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Supplemental Information

Robust T Cell Response Toward Spike, Membrane, and Nucleocapsid SARS-CoV-2 Proteins Is Not Associated with Recovery in Critical COVID-19 Patients

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Supplementary tables

Supplement Table 1. Time points of sampling of pooled samples. Related to Table 1 and Figure 3.

	Moderate	Severe	Critical	P=
Number of samples	32	16	17	
Time since positive test (median [range])	5 [-2-42]	8 [1-21]	9 [1-35]	ns
Time since hospital admission (median [range])	6.5 [1-79]	8.5 [1-57]	17 [1-69]	ns

Comparison was done with Kruskal-Wallis test. P<0.05 were considered significant.

Supplement Table 2. Characteristics of COVID-19 patients and unexposed donors. Related to Figure 1.

	COVID-19	**Unexposed donors**	**P=**
Number of patients	28 (73.7%)	10 (26.3%)	
Age (median [range])	69 [26-91]	55 [42-64]	0.002
Gender (Male/Female)	18/10 (36% / 64%)	5/5 (50% / 50%)	ns

Comparison of patient age was done with Mann-Whitney test. Comparison of patient gender was done with Fisher's exact test. $P<0.05$ were considered significant.

Supplement Table 3. Characteristics of SARS-CoV-2 PCR negative (cleared) and PCR positive (uncleared) patients. Related to Figure 4.

	SARS-CoV-2 cleared	**SARS-CoV-2 uncleared**	**P=**
Number of patients	11 (61.1%)	7 (38.9%)	
Age (median [range])	69 [44-85]	79 [58-88]	ns
Gender (Male/Female)	6/5 (54.5% / 45.5%)	3/4 (43% / 57%)	ns
Sample group initial (moderate/severe/critical)	5/3/3 (45% / 27% / 27%)	4/2/1 (57% / 29% / 14%)	
Sample group follow up (moderate/severe/critical)	8/0/3 (73% / 0% / 27%)	5/1/1 (71% / 14% / 14%)	
Time since positive test initial (median [range])	2 [1-14]	1 [1-9]	ns
Time since hospital admission initial (median [range])	2 [1-50]	1 [1-19]	ns
Time since positive test follow up (median [range])	30 [4-41]	9 [7-22]	ns
Time since hospital admission follow up (median [range])	31 [4-79]	8 [3-25]	ns
Time between initial and follow up (median [range])	21 [2-29]	6 [2-21]	ns

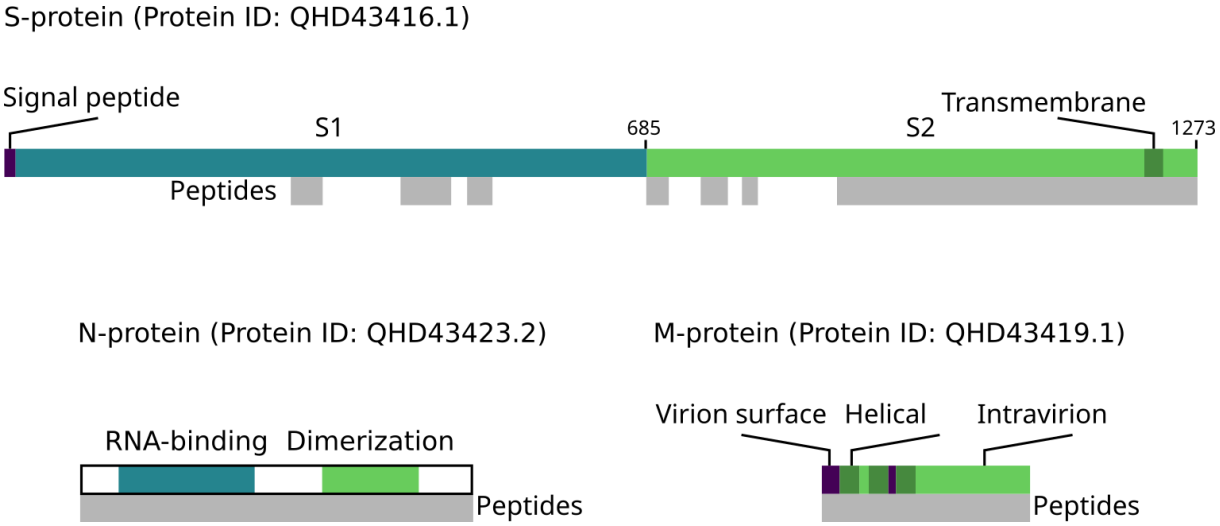
Comparison of patient gender was done with Fisher's exact test. All other comparisons were done with Mann-Whitney test. $P<0.05$ were considered significant.

Supplement Table 4. Characteristics of COVID-19 non-critical, recovered critical and critical patients who deceased. Related to Figure 5.

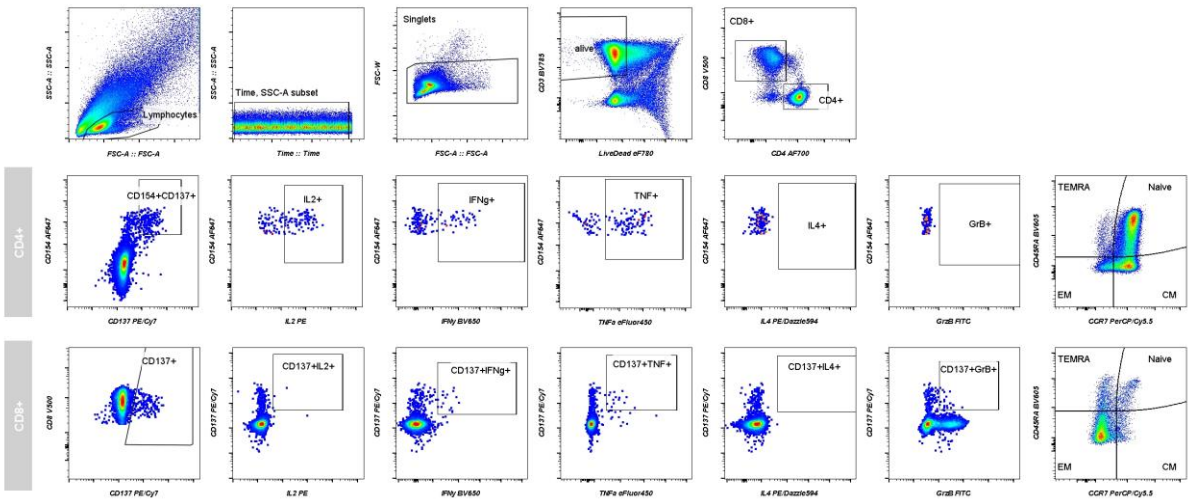
	COVID-19 non-critical	**COVID-19 recovered critical**	**COVID-19 deceased critical**	**P=**
Number of patients	15 (58%)	5 (19%)	6 (23%)	
Age (median [range])	81 [66-83]	58 [58-69]	63.5 [48-74]	ns
Gender (Male/Female)	6/9 (40% / 60%)	5/0 (100% / 0%)	6/0 (100% / 0%)	0.005
Sample group initial (moderate/severe/critical)	7/8/0 (47% / 53% / 0%)	0/0/5 (0% / 0% / 100%)	0/0/6 (0% / 0% / 100%)	
Sample group follow up (moderate/severe/critical)	14/1/0 (93% / 7% / 0%)	0/0/5 (0% / 0% / 100%)	NA	
Time since positive test initial (median [range])	4.5 [1-21]	10 [1-14]	4 [2-9]	ns
Time since hospital admission initial (median [range])	4 [1-21]	15 [1-18]	7 [3-69]	ns
Time since positive test follow up (median [range])	8.5 [4-42]	31 [8-35]	NA	ns
Time since hospital admission follow up (median [range])	8 [3-46]	32 [20-39]	NA	0.0341
Time between initial and follow up (median [range])	4 [2-25]	21 [3-21]	NA	ns

Comparison of patient gender was done with Fisher's exact test. Comparison of patient age and of initial samples was done with Kruskal-Wallis-test. Comparison of follow up samples was done with Mann-Whitney test. $P<0.05$ were considered significant.

Supplementary Figures

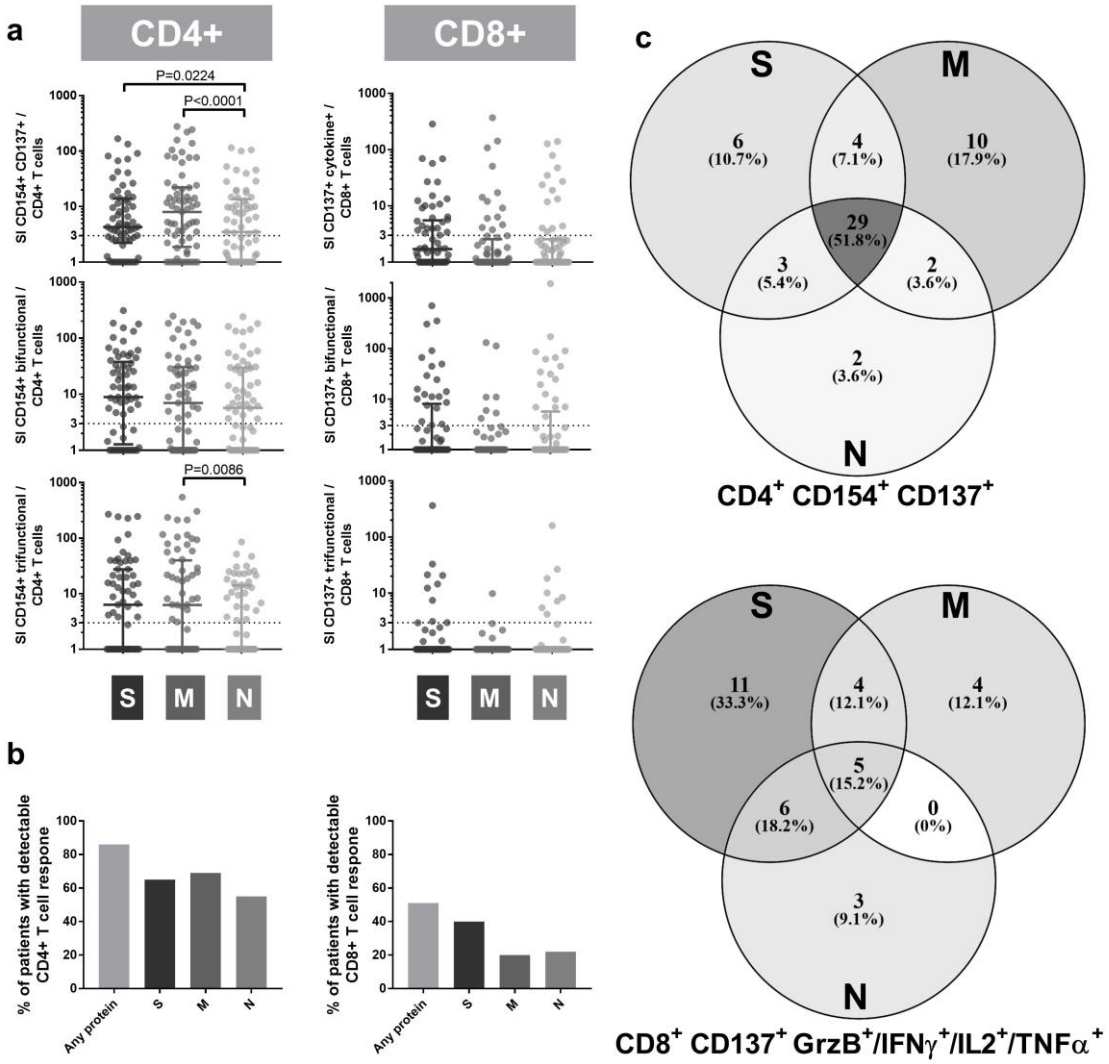


Supplementary figure S1: Schematics of the spike (S)-, nucleocapsid (N)- and membrane (M)-SARS-CoV-2 proteins. Related to Figure 1-5.
The S-protein overlapping peptide pool (OPP) contained 15mer peptide with 11 amino acid (AA) overlaps spanning the S-protein regions 304-338, 421-475, 492-519, 683-707, 741-770, 785-802, and 885-1273. The N- and M-protein OPPs also contained 15mer peptides with 11 AA covering the whole proteins.



Supplementary figure S2: Gating-strategy for the identification of SARS-CoV-2-reactive CD4$^+$ and CD8$^+$ T cells including cytokine and effector molecule expression as well as memory phenotype characterization. Related to Figure 1-5.

PBMC were stimulated with SARS-CoV-2-overlapping-peptide-pools for 16h. Brefeldin A was added after 2h to block the cytokine secretion. Representative plots illustrate the gating strategy. (top row) Lymphocytes were identified, doublets were excluded by forward scatter (FSC) width (W) and area (A) signals, live CD3$^+$ T cells were identified and subdivided into CD4$^+$ and CD8$^+$ T cells. (middle row) CD3$^+$CD4$^+$CD154$^+$ CD137$^+$ living lymphocytes were considered antigen-reactive CD4$^+$ T cells. These cells were further analyzed for the expression of interleukin (IL) 2, interferon γ (IFNγ), tumor necrosis factor (TNFα), IL4 and granzyme B (GrzB). (bottom row) CD3$^+$CD8$^+$CD137$^+$ living lymphocytes that produced at least one of the cytokines IL2, IFNγ, TNFα, or the effector molecule GrzB were considered as antigen-reactive CD8$^+$ T cells (CD8$^+$ CD137$^+$ cytokine$^+$). Antigen-reactive cytokine and effector molecule production was analyzed by the frequency of CD3$^+$CD8$^+$CD137$^+$ IL2/IFNγ/TNFα/IL4 or GrzB producers. Memory phenotype was analyzed for CD154$^+$ CD137$^+$ CD4$^+$ and CD137$^+$ cytokine$^+$ CD8$^+$ T cells as CD45RA$^+$ CCR7$^+$ (T_{NAIVE}), CD45RA$^-$ CCR7$^+$ (T_{CM}), CD45RA$^-$ CCR7$^-$ (T_{EM}) and CD45RA$^+$CCR7$^+$ (T_{EMRA}). Plots show pseudocolor plots. Large dots are used for SARS-CoV-2 specific T cells for better visibility.



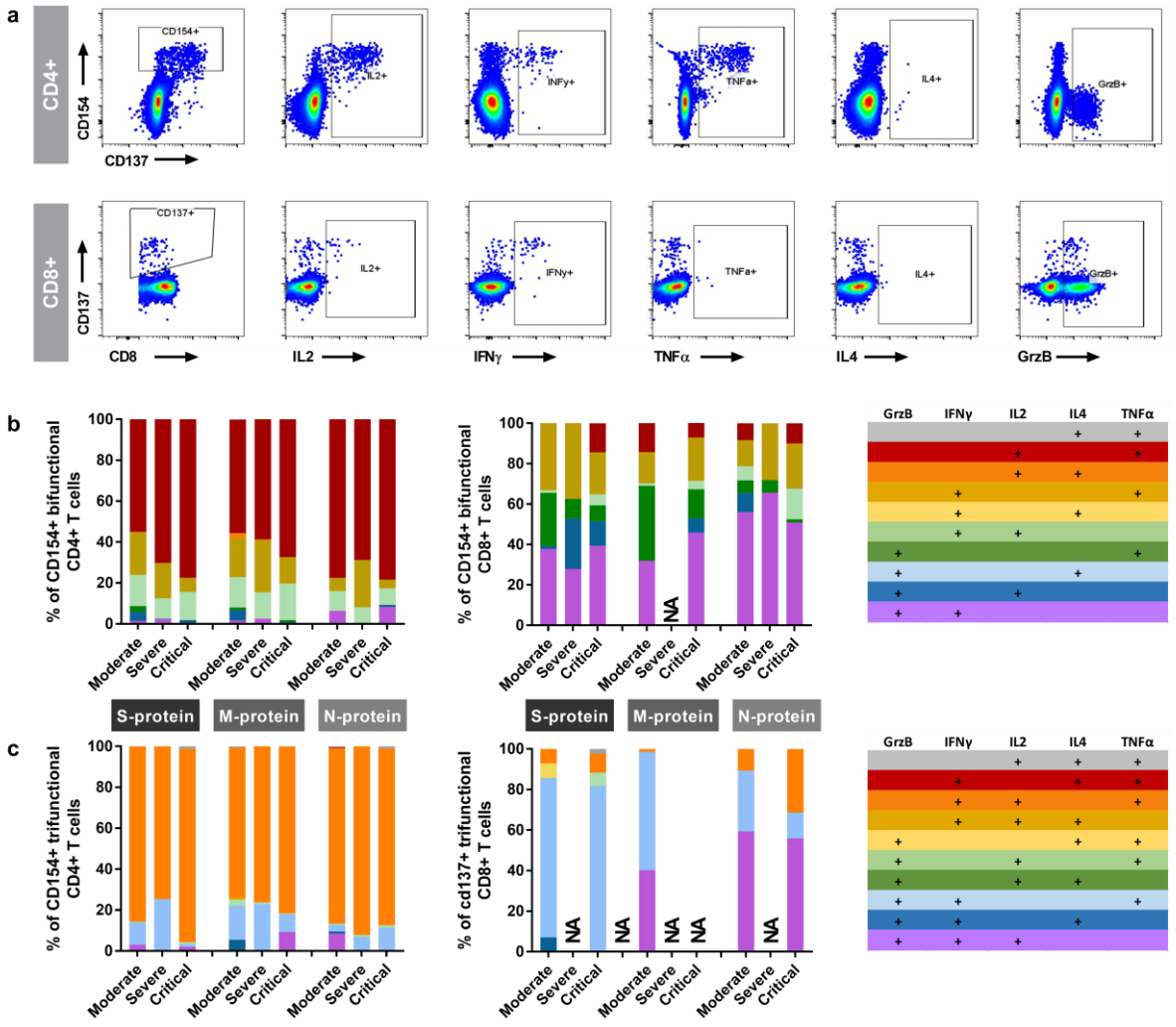
Supplementary figure S3: SARS-CoV-2-reactive T cells are induced by the S-, M- and N- protein with inter-individual pattern in pooled samples. Related to Figure 1.

Peripheral blood mononuclear cells (PBMC) isolated from 65 blood samples collected from 28 COVID-19 patients with moderate, severe or critical disease were compared to each other. PBMC were stimulated for 16 hours with S-, M-, or N-protein OPP. Antigen-reactive T cells were determined by flow cytometry and identified according to the gating strategy presented in Fig. 1 and supplementary figure S2.

a) Stimulation index (SI) of $CD154^+$ $CD137^+$ $CD4^+$ T cells (SARS-COV-2-specific $CD4^+$ T cells), $CD137^+$ IL2, IFNγ, TNFα and/or GrzB ($cytokine^+$) $CD8^+$ T cells (SARS-COV-2-specific $CD8^+$ T cells) and bifunctional and trifunctional $CD154^+$ $CD4^+$ and $CD137^+$ $CD8^+$ T cells. Bi- and trifunctional T cells were calculated by Boolean gating of IL2-, IFNγ-, TNFα, IL4-, and GrzB-production. SI was calculated by dividing the measured T cell subset response by the respective response in the DMSO control. Values above 3 were considered detectable in the following analyses. Scatter plots show line at median, error bars represent interquartile range. Statistical comparison was done with Friedman test and Dunn's multiple comparisons test. $P<0.05$ were considered significant.

b) Frequency of patient samples with detectable (SI > 3) $CD4^+$ (left) and $CD8^+$ (right) T cell responses after stimulation with S-, M-, or N-protein (total of 65 samples of 28 COVID-19 patients).

c) Venn diagrams of 65 COVID-19 patient samples with detectable (SI > 3) SARS-Cov-2-reactive $CD4^+$ or $CD8^+$ T cells after stimulation with S-, M- or N-protein. 56 of COVID-19 samples within $CD4^+$ T cells and 33 COVID-19 samples within $CD8^+$ T cells showed T cell reactivity towards at least one of the tested SARS-CoV-2-S, M, and N-proteins.

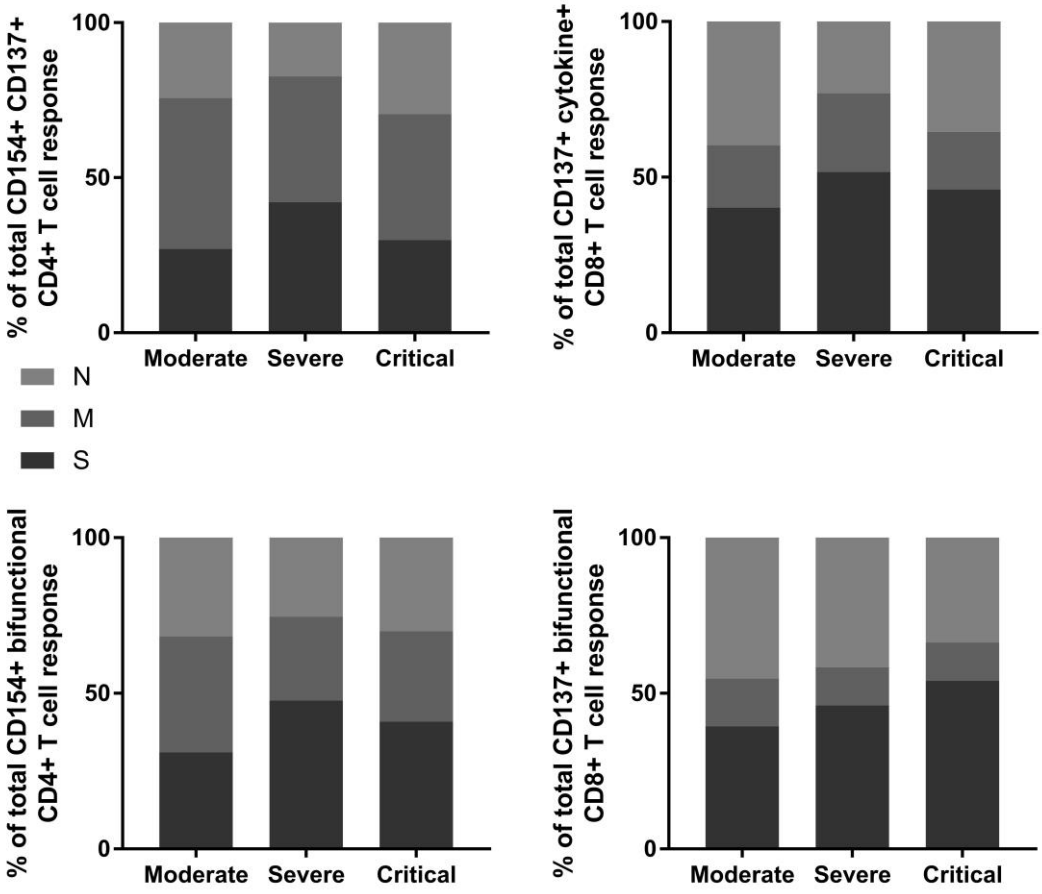


Supplementary figure S4: Composition of polyfunctional SARS-CoV-2-reactive T cells shows T_H1 characteristics of $CD4^+$ T cells and $CD8^+$ T cells with cytotoxic capacity in pooled samples. Related to Figure 3.

PBMC were isolated from blood samples of 28 COVID-19 patients at one or multiple time points after diagnosis (n=65 samples). PBMC were incubated for 16h with SARS-CoV-2 spike (S)-, membrane (M)-, or nucleocapsid (N)-protein overlapping peptide pools and analyzed with flow cytometry.

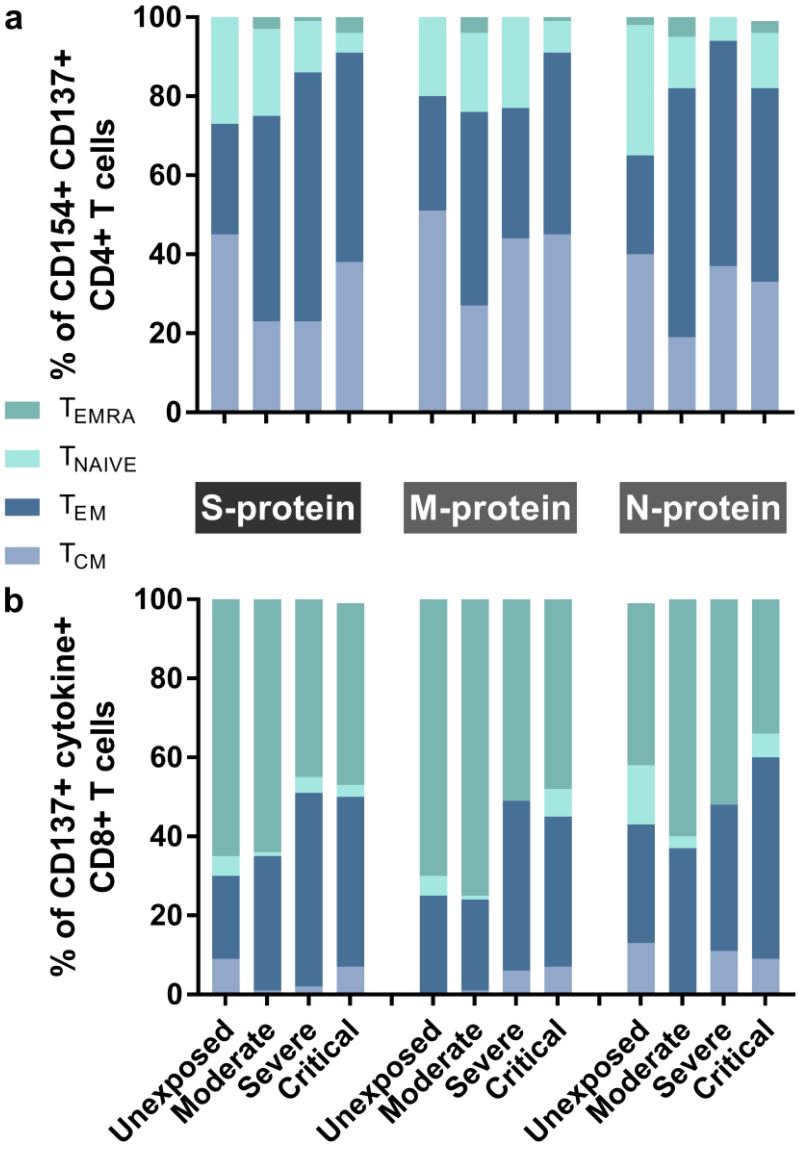
a) Representative gating of $CD4^+$ $CD154^+$, $CD8^+$ $CD137^+$ and interleukin (IL) 2-, interferon γ (IFNγ)-, tumor necrosis factor α (TNFα)-, IL4-, and granzyme B (GrzB)- producing $CD4^+$ (upper row) or $CD8^+$ (lower row) T cells.

b-c) Composition of bi- and trifunctional $CD154^+$ $CD4^+$ and $CD137^+$ $CD8^+$ T cells after stimulation with S-, M- or N-protein of patient samples with different COVID-19 disease severity (n=32 moderate, n= 16 severe and n=17 critical COVID-19 samples). Polyfunctional cells were calculated using Boolean gating. The relative share of each subset of the total bi- or trifunctional T cell response was calculated for each combination of peptide pool stimulation and clinical classification. To avoid skewed presentation, analysis was not done if in less than 5 individuals of a COVID-19 severity sample group a polyfunctional response could be measured (NA = not applicable).



Supplementary figure S5: Total responses towards SARS-CoV-2 spike (S)-, membrane (M)- and nucleocapsid (N)-overlapping peptide pools are not skewed in different COVID-19 severity groups in pooled samples. Related to Figure 3.

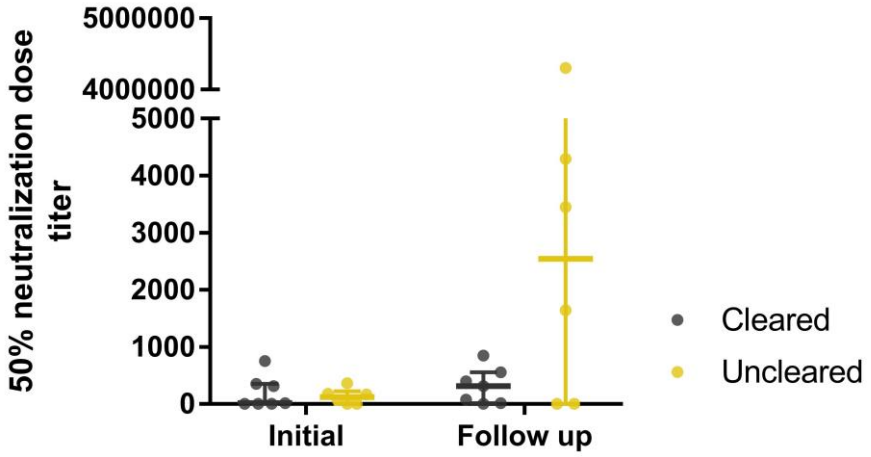
T cell responses of 32 moderate, 16 severe and 17 critical COVID-19 patient samples towards overlapping peptide pools of S-, M- and N-protein were analyzed. The mean relative share of each peptide response of the total response was analyzed for $CD154^+$ $CD137^+$ $CD4^+$ T cells, $CD154^+$ bifunctional T cells, $CD137^+$ IL2, IFNγ, TNFα and/or GrzB producing $CD8^+$ T cells ($CD137^+$ $cytokine^+$) and $CD137^+$ bifunctional $CD8^+$ T cells.



Supplementary figure S6: SARS-CoV-2-reactive T cells are of advanced differentiation stage phenotype in pooled samples. Related to Figure 3.

Blood was drawn from 28 COVID-19 patients at one or multiple time points after a positive SARS-CoV-2-infection test (n=65 samples) and of 10 donors before the COVID-19 pandemic. Severity of COVID-19 was assessed at the time of sampling as per the guidelines of the German Robert-Koch-Institute and samples grouped accordingly (n=32 moderate, n= 16 severe and n=17 critical COVID-19 samples). Peripheral blood mononuclear cells were stimulated for 16h with S-, M-, or N-protein overlapping peptide pools and analyzed by flow cytometry. The gating strategy to identify SARS-CoV-2 S-, M-, or N-protein reactive T cells and the memory phenotype is presented in Fig. S2.

a-b) Mean frequency of T_{NAIVE} ($CCR7^+$ $CD45RA^+$), T_{CM} ($CCR7^+$ $CD45RA^-$), T_{EM} ($CCR7^-$ $CD45RA^-$), and T_{EMRA} ($CCR7^-$ $CD45RA^+$) among $CD154^+$ $CD137^+$ $CD4^+$ (a) or $CD137^+$ cytokine$^+$ $CD8^+$ T cells.



Supplementary figure S7: Patients that stayed SARS-CoV-2 PCR positive have higher neutralizing antibody titers. Related to Figure 4.
Analysis of SARS-CoV-2 neutralizing antibodies in samples of patients who cleared the virus collected before (initial) and after (follow up) clearance (n=11 patients) and in samples of patients who failed to clear the virus in the observation period (initial = first sample; follow up = last obtained sample) (n=7 patients). Statistics were done with Kruskal-Wallis test with Dunn's multiple comparisons test. $P<0.05$ were considered significant. Scatter plots with line at median, error bars show interquartile range.