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Circulating effector γδ T cell populations are associated with acute coronavirus disease 19 in unvaccinated individuals

Anouk von Borstel[1], Thi HO Nguyen[2], Louise C Rowntree[2], Thomas M Ashhurst[3,4], Lilith F Allen[2], Lauren J Howson[1], Natasha E Holmes[5,6,7,8], Olivia C Smibert[5,9,10], Jason A Trubiano[8,11], Claire L Gordon[2,5], Allen C Cheng[12,13], Stephen J Kent[2,14,15], Jamie Rossjohn[1,16,17], Katherine Kedzierska[2,18] & Martin S Davey[1,19]

1 Infection and Immunity Program and Department of Biochemistry and Molecular Biology, Biomedicine Discovery Institute, Monash University, Clayton, VIC, Australia
2 Department of Microbiology and Immunology, University of Melbourne, at the Peter Doherty Institute for Infection and Immunity, Melbourne, VIC, Australia
3 Sydney Cytometry Core Research Facility, Charles Perkins Centre, Centenary Institute and University of Sydney, Sydney, NSW, Australia
4 Marie Bashir Institute for Infectious Diseases and Biosecurity, University of Sydney, Sydney, NSW, Australia
5 Department of Infectious Diseases, Austin Health, Heidelberg, VIC, Australia
6 Department of Critical Care, University of Melbourne, Parkville, VIC, Australia
7 Data Analytics Research and Evaluation (DARE) Centre, Austin Health and University of Melbourne, Heidelberg, VIC, Australia
8 Centre for Antibiotic Allergy and Research, Department of Infectious Diseases, Austin Health, Heidelberg, VIC, Australia
9 Department of Infectious Diseases, Peter MacCallum Cancer Centre, Melbourne, VIC, Australia
10 National Centre for Infections in Cancer, Peter MacCallum Cancer Centre, Melbourne, VIC, Australia
11 Department of Medicine (Austin Health), University of Melbourne, Heidelberg, VIC, Australia
12 Infection Prevention and Healthcare Epidemiology Unit, Alfred Health, Melbourne, VIC, Australia
13 School of Public Health and Preventive Medicine, Monash University, Melbourne, VIC, Australia
14 ARC Centre of Excellence in Convergent Bio-Nano Science and Technology, University of Melbourne, Melbourne, VIC, Australia
15 Melbourne Sexual Health Centre, Infectious Diseases Department, Alfred Health, Central Clinical School, Monash University, Melbourne, VIC, Australia
16 Australian Research Council Centre of Excellence in Advanced Molecular Imaging, Monash University, Clayton, VIC, Australia
17 Institute of Infection and Immunity, Cardiff University School of Medicine, Heath Park, Cardiff, UK
18 Global Station for Zoonosis Control, Global Institution for Collaborative Research and Education (GI-CoRE), Hokkaido University, Sapporo, Japan
19 Division of Biomedical Sciences, Warwick Medical School, University of Warwick, Coventry, UK

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Correspondence
Katherine Kedzierska, Department of Microbiology and Immunology, University of Melbourne, at the Peter Doherty Institute for Infection and Immunity, Melbourne, VIC 3000, Australia.
E-mail: kkedz@unimelb.edu.au

Martin S Davey, Division of Biomedical Sciences, Warwick Medical School, University of Warwick, Coventry CV4 7HL, UK
E-mail: martin.davey@warwick.ac.uk

Abstract

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection causes severe coronavirus disease 2019 (COVID-19) in a small proportion of infected individuals. The immune system plays an important role in the defense against SARS-CoV-2, but our understanding of the cellular immune parameters that contribute to severe COVID-19 disease is incomplete. Here, we show that populations of effector γδ T cells are associated with COVID-19 in unvaccinated patients with acute disease. We found that circulating $CD27^{neg}CD45RA^{+}CX3CR1^{+}$ $V\delta1_{effector}$ cells expressing Granzymes (Gzms) were enriched in COVID-19 patients with acute disease. Moreover, higher frequencies of $GzmB^{+}$ $V\delta2^{+}$ T cells were observed in acute COVID-19 patients. SARS-CoV-2 infection did not alter the γδ T cell receptor repertoire of either $V\delta1^{+}$ or $V\delta2^{+}$ subsets. Our work demonstrates an association between effector populations of γδ T cells and acute COVID-19 in unvaccinated individuals.

Present address
Lauren J Howson, Immunology Division,
Walter and Eliza Hall Institute of Medical
Research, Parkville, VIC 3052, Australia

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INTRODUCTION

Coronavirus disease 2019 (COVID-19) remains a global burden with over 663 million confirmed cases and approximately 6.7 million deaths have been reported to the WHO to date.[1] Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection can result in severe lung inflammation (i.e. acute respiratory distress syndrome (ARDS)).[2] However, we do not completely understand the immune parameters involved in the disease pathogenesis. SARS-CoV-2 enters respiratory epithelial cells *via* the ACE2 receptor upon inhalation and initiates replication.[2] SARS-CoV-2 antigens (Ags) are presented by major histocompatibility complex (MHC) I that are recognized by SARS-CoV-2-specific cytotoxic $CD8^+$ T cells.[3,4] Further Ag presentation induces both cellular and humoral immune cells. In COVID-19 patients with ARDS, a cytokine storm occurs as a result of an uncontrolled systemic immune response, which is the most common cause of death.[5] It is composed of large amounts of interferon (IFN)-α, IFN-γ, tumor necrosis factor (TNF) α, tumor growth factor (TGF) β, and interleukin (IL)-1β, -6, -12, -18, -33 as well as many chemokines produced by effector immune cells.[4] While both innate and adaptive immune responses play important roles in the defense against SARS-CoV-2, the role of γδ T cells is poorly understood.

γδ T cells are a unique population of unconventional lymphocytes. Circulating γδ T cells represent 5–10% of all $CD3^+$ T cells and express a T cell receptor (TCR), mostly consisting of Vγ9 paired to Vδ2,[6] termed $V\delta2^+$ T cells. $V\delta2^+$ T cells activate rapidly upon exposure to the phosphoAg (pAg) (E)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP).[7] This pAg is produced by an array of microbes including *Plasmodium falciparum* and *Mycobacterium tuberculosis*. The $V\delta2^+$ T cell subset possesses a γδTCR repertoire that contains public (i.e. shared) complementarity determining region 3 (CDR3) sequences.[6] $V\delta2^-$ γδT cells on the other hand, are present at high frequencies in tissues where they are thought to play an important role in immune surveillance.[8] The majority of tissue-infiltrating cells express a Vδ1 TCR paired to various Vγ chains.[9] $V\delta1^+$ T cells show private (i.e. not shared) clonotypic γδ TCR expansions and differentiate from naïve into effector cells upon microbial challenges such as repeated *P. falciparum*[10] and cytomegalovirus (CMV)[9] infection.

γδ T cells have been implicated in the immune response to SARS-CoV-2 and are associated with COVID-19 severity.[11] Activated $HLA\text{-}DR^+CD38^+$ and $CD69^+$ γδ T cells were increased in the circulation of acute COVID-19 patients with severe disease, compared with convalescent patients and/or uninfected individuals.[11–13] Importantly, activated γδ T cells correlated with COVID-19 severity, with increased frequencies[11] and counts[14] found in severe *vs.* mild infection.[11] In addition, IL-6 and IL-18 levels correlated with activated γδ T cells, indicating that these cells may contribute to the cytokine storm that occurs in severe acute COVID-19. Only a few studies investigated the involvement of specific γδ T cell subsets in SARS-CoV-2 infection. Reduced circulating $V\delta2^+$ T cell frequencies[12] and numbers have been found in COVID-19 patients correlating with disease severity.[15] While no differences in $V\delta1^+$ T cells have been identified, a recent study showed that $TCR\delta1^+$ (TRDV1) CDR3 sequences showed evidence of clonal focusing in COVID-19 patients aged > 50 years.[16]

γδ T cell subsets have been implicated in controlling other viral infections such as CMV and severe acute respiratory syndrome (SARS). In CMV, $V\delta1^+$ T cells expanded rapidly and undergo γδ TCR clonotype expansion upon infection in both stem cell[17] and kidney[18–21] transplantation settings and have been implicated in the resolution of CMV infection.[22] Moreover, $V\delta1^+$ T cells expressed an effector phenotype (i.e. $CD27^{lo}CD45RA^+$ or $CD45RA^+CD28^-$) in CMV-seropositive patients.[9,18] In SARS patients, $V\delta2^+$ T cells expanded during convalescent disease.[23] Moreover, $V\delta2^+$ T cells lines mounted IFN-γ-dependent responses upon *in vitro* stimulation with IPP and IL-2. Supernatants from stimulated $V\delta2^+$ T cells lines decreased both viral load and infectious units from

SARS-infected Vero cells. In line with this, Vδ2$^+$ T cells derived from controls co-cultured with SARS-infected THP-1 cells had increased IFN-γ production and cytotoxicity. Importantly, it remains to be elucidated whether and how γδ T cell subsets and γδ TCR repertoires change in response to SARS-CoV-2 infections and whether this associates with COVID-19 severity.

To gain a deeper understanding of γδ T cell repertoire responses in SARS-CoV-2 infection and how each subset associates with disease severity in SARS-CoV-2 infection, we analyzed peripheral blood mononuclear cells (PBMCs) from acute and convalescent COVID-19 patients using multi-color flow cytometry and γδ TCR sequencing. An increased activated Vδ1$_{effector}$ population was observed in acute COVID-19 patients compared with convalescent patients and healthy controls (HCs). This population had increased cytotoxic potential in acute COVID-19 patients. In addition, an expanded cytotoxic Vδ2$_{effector}$ population was found in patients with acute COVID-19. However, no γδ TCR selection was identified for either subset and the repertoire did not change over time. Together, this study suggests that circulating effector populations of γδ T cells may be associated with acute COVID-19.

RESULTS

Activated effector γδ T cell frequencies are increased in acute COVID-19

To explore the relationship between circulating γδ T cells and acute COVID-19, we examined a cohort of 23 acute (median age of 53 years, range 27–73 years; 11 females) and 49 convalescent (median age of 57 years, range 22–76 years; 17 females) COVID-19 patients from VIC, Australia (Figure 1a) and as described before.[11] We compared this cohort of COVID-19 patients with 21 healthy controls (HCs) with no history of COVID-19. We observed significantly decreased total γδ T cell frequencies in convalescent patients compared with HCs (Figure 1b), and in line with previous findings.[12] We then examined T cell memory phenotypes of γδ T cells in our cohorts. We divided γδ T cells into naïve-like, central memory (CM), effector memory (EM), and terminally differentiated CD45RA-expressing EM (TEMRA) subsets based on CD27 and CD45RA expression using high dimensional FFT-accelerated interpolation-based t-SNE (FItSNE) dimensional reduction analyses (Figure 1c). Acute COVID-19 patients presented with significantly lower frequencies of naïve-like cells and CM cells compared with HCs and convalescent patients, respectively, but increased TEMRA frequencies in comparison with both HCs and convalescent patients (Figure 1d). We then examined γδ T cell activation status by measuring CD38 and HLA-DR cell surface expression (Supplementary figure 1a). CD38$^+$HLA-DR$^+$ γδ T cells were significantly increased in acute COVID-19 patients compared with both HCs and convalescent patients (Figure 1e). This activated γδ T cell phenotype was not restricted to a single memory population but increased across all subsets (Supplementary figure 1b). Together, these data demonstrate that acute SARS-CoV-2 infection results in an increased proportion of activated γδ T cells with a TEMRA phenotype.

Individuals with acute SARS-CoV-2 infection have increased circulating effector Vδ1$^+$ and Vδ2$^+$ T cells

Circulating γδ T cells comprise adaptive-like Vδ1$^+$ and innate-like Vδ2$^+$ T cell subsets. We examined whether acute COVID-19 disease was associated with specific subsets and phenotypes of γδ T cells. This was investigated using a multi-parameter flow cytometry panel in a sub-cohort of 18 COVID-19 patients, comprising nine acute (median age of 58 years, range 27–72 years; five females) and nine convalescent donors (median age of 40 years, range 19–74 years; four females; Table 1; Figure 2a). Absolute counts of γδ, Vδ1, and Vδ2 T cells were not different between patient groups (Supplementary figure 1c). As we observed an increase in TEMRA γδ T cell frequencies in acute COVID-19 patients, we investigated whether there was a skewing of effector populations in the γδ T cell subsets between patient groups. In the sub-cohort of acute COVID-19 patients the TEMRA population had significantly higher proportions of Vδ1$^+$ T cells, while Vδ2$^+$ T cell frequencies were not statistically different (Figure 2b). We then focused on effector populations of Vδ1$^+$ and Vδ2$^+$ γδ T cells that we have previously phenotyped in healthy adults.[9,24] CD27^{lo}CX3CR1$^+$ Vδ1$_{effector}$ T cell proportions were significantly increased in acute COVID-19 patients in comparison with the convalescent group (Figure 2c). Conversely, convalescent patients displayed higher frequencies of CD28$^+$CD27^{hi} Vδ1$_{naive}$ T cells compared with acute patients (Supplementary figure 1d). Within the Vδ2$^+$ T cell population, a significantly reduced CD27^{hi} and increased CD27^{lo} cell population was observed in acute COVID-19 patients compared with convalescent patients (Figure 2d). We also assessed the total αβ and CD8$^+$ T cell frequencies within CD3$^+$ T cells and they were not significantly different between both acute and convalescent groups (Supplementary figure 1e). Finally, we correlated γδ T cells and their subsets to age. Vδ1$_{effector}$ frequencies correlated with age, while total γδ

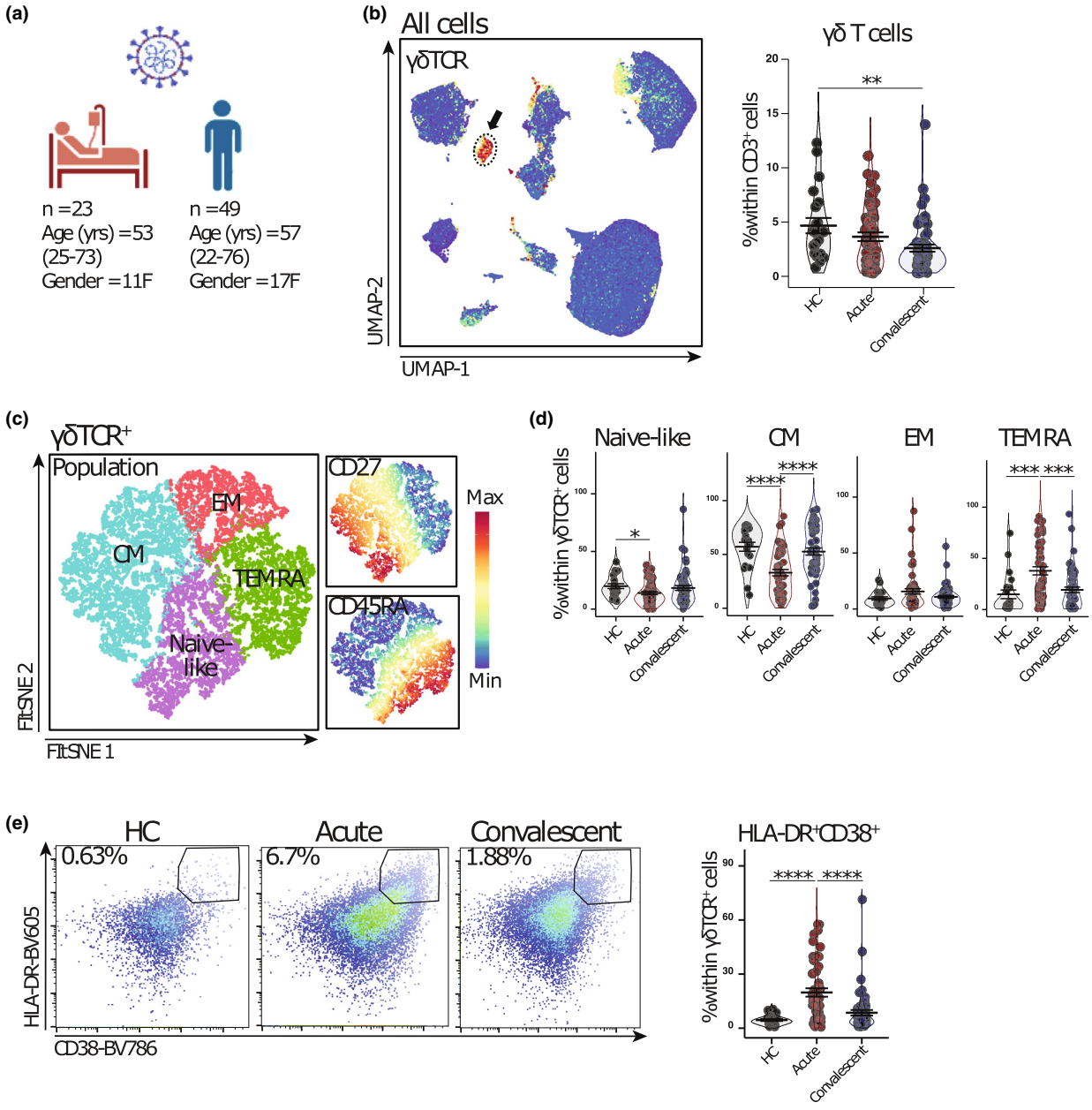

Figure 1. Flow Self-Organizing Map (FlowSOM) analyses of COVID-19 patient blood samples. **(a)** Schematic overview of the demographics of COVID-19 patients in our study sampled during acute disease (left) and at convalescence (right)[11] (created in Biorender). **(b)** UMAP plot of TCRγδ$^+$ cells from whole blood (left panel) and γδ T cell proportions within the CD3$^+$ T cell population in Australian healthy controls (HCs) (black; n = 21), acute (red; n = 23) and convalescent (blue; n = 49) COVID-19 patients. **(c)** FltSNE plots of cell surface expression of CD27 and CD45RA by γδ T cells (right panels). **(d)** Violin plots of naïve-like, CM, EM and TEMRA γδ T cell frequencies for HCs (black; n = 21), and acute (red; n = 23) and convalescent (blue; n = 49) COVID-19 patients. **(e)** Representative FACS plots (left) and summary graph (right) showing HLA-DR$^+$CD38$^+$ γδ T cell frequencies in HCs (black; n = 21), and acute (red; n = 23) and convalescent (blue; n = 49) COVID-19 patients. Statistical comparisons were performed using a Wilcoxon rank-sum test (equivalent to the Mann–Whitney U-test) with the *wilcox.test* function in R. The line is at the mean, and error bars represent SEM. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

T cells, total Vδ2$^+$ and Vδ1$^+$ T cells did not (Supplementary figure 1f). Together, our data suggest that COVID-19 does not alter or drive changes in γδ T cell populations but that the frequency of effector γδ T cell populations was elevated in patients that developed acute COVID-19.

Table 1. Demographic and clinical information

	Acute (n = 9)	Convalescent (n = 9)
Age (years), median (range)	58 (27–72)	40 (19–74)
Gender, *n* Females	5	4
CRP (mg L^{-1}), median (range)	112 (1.3–225)[a]	ND
Days from disease onset, median (range)	10 (6–16)	N/A
Days from ILI to hospitalization, median (range)	7 (5–11)	N/A
Total days in hospital, median (range)	9 (1–31)	N/A
Days from ILI to discharge, median (range)	16 (9–37)	N/A
Oxygen support, *n*	4	N/A

CRP, C-reactive protein; ILI, influenza-like illness; n, number; NH, non-hospitalized.
[a]Not determined for 2 donors.

Increased proportion of γδ T cells with cytolytic potential in COVID-19 patients with acute infection

γδ T cells are known to possess a pre-programmed cytolytic potential which increases after microbial exposure.[10] Cytolytic $V\delta1^+$ T cell frequencies, as a proportion of the entire $GzmA^+GzmB^+$ T cell population, were significantly higher in acute COVID-19 patients compared with convalescent patients, while cytolytic $V\delta2^+$ T cells did not differ (Figure 3a). We observed that $CD27^{lo}$ $V\delta2^+$ T cell proportions are increased in acute COVID-19 patients (Figure 2); thus, we gated on cytolytic cells within $CD27^-$ $V\delta2^+$ T cells to investigate differences between convalescent and acute COVID-19 patients. $GzmB^+CD27^-$ $V\delta2^+$ T cell proportions were significantly higher in acute COVID-19 patients, while $GzmA^+$ cells were not different (Figure 3b). Together these data show that γδ T cell subsets have increased cytolytic potential during acute SARS-CoV-2 infection.

γδ TCR repertoires remain stable after SARS-CoV-2 infection

Acute viral infection with CMV has been reported to drive the clonotypic selection within the γδ TCR repertoire.[9] We investigated whether acute SARS-CoV-2 infection drives changes in the γδ TCR repertoire. Vδ chain usage was skewed between acute and convalescent COVID-19 patients, with acute patient's γδ T cells expressing more Vδ1 chain sequences (Figure 4a). The $V\delta1^+$ (Figure 4b) and $V\delta2^+$ (Figure 4c) γδ TCR repertoires of acute patients showed no difference compared with convalescent patients. This was evident across visual (treeplots) and repertoire metrics (accumulated frequencies of the top 20 clonotypes and D75 index). Subsequently, we longitudinally mapped the top 20 γδ TCR clonotypes in a single acute patient and follow-up convalescent sample 20 days later (Figure 4d). The top 20 γδ TCR clonotypes in this individual were stable and did not change after SARS-CoV-2 infection. Taken together, these data suggest that COVID-19 may not drive the acute selection of specific γδ TCR clonotypes.

DISCUSSION

γδ T cells and their γδ TCR repertoires have been implicated in protective immune responses to viral infections such as CMV and SARS. However, the role of γδ T cell subsets (i.e. Vδ1 and $V\delta2^+$ T cells) in COVID-19 has remained unclear. It is also unknown if and how these subsets and their γδ TCR repertoires change after SARS-CoV-2 infection and associate with COVID-19 severity in unvaccinated patients. Here, we describe an association of increased circulating activated $V\delta1_{effector}$ and $V\delta2_{effector}$ T cells with disease severity in unvaccinated COVID-19 patients. While increased cytotoxic potential within both γδ T cell subsets was found in acute SARS-CoV-2 infection, no γδ TCR selection nor repertoire changes were observed over time.

COVID-19 patients with acute infection were overall lymphopenic and this finding is in line with previously published data.[14,25] Similarly, γδ T cell counts were decreased in patients with acute compared with mild disease.[14,25] Although our results deviate from this finding, likely due to smaller patient cohorts, increased γδ T cell frequencies were found in patients with acute COVID-19 compared with those with mild disease. While γδ T cell frequencies were not restored in convalescent patients, further longitudinal studies should be performed to investigate changes in their frequencies long-term post-COVID-19. γδ T cells, particularly $V\delta1^+$ T cells, are mostly tissue resident and infiltrate tissues such as the lungs. Two studies showed abundant γδ T cell numbers in the lungs and BALF from patients with SARS-CoV-2 infection.[26,27] While these findings should be confirmed in more patients, it is likely that γδ T cells are recruited to and implicated in local immune responses in the lung.

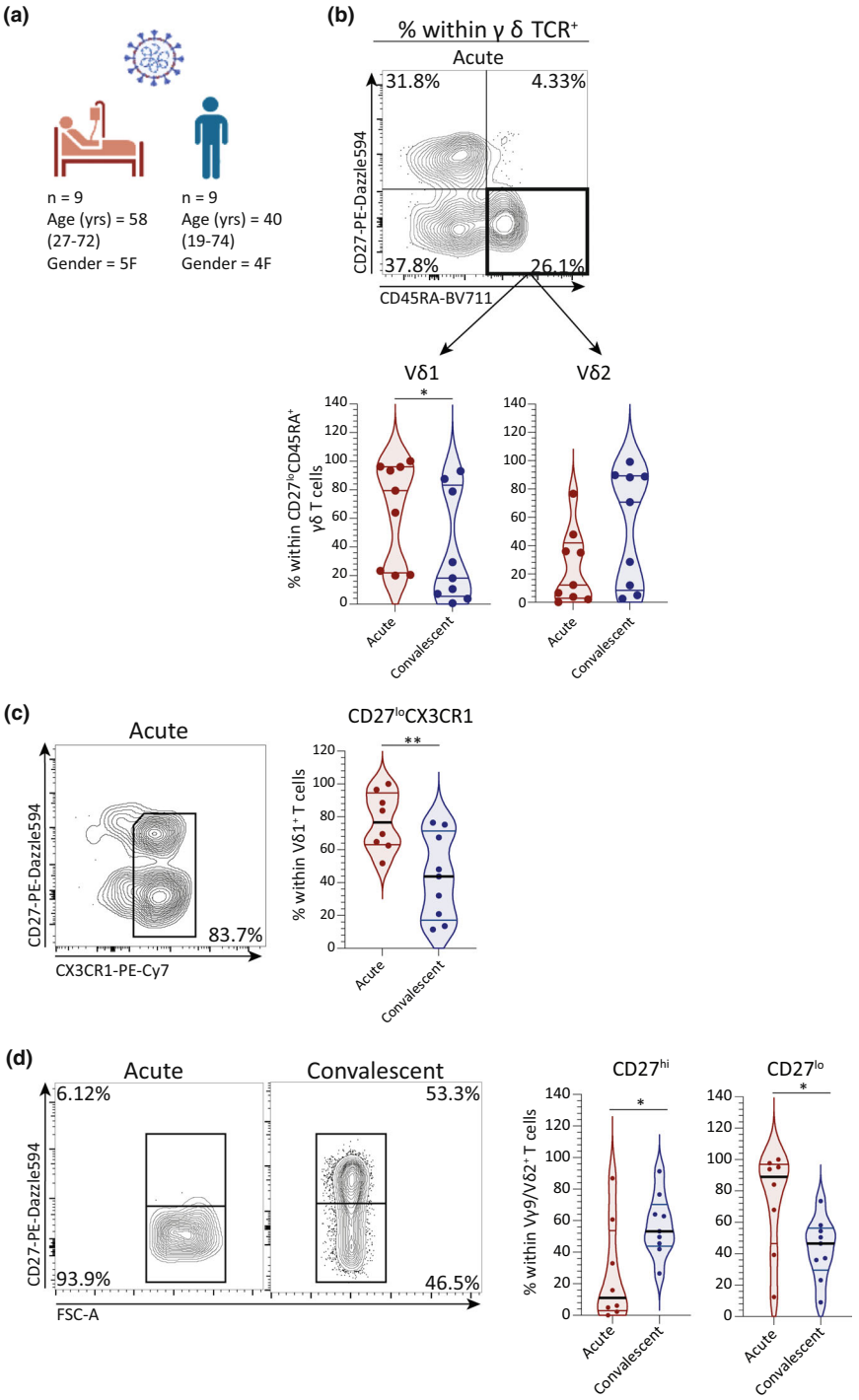

Figure 2. Vδ1$^+$ and Vγ9/Vδ2$^+$ T cell subsets in COVID-19 patients. **(a)** Schematic overview of the number of acute (red) and convalescent (blue) COVID-19 patients included in the second part of the study (created in Biorender). **(b)** Representative plot of the CD27^{lo}CD45RA$^+$ TEMRA γδ T cell population within an acute COVID-19 patient. Violin plots show frequencies of Vδ1$^+$ and Vδ2$^+$ T cells within the TEMRA population for acute (red; $n = 9$) and convalescent (blue; $n = 9$) patients. **(c)** Representative FACS plot gated on CD27^{lo}CX3CR1$^+$ Vδ1$_{effector}$ cells for an acute COVID-19 patient. Violin plot show frequencies of CD27^{lo}CX3CR1$^+$ Vδ1$_{effector}$ cells for acute (red; $n = 8$) and convalescent (blue; $n = 9$) patients. **(d)** Representative FACS plots gated on CD28$^+$CD27$^+$ and CD28$^-$CD27$^-$ Vγ9/Vδ2$^+$ T cells for an acute (left) and convalescent (right) COVID-19 patient. Violin plots show frequencies of both subsets in acute (red; $n = 8$) and convalescent (blue; $n = 9$) patients. Normality was tested using a Shapiro–Wilk test. Normally distributed data were tested using an unpaired *t*-test and non-Gaussian distributed data were tested using a Mann–Whitney *U*-test. *$P < 0.05$, **$P < 0.01$.

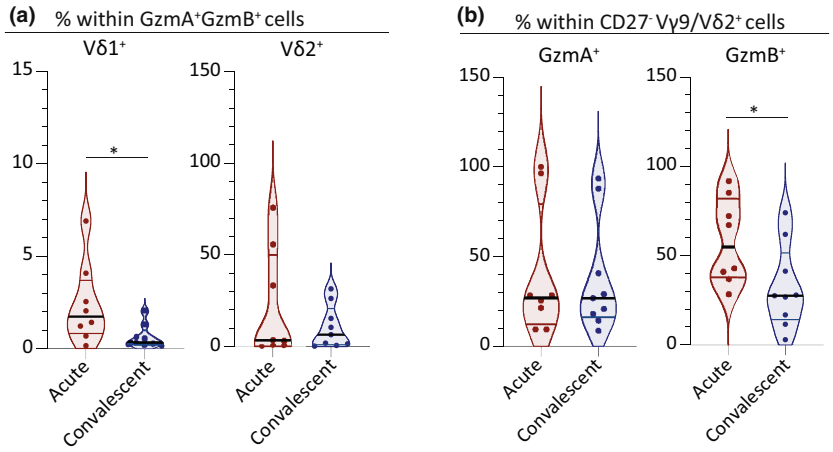

Figure 3. Increased cytolytic potential is found in acute COVID-19 patients. **(a)** Violin plots showing $V\delta1^+$ and $V\delta2^+$ T cell frequencies expressed within the total $GzmA^+GzmB^+$ T cell population within acute (red; $n = 8$) and convalescent (blue; $n = 9$) patients. **(b)** Violin plots of $GzmA^+$ and $GzmB^+$ frequencies gated within $CD27^-$ $V\gamma9/V\delta2_{effector}$ T cells within acute (red; $n = 8$) and convalescent (blue; $n = 9$) patients. Normality was tested using a Shapiro–Wilk test. All data were normally distributed and was further tested using an unpaired *t*-test. $*P < 0.05$.

Acute COVID-19 correlated with an expanded $V\delta1^+$ T cell frequency[25]; however, the $V\delta1^+$ T cells' phenotype was not characterized. In this study, we found an increased TEMRA γδ T cell population in unvaccinated patients with acute COVID-19, which were mainly $V\delta1^+$ T cells. Another study found increased naïve-like and decreased effector γδ T cells, while the TEMRA subset was not different.[14] Although this seems opposite to our findings, this study did not investigate the different γδ T cell subsets and designated naïve and effector cells based on CD45RA and CD62L expression. We observed increased frequencies of $CX3CR1^+$ $V\delta1_{effector}$ cells in patients with acute COVID-19. CX3CR1 plays a role in cell migration as its ligand (CX3CL1) is highly expressed in tissues, including lung tissue.[28,29] In addition, *in vitro* chemotaxis assays have shown that CX3CL1 induced migration of $CX3CR1^+$ cells with cytotoxic potential included γδ T cells.[30] However, there are no *in vivo* experiments demonstrating γδ T cell migration from the blood into tissues specifically in SARS-CoV-2 infection. Moreover, an increased presence of this subset could also predispose COVID-19 patients for acute disease. In our cohorts, $V\delta1_{effector}$ cells correlated with age, supporting earlier findings that $V\delta1^+$ T cells in the cord blood[9] and children[10] were mainly naïve (i.e. $CD27^{hi}$), while HCs had a mixture of naïve and $CD27^{lo}$ effector cells.[9] In addition, only $V\delta1_{effector}$ T cells acquire cytotoxic potential, regardless of age.[10] Interestingly, children (who have a naïve $V\delta1^+$ T cell repertoire) are at lower risk of contracting COVID-19.[31] Alternatively, an increased $V\delta1_{effector}$ population could also be the result of acute infection. It has been shown that $V\delta1^+$ T cells do not respond *in vitro* to SARS-CoV-2 nucleocapsid or spike proteins.[32] Therefore, they may become activated and respond to as yet unknown SARS-CoV-2 related Ags that drives their expansion. Further studies should investigate pre-COVID-19 samples to determine whether γδ T cell subsets predispose patients for acute disease.

There is emerging evidence of the adaptive functions of $V\delta1^+$ T cells in viral infections, which are similar to conventional αβ T cells.[33] While clonally expanded γδ TCRs were present in COVID-19 patients, we did not detect differences between acute and convalescent patient groups. Moreover, transition into convalescent disease in a single patient did not result in notable changes in their top 20 clonotypes. However, this may be due to the limited patient samples studied, as a recently published study did find clonotypic focusing of TRDV1 in a larger cohort of COVID-19 patients.[16] Here, TRDV1 γδ TCR clonotypic focusing was sustained over ~9 days after the first positive SARS-CoV-2 test, albeit only in patients aged > 50 years. No association with disease severity was found. The TRDV1 TCR repertoire was private and no specific CDR3 sequences were associated with SARS-CoV-2 exposure. The authors speculated that expanded TRDV1 TCRs are not specific for pathogen-derived Ags but respond to stress induced ligands.[34] Another option could be a response to cytokine stimulation, as conventional CD8 αβ T cells can expand clonally in response to IL-12 and IL-18.[34] Acute COVID-19 is associated with cytokine storms of mainly IL-6 and TNFα, but patients also showed elevated IL-12 levels.[35] It has been shown previously that *in vitro* stimulation of $CD27^{lo}$ $V\delta1^+$ T cells (which contained a set of focused

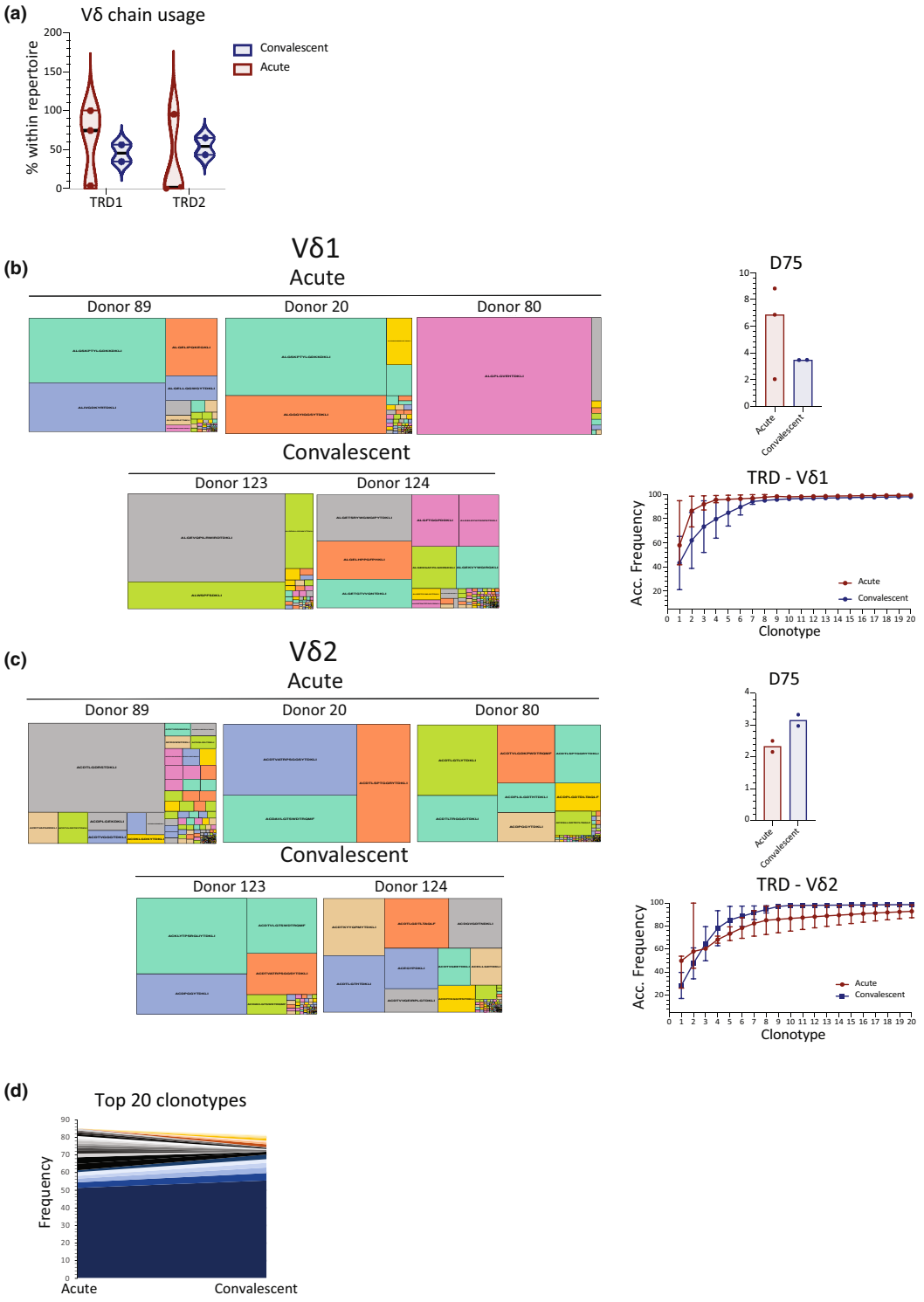

Figure 4. The γδ TCR repertoire in acute and convalescent COVID-19 patients. **(a)** Vδ chain usage in acute (red; *n* = 3) and convalescent (blue; *n* = 2) COVID-19 patients. Treeplots, accumulated frequencies of the top 20 clonotypes, and D75 is shown in acute (red; *n* = 3 or 2) and convalescent (blue; *n* = 2) COVID-19 patients for TRD from **(b)** Vδ1$^+$ and **(c)** Vδ2$^+$ T cells. Each color in the treeplots represents a different γδ TCR clonotype and the size represents the number of reads for each clonotype. **(d)** Sharing of the top 20 γδ TCR clonotypes in an acute COVID-19 patient (*n* = 1) during acute and convalescent disease state. Blue scale = expanding clonotypes, Gray scale = contracting clonotypes, Orange-Yellow scale = new clonotypes.

γδ TCR clones) with IL-15 resulted in proliferation of several clones.[9] While no differences in IL-15 levels have been found in COVID-19, increased levels were reported in patients with Middle East respiratory syndrome coronavirus (MERS-CoV) compared with controls.[36] Future studies should sequence $V\delta1^+$ TCR repertoires from COVID-19 patients with varying disease intensities and pre-COVID-19 samples to elucidate whether and how cytokines and/or SARS-CoV-2 drives changes herein.

$V\delta2^+$ T cells are involved in immune responses, particularly against microbes mainly *via* recognition of their Ag HMBPP. While no associations of $V\delta2^+$ T cells with disease severity in COVID-19 were found in our study, reduced cell counts were found in acute COVID-19.[25] Nevertheless, they have been implicated in protective immune responses in COVID-19 as patients who did not survive the infection had lower $V\delta2^+$ T cell counts than those who survived.[37] $V\delta2^+$ T cells may have direct anti-viral potential in COVID-19 as they rapidly activated (i.e. increased CD69 expression and IFN-γ production) in PBMC cultures stimulated with influenza A.[38] Inhibition of the mevalonate pathway and isopentyl pyrophosphate (IPP; i.e. mammalian equivalent of HMBPP) synthesis in PBMCs significantly reduced IFN-γ production by $V\delta2^+$ T cells in response to influenza A. In addition, another study confirmed these results and also showed that these cells had enhanced cytolytic potential, killed influenza-infected cells (A and B) and utilized a public Vγ9 TCR CDR3 paired to various Vδ2 CDR3s.[39] Transfected Vδ2 TCRs from COVID-19 patients activated in response to epitopes that were present on non-structural protein 8 (NSP8), a protein encoded in the SARS-CoV-2 genome.[40] However, we did not find any evidence of disease severity being associated with expansions of the similar TCRs in COVID-19. We showed that $CD27^-$ $V\delta2_{effector}$ T cell proportions correlated with disease severity. They are composed of a larger $GzmB^+$ population, which is in line with previously published data.[41] These cells were also more responsive to HMBPP stimulation in this study. Together this suggests that while $V\delta2^+$ T cells may not directly recognize virus infected cells, infection does have the potential to induce IPP accumulation and to drive the $V\delta2^+$ T cell response.

A limitation of this study is that we were unable to include a pre-COVID-19 sample, and thus cannot compare with a baseline measurement how COVID-19 changes the γδ T cell population. Future studies should aim to investigate γδ T cell subsets in a pre-COVID-19 sample and also longitudinally in patients with both acute and mild acute COVID-19 to study whether disease severity is associated with long-term changes in the γδ T cell compartment. Another limitation is that tissue-resident γδ T cells were not analyzed. As $V\delta2^-$ γδ T cell subsets are mostly tissue resident, and SARS-CoV-2 initially infects the mucosal lining of the respiratory tract,[2] tissue infiltrating γδ T cells should be studied in the context of COVID-19 to detect tissue-specific effects of the infection and association with disease severity. Other avenues that could also be explored are the potentially long-term impacts of one or multiple vaccinations against COVID-19, and infection with different SARS-CoV-2 variants, on the γδ T cell population.

In summary, an association was found between disease severity and activated TEMRA γδ T cells. These cells consisted mainly of cytotoxic $V\delta1_{effector}$ T cells in patients with acute SARS-CoV-2 infection. Moreover, $V\delta2_{effector}$ T cells with cytotoxic potential were increased in acute COVID-19. Our study suggests that γδ T cell subsets may predispose patients for acute disease; however, further research should be performed to investigate whether vaccination against COVID-19 changes the γδ T cell compartment to promote mild disease.

METHODS

Cohort and ethics statement

Acute and convalescent COVID-19 patients were recruited with written consent *via* the University of Melbourne (#2057366, #2056901, #2056689, #2056761, #1442952, #1955465, and #1443389), Austin Hospital (HREC/63201/Austin-2020) and Alfred Hospital (#280/14), as previously described,[3] in accordance with the Declaration of Helsinki Principles and the Australian National Health and Medical Research Council Code of Practice (Table 1). Healthy pre-pandemic buffy packs were obtained from the Australian Red Cross LifeBlood (2015#8, West Melbourne, Australia; Table 1). PBMCs or flow-through fractions following tetramer-associated magnetic enrichment (TAME) from our previous study[3] were used for the assessment of γδ T cell subsets in this study and were selected based on available PBMCs (MUHREC #26340).

Peripheral blood mononuclear cell isolation

Peripheral blood was layered over Ficoll-Paque (GE Healthcare, Chicago, IL, USA) or lymphoprep (STEMCELL Technologies, Vancouver, Canada) and centrifuged for 20 min at 600–800× g at room temperature without brake. The PBMC layer was removed and washed twice in Roswell Memorial Park Institute (RPMI) 1640 medium for 10 min at 400× *g* at 4°C. Cells were frozen at −80°C in fetal calf serum (FCS) and 10% dimethyl sulfoxide (both Sigma-Aldrich, St Louis, MO, USA) and stored in liquid nitrogen.

Computational flow cytometry analysis

Computational analysis of γδ T cell data was performed on previously published dataset of healthy and COVID-19 acute

and convalescent blood samples[11] using FlowSOM and the Specter R package[42] (https://github.com/ImmuneDynamics/Spectre), as described previously in detail.[11] A gating strategy is provided in Supplementary figure 2a. Cellular expression of CD27, CD45RA, CD38 and HLA-DR was determined on the γδ T cell cluster and plotted by FltSNE plots for Figure 1. Subsets of γδ T cells included naïve-like $CD45RA^{+}CD27^{+}$, TEMRA $CD45RA^{+}CD27^{-}$, CM $CD45RA^{-}CD27^{+}$ and EM $CD45RA^{-}CD27^{-}$ subsets were evaluated for CD38 and HLA-DR expression using manual gating in FlowJo. Volcano plots were created in R, where comparisons were performed using a Wilcoxon rank-sum test (equivalent to the Mann–Whitney *U*-test) with the *wilcox.test* function in R. Statistics displayed in volcano plots were corrected with a False Discovery Rate (FDR) adjustment.

Antibodies and flow cytometry

Staining for γδ T cell subsets was performed as described previously.[43] In short, frozen PBMCs were thawed and washed twice in PBS. The cells were first stained with Zombie Aqua (1:500 in PBS; BioLegend, San Diego, CA, USA), washed, and then split and stained with both the γδ T cell and Gzm panel (Supplementary table 2). Intracellular staining continued by fixing and permeabilizing PBMCs using the Foxp3/Transcription factor staining buffer set (eBioscience, San Diego, CA, USA) and incubation with intracellular antibodies diluted in permeabilization buffer. An acute COVID-19 patient had to be excluded from subsequent γδ T cell subset and Gzm analyses as no anti-CD27 antibody was added and no Gzm panel staining was performed. Absolute cell counts were quantified in a selection of samples (n = 2 acute and n = 5 convalescent COVID-19 patients) using accucheck counting beads (Thermo Fisher Scientific, Waltham, MA, USA). A representative gating strategy is shown in Supplementary figure 2b. All samples were acquired on a Fortessa X20 flow cytometer and resulting FCS files were analyzed using FlowJo v10 (both BD Biosciences, Franklin Lakes, NJ, USA).

Bulk cell sorting and RNA-based TCR repertoire analysis

Part of the PBMCs from three acute (for one donor also a convalescent sample was analyzed) and two convalescent COVID-19 patients were stained for bulk sorting as described previously.[10] In short, PBMCs were stained with Zombie Aqua dye (BioLegend) and then incubated with antibodies indicated in the bulk sort panel (Supplementary table 1) for 20 min on ice. The cells were then bulk sorted according to the sort strategy described in Supplementary figure 2c into RNA*later* (Sigma Aldrich) using a FACS ARIA II Fusion (BD Biosciences). Total cell numbers sorted for each donor are given in Supplementary table 2. RNA was purified using a RNeasy plus micro kit (Qiagen, Hilden, Germany) and the human TCRδ chain iR profile kits (iRepertoire Inc., Huntsville, AL, USA) were used to perform amplicon rescued multiplex (ARM)-PCR to generate complementarity determining region (CDR) 3 libraries for sequencing following the manufacturer's instructions. Sequencing was performed using an Illumina MiSeq (Monash Health Translation Precinct Medical Genomics Facility, Clayton, VIC, Australia). We used iRweb tools (iRepertoire Inc.) to assign CDR3 sequences, variable (V), diversity (D), and junction (J) gene usage, calculate diversity indexes (DI; this metric considers the clonal frequency to occupy 50% of the total repertoire (D50) and abundance of unique CDR3 sequences (Shannon entropy)) and plot tree maps.

Statistical analysis

Tabulated data were plotted and analyzed in GraphPad PRISM 9 (GraphPad Software, San Diego, CA, USA). Each data set was assessed for normality using Shapiro–Wilk normality test. Differences between cohorts were analyzed by an unpaired *t*-test for normally distributed data or a Mann–Whitney *U*-test for non-parametric data. Correlations were analyzed using a Pearson r-test. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$.

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AUTHOR CONTRIBUTIONS

Anouk von Borstel: Conceptualization; data curation; formal analysis; investigation; visualization; writing – original draft. **Thi HO Nguyen:** Conceptualization; data curation; formal analysis; investigation; visualization. **Louise C Rowntree:** Conceptualization; data curation; formal analysis. **Thomas M Ashhurst:** Data curation; formal analysis; investigation; visualization. **Lilith F Allen:** Resources. **Lauren J Howson:** Investigation. **Natasha E Holmes:** Resources. **Olivia C Smibert:** Resources. **Jason A Trubiano:** Resources. **Claire L Gordon:** Resources. **Allen C Cheng:** Resources. **Stephen J Kent:** Funding acquisition; resources. **Jamie Rossjohn:** Conceptualization; supervision. **Katherine Kedzierska:** Conceptualization; funding acquisition; project administration; supervision. **Martin S Davey:** Conceptualization; funding acquisition; project administration; supervision; formal analysis; writing – original draft.

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CONFLICT OF INTEREST

The authors declare no competing interests.

DATA AVAILABILITY STATEMENT

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

REFERENCES

1. WHO. WHO Coronavirus (COVID-19) Dashboard; 2023. https://covid19.who.int/. Accessed January 2023.
2. Hu B, Guo H, Zhou P, Shi ZL. Characteristics of SARS-CoV-2 and COVID-19. *Nat Rev Microbiol* 2021; **19**: 141–154.
3. Nguyen THO, Rowntree LC, Petersen J, *et al.* $CD8^+$ T cells specific for an immunodominant SARS-CoV-2 nucleocapsid epitope display high naive precursor frequency and TCR promiscuity. *Immunity* 2021; **54**: 1066–1082.
4. Li X, Geng M, Peng Y, Meng L, Lu S. Molecular immune pathogenesis and diagnosis of COVID-19. *J Pharm Anal* 2020; **10**: 102–108.
5. Huang C, Wang Y, Li X, *et al.* Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet* 2020; **395**: 497–506.
6. Morita CT, Jin C, Sarikonda G, *et al.* Nonpeptide antigens, presentation mechanisms, and immunological memory of human Vγ2Vδ2 T cells: discriminating friend from foe through the recognition of prenyl pyrophosphate antigens. *Immunol Rev* 2007; **215**: 59–76.
7. Hintz M, Reichenberg A, Altincicek B, *et al.* Identification of (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate as a major activator for human γδ T cells in *Escherichia coli*. *FEBS Lett* 2001; **509**: 317–322.
8. Hayday AC. γδ T cell update: adaptate orchestrators of immune surveillance. *J Immunol* 2019; **203**: 311–320.
9. Davey MS, Willcox CR, Joyce SP, *et al.* Clonal selection in the human Vδ1 T cell repertoire indicates γδ TCR-dependent adaptive immune surveillance. *Nat Commun* 2017; **8**: 1–15.
10. von Borstel A, Chevour P, Arsovski D, *et al.* Repeated *Plasmodium falciparum* infection drives clonal expansion of an adaptive human γδ T cell repertoire. *Sci Transl Med* 2021; **13**: eabe7430.
11. Koutsakos M, Rowntree LC, Hensen L, *et al.* Integrated immune dynamics define correlates of COVID-19 severity and antibody responses. *Cell Rep Med* 2021; **2**: 100208.
12. Jouan Y, Guillon A, Gonzalez L, *et al.* Phenotypical and functional alteration of unconventional T cells in severe COVID-19 patients. *J Exp Med* 2020; **217**: e20200872.
13. Lei L, Qian H, Yang X, *et al.* The phenotypic changes of γδ T cells in COVID-19 patients. *J Cell Mol Med* 2020; **24**: 11603–11606.
14. Odak I, Barros-Martins J, Bošnjak B, *et al.* Reappearance of effector T cells is associated with recovery from COVID-19. *EBioMedicine* 2020; **57**: 102885.
15. Carissimo G, Xu W, Kwok I, *et al.* Whole blood immunophenotyping uncovers immature neutrophil-to-VD2 T-cell ratio as an early marker for severe COVID-19. *Nat Commun* 2020; **11**: 5243.
16. Joseph M, Wu Y, Dannebaum R, *et al.* Global patterns of antigen receptor repertoire disruption across adaptive immune compartments in COVID-19. *Proc Natl Acad Sci USA* 2022; **119**: e2201541119.
17. Ravens S, Schultze-Florey C, Raha S, *et al.* Human γδ T cells are quickly reconstituted after stem-cell transplantation and show adaptive clonal expansion in response to viral infection. *Nat Immunol* 2017; **18**: 393–401.
18. Déchanet J, Merville P, Lim A, *et al.* Implication of γδ T cells in the human immune response to cytomegalovirus. *J Clin Invest* 1999; **103**: 1437–1449.
19. Couzi L, Pitard V, Netzer S, *et al.* Common features of γδ T cells and $CD8^+$ αβ T cells responding to human cytomegalovirus infection in kidney transplant recipients. *J Infect Dis* 2009; **200**: 1415–1424.
20. Couzi L, Pitard V, Sicard X, *et al.* Antibody-dependent anti-cytomegalovirus activity of human γδ T cells expressing CD16 (FcγRIIIa). *Blood* 2012; **119**: 1418–1427.
21. Couzi L, Pitard V, Moreau JF, Merville P, Déchanet-Merville J. Direct and indirect effects of cytomegalovirus-induced γδ T cells after kidney transplantation. *Front Immunol* 2015; **6**: 3.
22. Lafarge X, Merville P, Cazin MC, *et al.* Cytomegalovirus infection in transplant recipients resolves when circulating γδ T lymphocytes expand, suggesting a protective antiviral role. *J Infect Dis* 2001; **184**: 533–541.
23. Poccia F, Agrati C, Castilletti C, *et al.* Anti-severe acute respiratory syndrome coronavirus immune responses: The role played by Vγ9Vδ2 T cells. *J Infect Dis* 2006; **193**: 1244–1249.
24. Davey MS, Willcox CR, Hunter S, *et al.* The human $V\delta2^+$ T-cell compartment comprises distinct innate-like $V\gamma9^+$ and adaptive $V\gamma9^-$ subsets. *Nat Commun* 2018; **9**: 1760.
25. Laing AG, Lorenc A, Del Molino Del Barrio I, *et al.* A dynamic COVID-19 immune signature includes associations with poor prognosis. *Nat Med* 2020; **26**: 1623–1635.

26. Chen XJ, Li K, Xu L, *et al.* Novel insight from the first lung transplant of a COVID-19 patient. *Eur J Clin Invest* 2021; **51**: e13443.
27. Cerapio JP, Perrier M, Pont F, *et al.* Single-cell RNAseq profiling of human γδ T lymphocytes in virus-related cancers and COVID-19 disease. *Viruses* 2021; **13**: 2212.
28. Uhlén M, Fagerberg L, Hallström BM, *et al.* Tissue-based map of the human proteome. *Science* 2015; **347**: 1260419.
29. Karlsson M, Zhang C, Méar L, *et al.* A single-cell type transcriptomics map of human tissues. *Sci Adv* 2021; **7**: eabh2169.
30. Nishimura M, Umehara H, Nakayama T, *et al.* Dual functions of Fractalkine/CX3C ligand 1 in trafficking of Perforin$^+$/Granzyme B$^+$ cytotoxic effector lymphocytes that are defined by CX3CR1 expression. *J Immunol* 2002; **168**: 6173–6180.
31. Zimmermann P, Curtis N. Why is COVID-19 less severe in children? A review of the proposed mechanisms underlying the age-related difference in severity of SARS-CoV-2 infections. *Arch Dis Child* 2021; **106**: 429–439.
32. Singh K, Cogan S, Elekes S, *et al.* SARS-CoV-2 spike and nucleocapsid proteins fail to activate human dendritic cells or γδ T cells. *PLoS One* 2022; **17**: e0271463.
33. Davey MS, Willcox CR, Baker AT, Hunter S, Willcox BE. Recasting human Vδ1 lymphocytes in an adaptive role. *Trends Immunol* 2018; **39**: 446–459.
34. Raué HP, Beadling C, Haun J, Slifka MK. Cytokine-mediated programmed proliferation of virus-specific CD8$^+$ memory T cells. *Immunity* 2013; **38**: 131–139.
35. Hu B, Huang S, Yin L. The cytokine storm and COVID-19. *J Med Virol* 2021; **93**: 250–256.
36. Mahallawi WH, Khabour OF, Zhang Q, Makhdoum HM, Suliman BA. MERS-CoV infection in humans is associated with a pro-inflammatory Th1 and Th17 cytokine profile. *Cytokine* 2018; **104**: 8–13.
37. Rijkers G, Vervenne T, van der Pol P. More bricks in the wall against SARS-CoV-2 infection: involvement of γ9δ2 T cells. *Cell Mol Immunol* 2020; **17**: 771–772.
38. Jameson JM, Cruz J, Costanzo A, Terajima M, Ennis FA. A role for the mevalonate pathway in the induction of subtype cross-reactive immunity to influenza a virus by human γδ T lymphocytes. *Cell Immunol* 2010; **264**: 71–77.
39. Sant S, Jenkins MR, Dash P, *et al.* Human γδ T-cell receptor repertoire is shaped by influenza viruses, age and tissue compartmentalisation. *Clin Transl Immunol* 2019; **8**: e1079.
40. Du B, Guo Y, Li G, Zhu Y, Wang Y, Xi X. Non-structure protein ORF1ab (NSP8) in SARS-CoV-2 contains potential γδT cell epitopes. *Front Microbiol* 2022; **13**: 936272.
41. Ryan PL, Sumaria N, Holland CJ, *et al.* Heterogeneous yet stable Vδ2$^+$ T-cell profiles define distinct cytotoxic effector potentials in healthy human individuals. *Proc Natl Acad Sci USA* 2016; **113**: 14378–14383.
42. Ashhurst TM, Marsh-Wakefield F, Putri GH, *et al.* Integration, exploration, and analysis of high-dimensional single-cell cytometry data using spectre. *Cytometry A* 2021; **101**: 237–253.
43. Cossarizza A, Chang H, Radbruch A, *et al.* Guidelines for the use of flow cytometry and cell sorting in immunological studies (third edition). *Eur J Immunol* 2021; **51**: 2708–3145.

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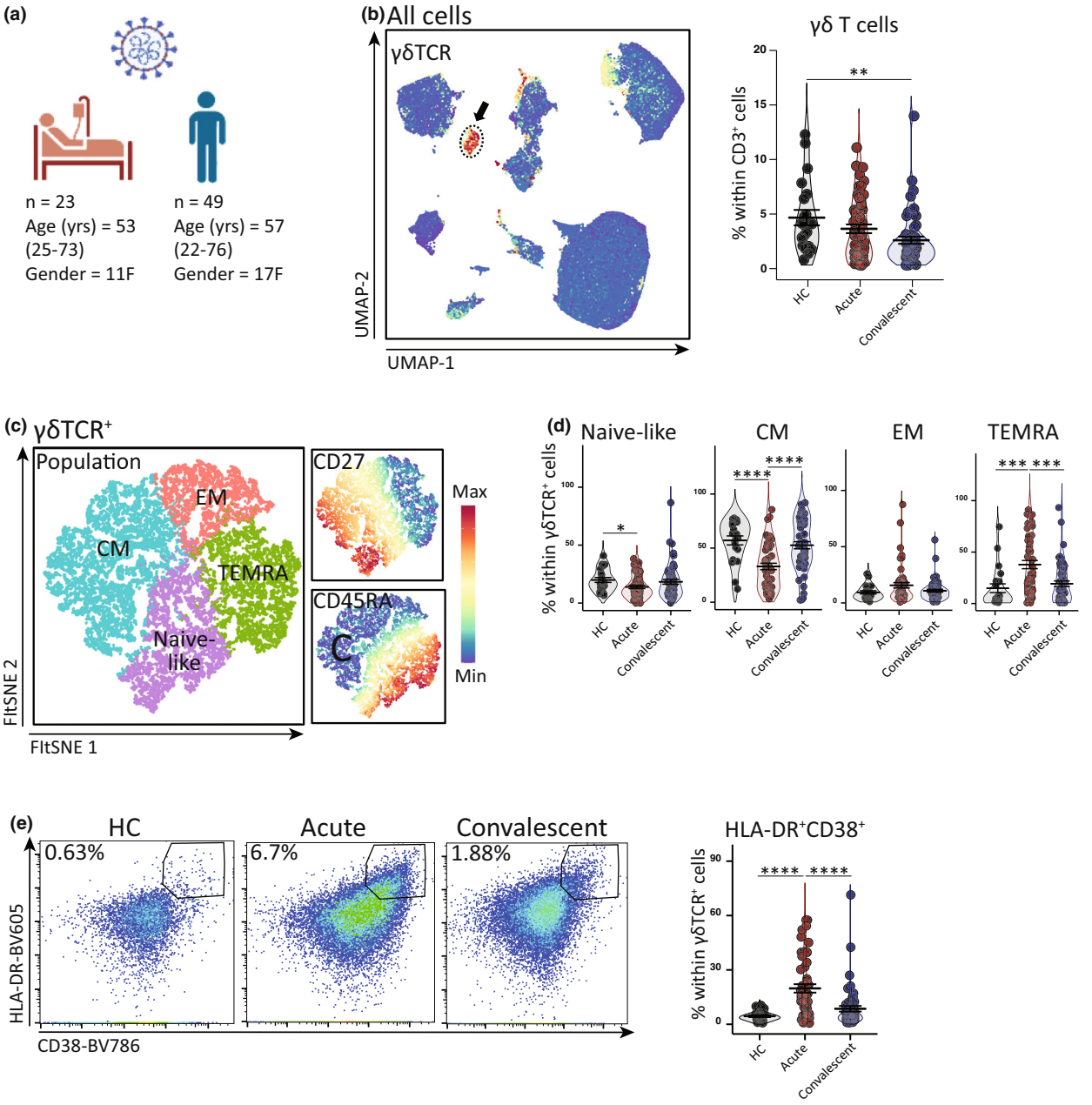

The immune system plays a role in controlling SARS-CoV-2 but our understanding of the immune compartments that contribute to COVID19 disease are incomplete. In this study we show that populations of effector gamma delta T cells are associated with acute COVID-19 in unvaccinated patients.