Contents lists available at ScienceDirect

EBioMedicine

Research paper

Reappearance of effector T cells is associated with recovery from COVID-19

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ARTICLE INFO

ABSTRACT

Background: Elucidating the role of T cell responses in COVID-19 is of utmost importance to understand the clearance of SARS-CoV-2 infection.

Methods: 30 hospitalized COVID-19 patients and 60 age- and gender-matched healthy controls (HC) participated in this study. We used two comprehensive 11-colour flow cytometric panels conforming to Good Laboratory Practice and approved for clinical diagnostics.

Findings: Absolute numbers of lymphocyte subsets were differentially decreased in COVID-19 patients according to clinical severity. In severe disease (SD) patients, all lymphocyte subsets were reduced, whilst in mild disease (MD) NK, NKT and $\gamma\delta$ T cells were at the level of HC. Additionally, we provide evidence of T cell activation in MD but not SD, when compared to HC. Follow up samples revealed a marked increase in effector T cells and memory subsets in convalescing but not in non-convalescing patients.

Interpretation: Our data suggest that activation and expansion of innate and adaptive lymphocytes play a major role in COVID-19. Additionally, recovery is associated with formation of T cell memory as suggested by the missing formation of effector and central memory T cells in SD but not in MD. Understanding T cell-responses in the context of clinical severity might serve as foundation to overcome the lack of effective anti-viral immune response in severely affected COVID-19 patients and can offer prognostic value as biomarker for disease outcome and control.

Funding: Funded by State of Lower Saxony grant 14−76,103−184CORONA-11/20 and German Research Foundation, Excellence Strategy − EXC2155"RESIST"−Project ID39087428, and DFG-SFB900/3−Project ID158989968, grants SFB900-B3, SFB900-B8.

1. Introduction

Cross-species infections are becoming more common due to increase in interlinking human and animal activities, furthered by great genetic diversity of viruses [1]. The threat of emerging global pandemics by new virus species and/or mutants has been significantly heightened in the wake of severe acute respiratory syndrome coronavirus (SARS-CoV) [2] and Middle East respiratory syndrome coronavirus (MERS-CoV) [3] outbreaks. Both of these zoonotic coronaviruses are highly infectious and have high fatality rates [4]. In December 2019, another type of coronavirus, later named SARS-CoV-2, was reported in patients with pneumonia, causing coronavirus disease 2019 (COVID-19) [5]. SARS-CoV-2 has since turned into a global pandemic with a total of 9 110 186 confirmed cases of COVID-19 and

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473 061 fatal cases as of June 24th 2020 according to recent WHO situation report [6].

Since the onset of the SARS-CoV-2 pandemic, research focuses on immune profiling of the infection, striving towards better patient management and treatment. As it is the case in the majority of viral infections, key players in battling the SARS-CoV-2 appear to be NK, T and B cells [7,8]. According to available data [9−12], COVID-19 patients with severe disease have increased levels of C-reactive protein and IL-6 as well as augmented neutrophil counts but reduced lymphocyte counts in blood. Moreover, NK cells and $CD8^+$ T cells in COVID-19 patients`appear to be functionally exhausted, indicated by increased expression of NKG2A [8] and lower production of IFN-γ, TNF-α and IL-2 [13]. Nevertheless, it is unclear whether and how profiling of T cell responses can be used as prognostic biomarker for disease outcome and control. Furthermore, no data is available on the role of $\gamma\delta$ T cells in anti-SARS-CoV-2 immune responses, although it has been demonstrated that these cells contribute to immunity against SARS-CoV and other viruses [14−16].

In the present study we analysed dynamics of NK, NKT, $\alpha\beta$ and $\gamma\delta$ T cells subsets in the peripheral blood of patients with mild and severe COVID-19 compared to gender- and age-matched controls. To reliably assess major lymphocyte subsets' profiles during successful immune response against SARS-CoV-2 infection, we developed two comprehensive Good Laboratory Practice (GLP)-conforming 11-colour flow cytometric panels approved for clinical diagnostics. Using those panels, we examined the composition of seven major lymphocyte populations in patients with mild and severe COVID-19 and

followed formation of effector and memory $\alpha\beta$ and $\gamma\delta$ T cells from consecutive blood samples of patients who did or did not clinically improve. We found that recovery from COVID-19 was closely associated with expansion and differentiation/maturation in $\alpha\beta$, but not $\gamma\delta$ T cells.

2. Materials and methods

2.1. Study participants

Patients with PCR-confirmed SARS-CoV-2 infection were recruited at Hannover Medical School from March 30th until April 16th 2020. Based on the clinical presentation, disease was classified as "mild" or "severe" for every single patient at admission. Mild disease was defined for patients with stable lung parameters with no oxygen flow or of up to 3 litres per minute. In contrast, severe disease was defined as oxygen flow equal or greater than 6 litres per minute to maintain a $SpO_2 > 90\%$, or non-invasive or invasive ventilation. Patient characteristics are shown in Table 1. To assess the impact of infection on lymphocyte subsets, age- and gender-matched healthy controls (HC) were selected for every patient in a 2:1 control-to-patient ratio. Those patients of 56 years of age and older were gender-matched to the group of 56−69 year old healthy controls. Healthy controls were recruited through the Institute of Transfusion Medicine in October and November 2019, prior to SARS-CoV-2 outbreak. Healthy control characteristics are listed in Supplementary Table 1. The study was approved by the institutional review board at Hannover Medical School (#9001_BO_K2020 and #8606_BO_K2019) and informed consent was obtained from all patients and healthy controls.

2.2. Flow cytometry

EDTA-anticoagulated whole blood was stained with the antibodies as listed in Supplementary Table 2. For analysis of absolute cell counts BD TruCount™ tubes were used, and cells were analysed in a lyse-no-wash fashion according to manufacturer's instructions. For analysis of activation markers, cells were analysed in a lyse-wash fashion. Stained cells were acquired on BD FACS Lyric, standardized for clinical cell analysis. Flow cytometry data were analysed using FCSExpress 7 (De Novo Software).

2.3. Statistical analysis

Data were analysed using Prism 7.05 (GraphPad). Patient groups were pairwise compared either to each other or to the reference group of healthy controls. Where data was normally distributed, unpaired t-test was used and where that was not the case, Mann-Whitney U test was applied. For categorical variables Fisher`s exact test was used. For analysis of longitudinal changes two-tailed paired t-test was applied.

3. Results

Our cohort of 30 hospitalized COVID-19 patients was predominantly male (80%), of older age (mean 61 years, range 19−91) and 93% had mostly mild pre-existing medical conditions. Patients were classified upon admission as mild ($n = 15$) and as severe ($n = 15$, Table 1). Patients with severe disease (SD) were slightly older and had a higher proportion of male gender, although both not significantly different from patients with mild disease (MD). Representative of the wider population [17], almost all (28/30) patients in our cohort had pre-existing medical conditions. Characteristics of healthy controls (HC) are listed in Supplementary Table 1.

Table 1
Patients' characteristics.

ID	Severity	Age	Gender	PEMC	cv PEMC	Symptoms	First Sampling*	Con-valescence	Convalescence Sampling*
001	mild	71	m	no	no	Fever, malaise	13	NA	NA
002	mild	68	m	yes	yes	Fever, diarrhoea	13	NA	NA
003	mild	64	m	yes	yes	Malaise	14	NA	NA
012	mild	48	f	yes	no	Fever, dry cough, dyspnoea	8	NA	NA
013	mild	85	m	yes	yes	Fever, dry cough	4	yes	17
014	mild	51	m	yes	yes	Fever, myalgia	16	NA	NA
016	mild	91	m	yes	yes	Dry cough, malaise	9	no	12
019	mild	48	f	yes	no	Dry cough	9	NA	NA
020	mild	57	f	yes	yes	Dyspnoea, myalgia, diarrhoea, emesis	8	NA	NA
021	mild	48	m	yes	yes	Fever, dry cough, myalgia	3	NA	NA
022	mild	75	m	yes	yes	Fever	8	no	18
023	mild	60	m	yes	yes	Dry cough, myalgia, cephalgia	10	NA	NA
027	mild	83	f	yes	yes	Fever, dry cough, dyspnoea, malaise	5	yes	12
028	mild	42	m	yes	no	Nausea, emesis	3	NA	NA
031	mild	79	m	yes	yes	Diarrhoea, nausea, emesis	2	no	7
004	severe	75	f	yes	yes	Fever, dry cough, dyspnoea	7	yes	17
005	severe	60	m	yes	yes	Fever, dry cough	20	NA	NA
006	severe	51	m	yes	no	Fever, diarrhoea, nausea	12	yes	19
007	severe	60	m	yes	no	Fever, dry cough, diarrhoea	16	no	33
008	severe	56	m	yes	yes	Fever, dry cough, dyspnoea	19	no	26
009	severe	68	m	yes	no	Dry cough, diarrhoea	18	yes	25
010	severe	69	m	yes	yes	Dry cough, dyspnoea	21	no	38
011	severe	45	m	yes	yes	Dry cough, dyspnoea	5	no	12
015	severe	47	m	yes	yes	Fever, dry cough, dyspnoea	16	no	31
017	severe	19	m	yes	no	Malaise	5	yes	19
018	severe	82	f	yes	yes	Dry cough	7	NA	NA
025	severe	65	m	yes	yes	Fever, dry cough, dyspnoea	8	yes	16
026	severe	71	m	yes	yes	Fever, dry cough	13	no	18
029	severe	53	m	yes	no	Fever, dry cough, diarrhoea, nausea	8	no	15
030	severe	39	m	no	no	Fever, dyspnoea	32	NA	NA

Abbreviations: PEMC: pre-existing medical conditions, cv: cardio vascular, m: male, f: female, NA: not available.

* Days after onset of symptoms.

3.1. Severe COVID-19 patients show reduced counts of lymphocyte subsets

Using BD TruCount™ technology, we observed significantly reduced lymphocyte numbers in COVID-19 patients, inversely correlated with disease severity (Supplementary Fig. 1).

To further characterize the composition of the lymphocyte pool, we developed two comprehensive GLP-conforming 11-colour comprehensive flow cytometric mAb panels, which were approved in-house for clinical diagnostics. The first panel of mAb used in this study (Supplementary Fig. 2a) allowed us to profile NK, NKT, B, $\alpha\beta$ and $\gamma\delta$ T cell numbers in blood of HC and COVID-19 patients with MD or SD. Compared to MD patients, we found strikingly lower numbers of NK, NKT, $\gamma\delta$-, and CD8$^+$ cells in the blood of SD COVID-19 patients (Fig. 1). $\gamma\delta^+$Vγ9$^+$ cells are reported to be responsive to phospho-antigens [18,19] and we noticed a trend to reduced numbers of $\gamma\delta^+$Vγ9$^+$ in SD patients compared to MD (P = 0.0502, two-tailed Student's t-test), going in hand with recently published data [20]. In addition, all subsets investigated were significantly reduced in SD patients compared to HC (Fig. 1). Interestingly, MD patients had similar levels of NK, NKT, $\gamma\delta$-, conventional CD4$^+$ (CD4$_{conv}$) and CD8$^+$ cells as compared to HC. Of note, both MD and SD patients showed a marked reduction of regulatory T cells (Fig. 1). Together, these data provide a detailed depiction of reduced lymphocyte subsets in mild and severe COVID-19 patients, employing certified counting beads for analysis of absolute cell numbers.

3.2. Patients with severe COVID-19 infection lack generation of effector and central memory $\alpha\beta$ CD4$_{conv}$ and CD8$^+$ cells

To characterize the involvement of different subsets of $\alpha\beta$ CD4$_{conv}$, $\alpha\beta$ CD8$^+$, and $\gamma\delta$ T cells based on their antigen experience [21,22], we developed a staining panel dedicated to identifying four distinct populations based on CD62L and CD45RA expression (Supplementary Fig. 2b). Looking at the distribution of CD45RA$^+$CD62L$^+$ on conventional CD4$^+$ T cells (CD4$_{conv}$) we defined naïve (CD4$_{naïve}$, CD45RA$^+$CD62L$^+$), effector/effector memory (CD4$_{eff/em}$, CD45RA$^-$CD62L$^-$), terminally differentiated cells (CD4$_{temra}$, CD45RA$^+$CD62L$^-$) and central memory (CD4$_{cm}$, CD45RA$^-$CD62L$^+$). Based on this allocation, we observed a marked decrease of CD4$_{eff/em}$ in SD compared to MD patients (Fig. 2a). In addition, both COVID-19 groups had increased frequencies of CD4$_{temra}$ and decreased CD4$_{eff/em}$ compared to HC (Fig. 2a). The same observations were made when absolute cell counts were calculated (Supplementary Fig. 3a). Investigating the memory phenotypes of $\alpha\beta$ CD8$^+$ cells revealed a striking increase in frequencies of CD8$_{eff/em}$ cells, as well as a decrease of CD8$_{naïve}$ T cells in MD compared to HC and SD (Fig. 2b). These observations indicate successful mobilization and/or differentiation of cytotoxic lymphocytes (CTL) in MD patients. Compared to MD, patients with SD had higher frequency of CD8$_{naïve}$ T cells and lower frequency of CD8$_{eff/em}$ cells, implying deficits in successfully mounting CTL responses in SD patients or increased recruitment to the peripheral organs. Furthermore, we found similar frequencies of CD8$_{cm}$ and CD8$_{temra}$ in samples from MD and HC but a decrease of these cell populations in SD (Fig. 2b), again hinting towards an impaired CTL response in patients with SD. The absolute numbers of CD8 memory subsets was changed similarly with the exception of CD8$_{naïve}$ T cells in SD which were similar to those of MD patients (Supplementary Fig. 3b).

3.3. $\gamma\delta$ T cells from COVID-19 patients exhibit a distinct memory signature

Human $\gamma\delta$ T cells have been shown to be important for regulating immune responses to CMV, HCV and SARS-CoV infections [14−16]. Using a panel of 11 specific mAb (Supplementary Fig. 2b), we found that expression of CD45RA and CD62L on $\gamma\delta$ cells would also allow
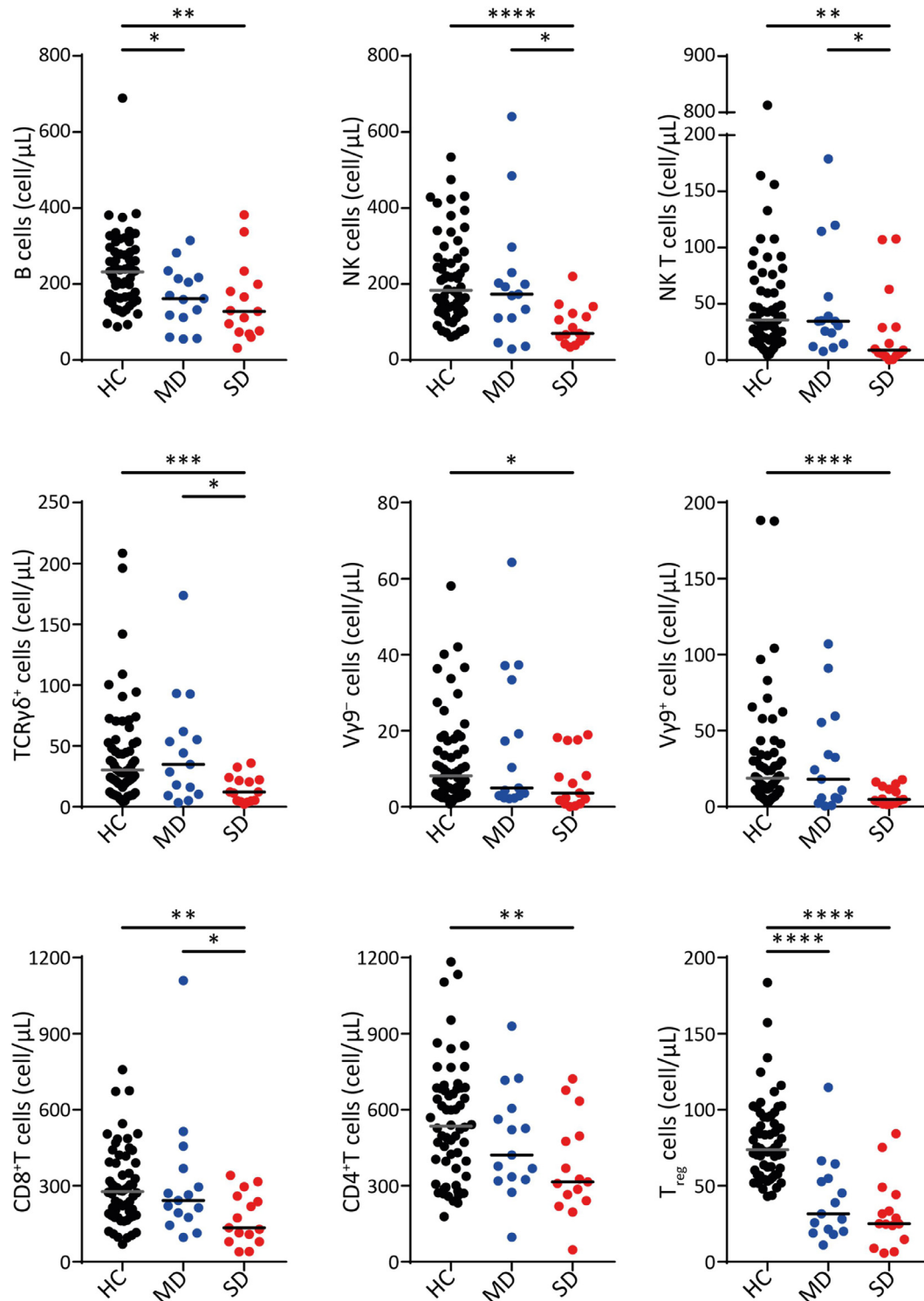
Fig. 1. Absolute numbers of lymphocyte subsets in healthy controls and mild and severe COVID-19 patients.

Lines represent median. Statistical analysis was performed by Mann-Whitney U test or Student's t-test where applicable. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$; ns: not significant; HC: Healthy Control; MD: Mild Disease; SD: Severe Disease.

their categorization into four subsets as defined above for $\alpha\beta$ T cells. Interestingly, we revealed a marked increase in both frequencies and absolute cell numbers of naïve-like $\gamma\delta$ ($\gamma\delta_{naïve-l}$) cells, and a proportional decrease in effector-like $\gamma\delta$ ($\gamma\delta_{eff-l}$) cells in both groups of patients suffering from COVID-19 (Fig. 3). These data suggest that $\gamma\delta_{eff-l}$ T cells might be recruited from the blood to peripheral organs such as the lungs of COVID-19 patients and that these cells might participate in the immune response against SARS-CoV-2 infection.

3.4. Early establishment of T cell memory suggests better clinical outcome

To evaluate the hypothesis that convalescence from COVID-19 is related to establishment of successful T cell immunity, we evaluated the signature of $CD4_{conv}$ and $CD8^+$ cells based on the clinical course of the disease. To that end, we collected longitudinal samples of 5 MD and 12 SD patients. We defined
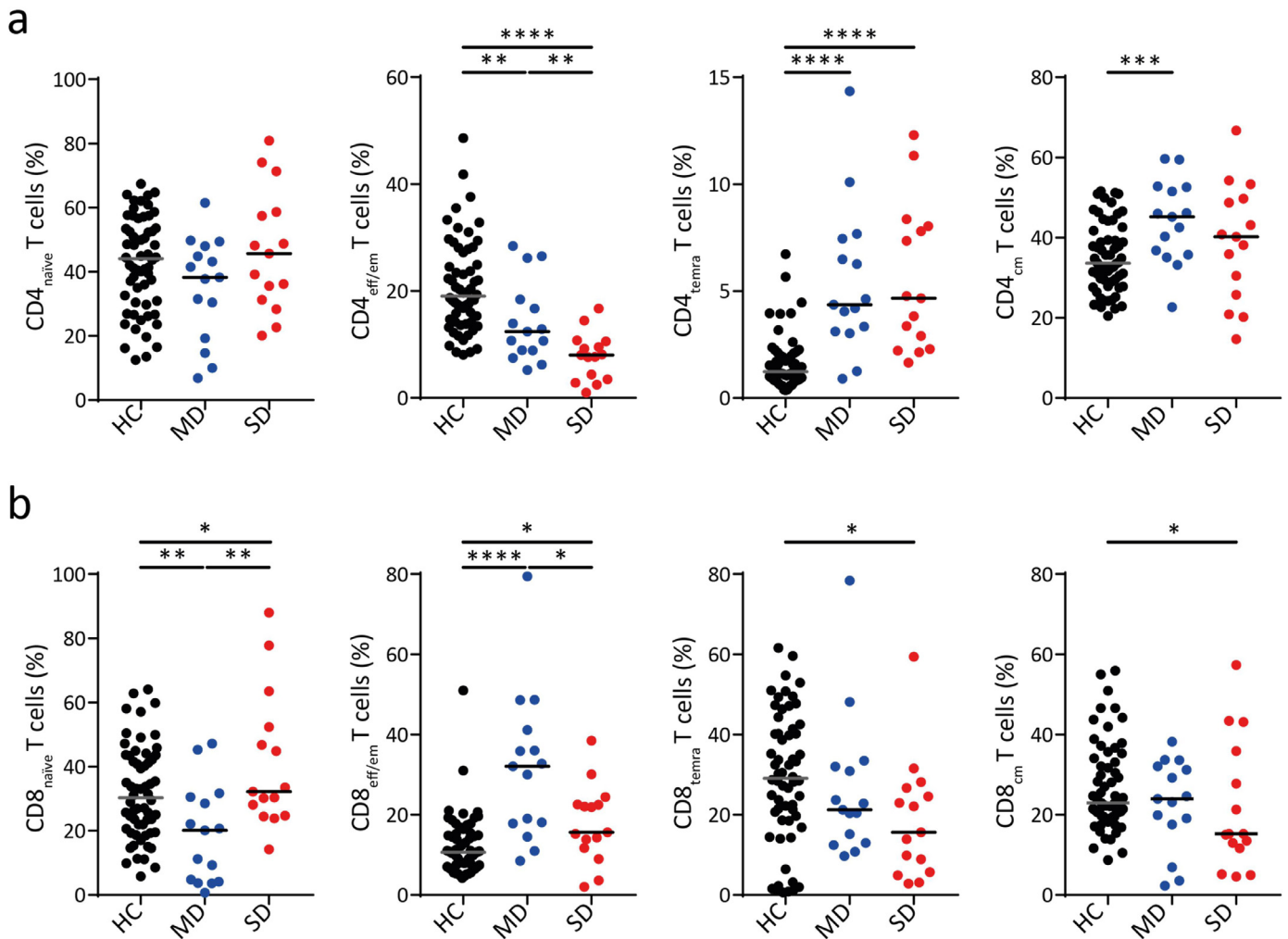
Fig. 2. Mild COVID-19 disease is paralleled by increased levels of effector and memory T cells.
Gated on CD4$_{conv}$ (a). Gated on CD8$^+$ T cells (b). Lines represent median. Statistical analysis was performed by Mann-Whitney U test or Student's t-test where applicable.
$^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$; ns: not significant; HC: Healthy Control. MD: Mild Disease; SD: Severe Disease.

convalescence as an improvement of lung function (e.g. reduction of oxygen flow or ventilation parameters), excluding transient variations, thus only taking long-lasting clinical improvement in consideration. Convalescence rate in MD and SD patients occurred at similar frequencies (2/5 vs 5/12) while the period of follow-up was slightly longer in SD patients without reaching statistical significance (SD: 10.08 vs MD: 7.60 days; Table 1). To analyse the immune responses in a longitudinal manner, we selected convalescing (n = 7) and non-convalescing (n = 10) MD and SD patients. In all convalescing patients irrespective of the disease severity, we observed a strong decrease of the frequency of CD4$_{naïve}$ and a marked increase of CD4$_{eff/em}$ and CD4$_{cm}$ populations (Fig. 4a) suggesting a turnover of naïve to memory/effector cells. The same observations were made for all convalescing patients regarding the relative distribution of naïve and memory CD8$^+$ T cell subsets (Fig. 4b). Notably, no significant changes could be observed for any of the CD4$_{conv}$ and CD8$^+$ subpopulations in non-convalescing patients (Fig. 4). We also failed to observe any significant alterations in frequencies of the different subtypes of $\gamma\delta$ cells in relation to disease clearance (Supplementary Fig. 4). We therefore suggest that the alterations observed for CD4$_{conv}$ and CD8$^+$ $\alpha\beta$ T cells in convalescing patients that recover from COVID-19 are linked to differentiation of effector/memory $\alpha\beta$ T cells.

4. Discussion

From an immunological perspective, adaptive anti-viral responses occur in two layers. First, a NK, NKT and CD8$^+$ T cell response is programmed to prevent the disease to proceed to a severe phase. Second, helper T cells are poised to influence and program B cells for specific neutralizing antibody formation, conferring long-lasting humoral immunity [23]. The establishment of immunity to SARS-CoV-2 has been associated with the presence of neutralizing IgG antibodies in blood sera [24,25]. However, some viruses such as influenza are notoriously known to be resilient to establish humoral immunity [26]. This might also be the case for SARS-CoV-2. We therefore studied changes in T cell subsets in COVID-19 patients.

Applying two comprehensive GLP-conforming 11-colour flow cytometric panels approved for clinical diagnostics, this prospective single-centre study from Germany identified profound changes in absolute numbers in seven different lymphocyte subsets in hospitalized COVID-19 patients with mild and severe disease. These findings were in line with and extended the current literature on COVID-19-induced lymphocytopenia [9–12]. Additionally, we revealed changes in the formation of different T memory cell subsets comparing mildly and severely affected COVID-19 patients with healthy controls. Moreover, in a longitudinal approach we showed that convalescing – but not non-convalescing – patients develop increased frequencies of
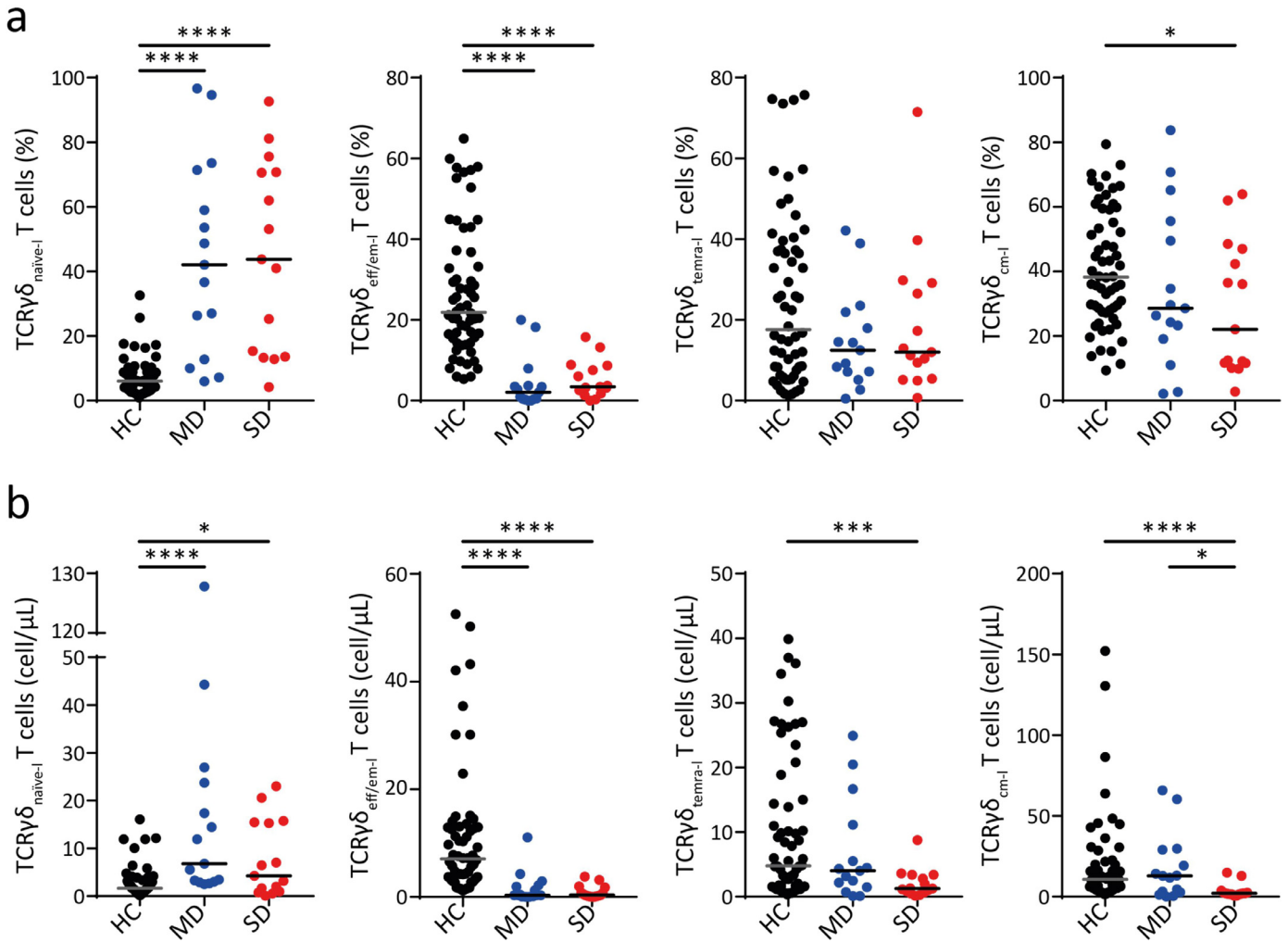
Fig. 3. $\gamma\delta$ T cells from COVID-19 patients exhibit a distinct memory signature.
Gated on $\gamma\delta$ T cells (a). Absolute numbers of $\gamma\delta$ T cell subpopulations (b). Lines represent median. Statistical analysis was performed by Mann-Whitney *U* test or Student's t-test where applicable. *$p< 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$; HC: Healthy Control. MD: Mild Disease; SD: Severe Disease.

different subsets of $CD4_{conv}$ and $CD8^+$T cell showing memory/effector phenotypes. Together, our findings indicate that mounting an efficient T cell response allows hospitalized patients to combat and overcome SARS-CoV-2 infections.

In agreement with recent reports, we observed a general decrease of lymphocytes in all COVID-19 patients that was more pronounced in severe cases [8−10,12]. Such a reduction of immune cells in the blood of SD patients might be due to several causes. It is conceivable that infection with SARS-CoV-2 directly affects differentiation and/or production in and release from primary lymphoid organs. Although this virus in general is not suspected to infect and destroy immune cells or their progenitors to a large degree, it seems possible that such effects might be secondary and caused by massive release in inflammatory mediators as a response to the infection [9,10]. Alternatively, failure to observe lymphocytes in the blood might be due to their homing/recruitment to the lung or other infected organs [11]. COVID-19 patients with a mild clinical course did not show reduction in NK, NKT and $\gamma\delta$ T cells counts. NK cells are part of the innate immunity and their significance in clearance of viral infections has been well documented [27,28]. It is thus reasonable to assume that these cells contribute to keeping the disease from progressing, as suggested before [8]. The observation that these innate and innate-like immune cells are present in regular numbers at early stages of the disease might also indicate that this arm of the immune system helps to contain early virus infection.

Compared to HC, COVID-19 patients showed already altered effector/effector memory and naïve T cell proportions at hospital admission. Increased frequencies of effector and memory populations in MD, but not SD, suggest that MD patients already launched a broad adaptive immune response at that time point, whilst this was presumably not the case in severely affected patients. Along the same line, a shift towards further activation of $\alpha\beta$ T cells coincided with convalescence, also hinting at the presence of SARS-CoV-2 responsive T cells within this pool. These findings are in line with very recent reports describing the role of T cell immunity in COVID-19 patients [29−32].

Data of the present study highlight the role of T cells in successfully controlling SARS-CoV-2 infections. Different sampling times of follow-up samples, as well as relatively small patient sample size remain the main limitation of this study. Additionally, combination of these highly standardized data with more in-depth analysis of SARS-CoV-2 specific responses might provide a more comprehensive overview of immune responses in COVID-19. Along those lines, a long-term longitudinal evaluation of a variety of immune subsets as recently reported in a pre-published study [33] would be of great value in our understanding of dynamics of anti-SARS-CoV-2 responses. More research is warranted, preferably in larger number of patients to corroborate findings of this study. Such research might also reveal implications for vaccine design and assessment of successful immune response in vaccine trials. Furthermore, standardized
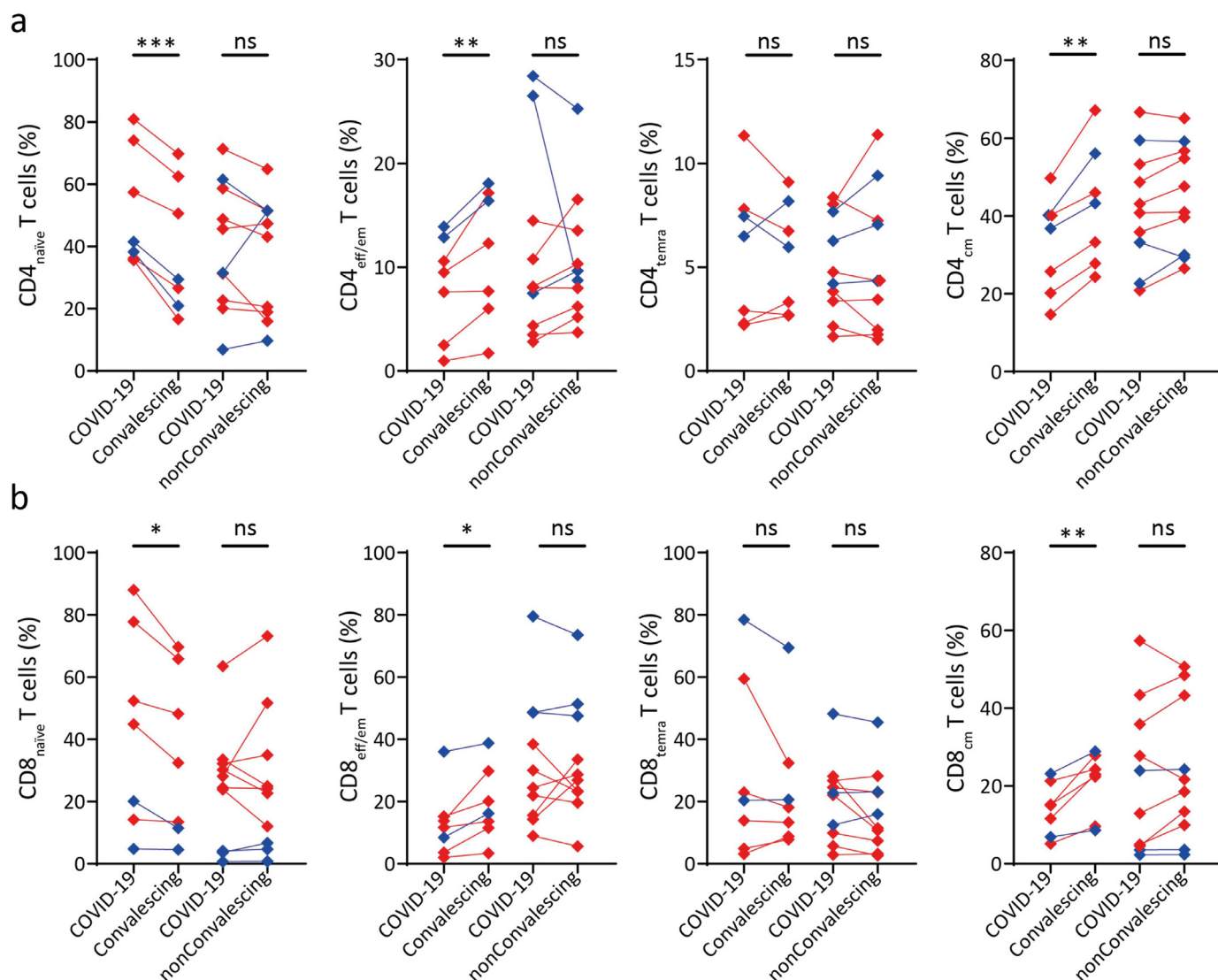
Fig. 4. Increase of effector/memory T cells suggests better clinical outcome.
Gated on CD4$_{conv}$ (a). Gated on CD8$^+$ (b). Graphs show patients' memory subsets' values in convalescing vs non-convalescing patients. Patients who were diagnosed with MD at the point of admission are displayed with blue lines. Patients with SD at the point of admission are depicted with red lines. Statistical analysis was performed paired Student's t-test. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; ns: not significant.

comprehensive lymphocyte subset analyses by flow cytometry can be easily implemented and, after stringent validation might serve as a biomarker for disease outcome and control.

Author contributions

IO, CK and CRSF designed the study, KS and SD obtained written informed consent from all patients, LG obtained written informed consent from all healthy controls, CRSF and IO performed experiments and analysed experimental data; IO, CRSF, ImP, RF and CK interpreted the data, IO and CRSF wrote the manuscript, JBM, BB, KS, SD, OW, MB, MMH, IsP, TW, MC, MS, LG, RB, AG, ImP, RF and CK reviewed and edited the manuscript. All authors read and approved the final version of the manuscript.

Declaration of Competing Interests

All authors declare no conflict of interest regarding this work.

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ebiom.2020.102885.

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