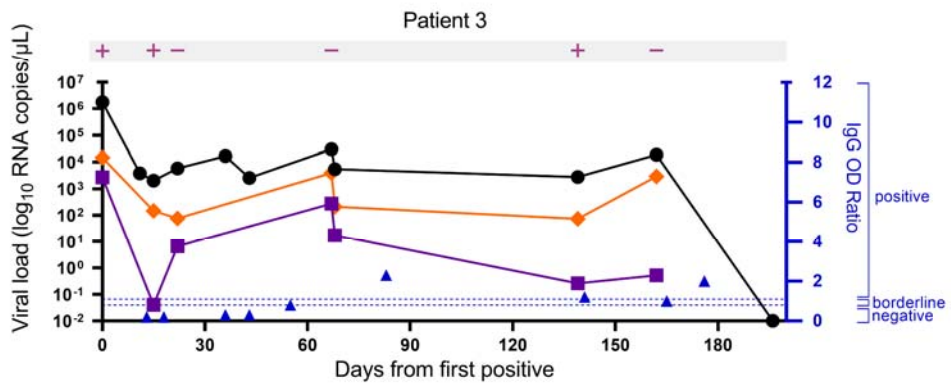
605 **Figure 3.**

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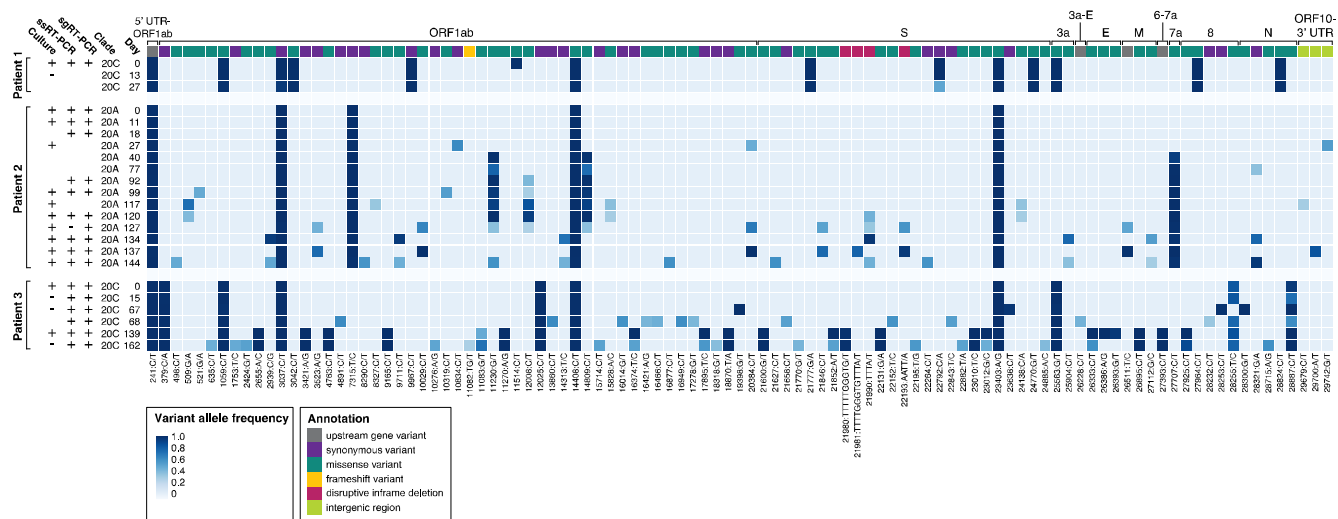
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Figure 4.

618   **Table 1.** Demographic, oncological, and clinical characteristics of study patients.

	Patient 1	Patient 2	Patient 3
Demographic characteristics			
Age range (years)	0-5	20-25	0-5
Sex	Female	Male	Male
Body Mass Index	16.6 kg/m^2	27 kg/m^2	16.3 kg/m^2
Oncological characteristics			
Oncological diagnosis	SR B-ALL	HR B-ALL, refractory	HR B-ALL
Other coexisting medical conditions	None	Obstructive sleep apnea Hypothyroidism Intermittent Asthma	None
Oncological treatment	Chemotherapy	Lymphodepleting chemotherapy and CAR-T cell therapy	Chemotherapy
Phases of oncological treatment during viral shedding	-Induction -Consolidation -Interim maintenance -Delayed intensification	Post- CAR-T cell therapy	-Delayed intensification -Maintenance
Active immunosuppressive or immunomodulatory drugs?	SR B-ALL chemotherapy per COG AALL0932: Dexamethasone, vincristine, peg-asparaginase, methotrexate, 6-MP, doxorubicin	Cyclophosphamide and Fludarabine as lymphodepleting agents prior to CAR-T cell therapy	HR B-ALL chemotherapy per COG AALL0232: Cyclophosphamide, cytarabine, thioguanine, vincristine, dexamethasone, methotrexate, mercaptopurine
Chemotherapy	Yes (detailed above)	Yes (detailed above)	Yes (detailed above)
Corticosteroids	Yes (per chemotherapy protocol)	No	Yes (per chemotherapy protocol)
CAR-T therapy	No	Yes, Tisagenlecleucel	No
Clinical, laboratory and imaging data			
Days of PCR positivity	46 (inconclusive/positive again day 116-117)	165+ from time of report	162
Symptomatic at time of initial positivity	No	No	Yes
Symptomatic at any positive time point	Yes	Yes	Yes
Symptoms			
Fever	Yes	Yes	Yes
Malaise	Yes	Yes	Yes
Cough	Yes	Yes	Yes
Sore throat	Yes	No	Yes
Headache	No	No	No
Shortness of breath	No	Yes	Yes
Congestion/rhinorrhea	No	Yes	Yes
Vomiting	Yes	Yes	Yes
Abdominal pain	Yes	Yes	Yes
Anosmia and Ageusia	Unable to assess	Unable to assess	Unable to assess

Other infectious disease findings	No	- *Clostridioides difficile* diarrhea - *Staphylococcus hominis* bacteremia (day +7)	Superimposed bacterial pneumonia (day 162, improved with antibiotics)
Laboratory data at initial positivity	WBC: 2.14 K/µL ANC: 0.74 K/µL ALC: 1.36 K/µL	WBC: 2.30 K/µL ANC: 1.59 K/µL ALC: 0.42 K/µL	WBC: 0.65 K/µL ANC: 0.00 K/µL ALC: 0.56 K/µL
Lymphocyte subsets	Not done	Day +7 to +158 (multiple) CD3: 75-1000 cells/µL CD4: 31-314 cells/µL CD8: 52-747 cells/µL CD19: <1 cell/µL throughout	Day +13 CD3: 1204 cells/µL CD4: 194 cells/µL CD8: 987 cells/µL CD19: 1 cells/µL
Chest CT scan	Not done	Bilateral patchy ground glass opacities involving all lobes, predominantly peripherally in lower lobes (similar in all 3 CT scans on days +38, +73 and +116)	Persistent multifocal pneumonia and scattered areas of ground-glass opacities, with shifting opacities increased in some areas but improved in others (CT scans on days +22, +47, +96, +166)
Treatment for COVID-19 infection			
Oxygen supplementation	No	Yes, intermittent (maximal 10L per minute via facemask)	Yes (maximal 5L per minute via facemask)
COVID-19 related ICU admissions	No	Yes (5 days)	No
Mechanical ventilation	No	No	No
Remdesivir	No	Yes, two 5 day courses	Yes, one 5 day course
Convalescent plasma	No	Yes (measured as ratio serum to calibrator absorbance) Day 78: 4.3 Day 103: 9.1 Day 110: 8.6 Day 123: 8.3 Day 130: 7.5 Day 137: 4.6 Day 144: 1.4 Day 158: 4.1 Day 165: 3.4 Day 172: 1.53	No

619 SR, standard risk; HR, high risk; B-ALL, B-cell acute lymphocytic leukemia; CAR-T,
620 chimeric antigen receptor T-cell therapy; COG, Children's Oncology Group; WBC, white
621 blood cells; ANC, absolute neutrophil count; ALC, absolute lymphocyte count; CT,
622 computerized tomography; ICU, intensive care unit

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