JCI The Journal of Clinical Investigation

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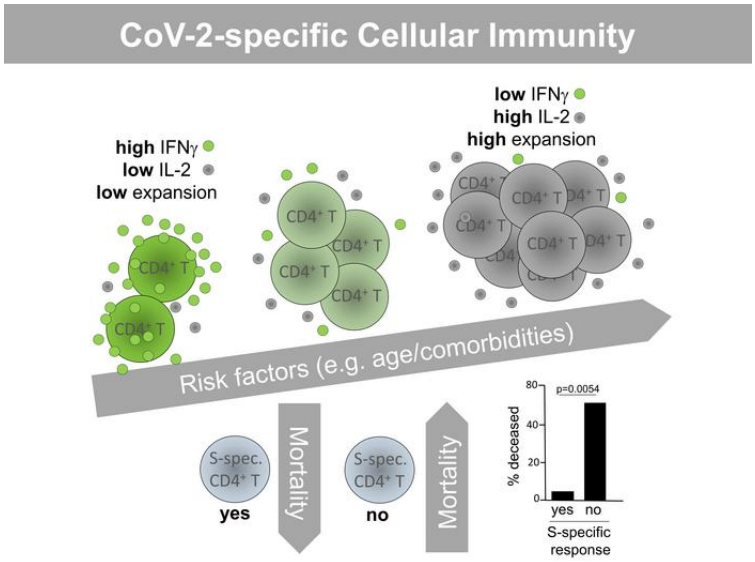
J Clin Invest. 2020. https://doi.org/10.1172/JCI140965.

Research In-Press Preview

Graphical abstract



SARS-CoV-2 Specific T-cell Responses and Correlations with COVID-19 Patient Predisposition

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The authors have declared that no conflict of interest exists

Abstract

COVID-19 has emerged as a global pandemic caused by SARS-CoV-2. So far, viral targets of cellular immunity and factors determining successful mounting of T-cell responses are poorly defined. We therefore analyzed cellular responses to membrane, nucleocapsid and spike protein in individuals suffering from moderate or severe infection and after recovery from mild disease. We demonstrate that the CoV-2 specific $CD4^+$ T-helper cell response is directed against all three proteins with comparable magnitude, ex vivo proliferation and portions of responding patients. However, deceased individuals were more frequently amongst non-responders. Higher patient age and comorbidity index correlated with increased frequencies of CoV-2 specific $CD4^+$ T-cells, harboring higher portions of IL-2-, but lower portions of IFNγ secreting cells. Diminished frequencies of membrane protein reactive $IFN\gamma^+$ T cells were particularly associated with higher Acute Physiology And Chronic Health Evaluation II scores in patients admitted to intensive care. CoV-2 specific T cells exhibited elevated PD-1 expression in active patients as compared to recovered individuals with previous mild disease. In summary, our data suggest a link between individual patient predisposition with respect to age and comorbidity and impairment of CoV-2 specific Th1-type cellular immunity, thereby supporting a concept of altered T-cell function in patients at risk.

Introduction

Currently, the zoonotic Coronavirus Disease-2019 (COVID-19), caused by SARS-CoV-2, is dramatically challenging global health systems. COVID-19 exhibits a diverse spectrum of clinical manifestations, ranging from a moderate course of infection with no or mild symptoms to viral pneumonia and hospitalization particularly in patients at risk, ultimately requiring intensive care unit (ICU) admission in case of acute respiratory distress syndrome (ARDS) (1, 2). Factors determining clinical outcome include patient age, chronic pulmonary and heart disease as well as excessive production of the inflammatory mediators interleukin 6 (IL-6) and 8 (IL-8) (3, 4). With respect to the latter ones, the mechanisms driving such exacerbation of innate immunity are poorly understood. Similarly, the exact immunological correlates of protection from COVID-19 remain obscure: despite pioneering data from a simian CoV-2 infection model (5), the relative contribution of humoral and cellular responses to overall immunity has not been elucidated. Particularly, data on the SARS-CoV-2 specific T cell response is limited, including their viral targets, functional features and an association with clinical parameters. T-cell mediated immunity against 2002/2003 SARS-CoV, exhibiting high homology to CoV-2 (6), has been shown to be preferentially directed against viral spike protein (7) that is also the target of potent neutralizing antibodies (8). Furthermore, rodent models suggested a critical contribution of both SARS-CoV specific CD8 and CD4 responses to viral clearance (9-11). Recent bioinformatics approaches have predicted T cell epitopes in both membrane (M), nucleocapsid (N) and spike (S) protein of SARS-CoV-2 (12-15). We therefore phenotypically and functionally characterized the SARS-CoV-2 specific $CD4^+$ T cell response to all three proteins in patients with moderate and severe disease and mildly affected individuals after recovery. Our study provides important new information on the composition of anti-CoV-2 T-cell immunity, suggesting that patient-specific risk factors such as age and comorbidities pre-determine the CoV-2 specific T cell response both quantitatively and functionally.

Results

Study subjects

The study cohort consisted of 39 hospitalized, acutely CoV-2 infected individuals from whom 23 with severe disease were in intensive care and 16 with a moderate disease course in non-intensive care. Details of their characteristics are listed in Table I. Both groups did not show differences with respect to sex and ethnic background. However, ICU patients were significantly older, largely suffered from acute respiratory distress syndrome (ARDS), showed significantly higher pneumonia severity index (PSI) and Charlson Comorbidity Index (CCI) scores, experienced more often bacterial superinfections and were characterized by a longer infection history as reflected by number of days since symptoms onset. Furthermore, ICU treated individuals exhibited significantly elevated absolute counts of peripheral leukocytes and an increased CD4 to CD8 T cell ratio on the day of antigen-specific T cell analysis. Based on clinical routine, absolute lymphocyte and $CD4^+$ T cell counts were collected within ± 3 days around the date of specific T cell analysis, being below or close to the lower limit of the reference range, respectively, in both ICU- and non-ICU patients.

We further included a group of 7 convalescent individuals into this cross-sectional study to examine CoV-2 specific immunity in the non-acute/memory phase. Based on recruitment routines, these subjects all presented a previous mild disease course, principally bearing the potential to identify functional qualities associated with uncomplicated recovery.

Detection, quantification and functional characterization of SARS-CoV-2 specific T cells in active COVID-19 patients

To identify SARS-CoV2 reactive T cells, PBMC were individually stimulated with overlapping peptide pools spanning membrane glycoprotein, nucleocapsid phosphoprotein, or spike glycoprotein with the capacity to activate both $CD4^+$ and $CD8^+$ T cells irrespective of HLA-type (16). After pre-gating on live $CD3^+dump^-$ lymphocytes, antigen-reactive $CD4^+$ Th cells were identified based on co-expression of CD154 and CD137, as demonstrated earlier (17), allowing sensitive detection with low background, followed by subsequent analysis of

cytokine expression and ex vivo proliferation based on Ki67 staining (Fig.1A). A T cell response was considered positive when SARS-CoV-2 peptide mix stimulated cultures contained at least twofold higher frequencies of $CD154^+CD137$ $CD4^+$ T cells as compared to the unstimulated control with at least ten events. Based on limitations in cell numbers or immediate availability of reagents at the peak of the pandemic, not all analyses were conducted for all patients.

The overall portion of all acutely infected, hospitalized donors displaying specific $CD4^+$ T cell responses was similar for both M-, N- and S-peptide mixes, ranging from 60 % (N-protein) to 70 % (S-protein) (Fig. 1B). Within the group of responding patients, mean frequencies of antigen-reactive $CD4^+CD154^+CD137^+$ T cells were not significantly different for M-, N-, or S-antigen (Fig. 1B); however, males showed a higher relative magnitude of responses to N protein than females after relative quantification (Fig. 1B). With respect to their functionality, N-specific T cells comprised lower frequencies of $IFN\gamma^+$ and $IFN\gamma^+TNF\alpha^+$ bifunctional T cells than those specific for S- and M-protein with the latter comparisons being highly significant (Fig. 1C, left and middle). T cells reactive to M-, N- or S-protein showed high levels of ex vivo proliferation based on Ki67 expression, as compared to the total $CD4^+$ population (Fig. 1C).

Features associated with non-responders to CoV-2 antigen specific stimulation

Stratifying patients for their capacity to mount a T cell response or not, we found that non-responders exhibited a shorter infection history (as estimated by days since symptoms onset); this observation was statistically significant for M- and N-responses and showed a trend for S-responses (Fig. 2A). Next, we analysed CoV-2 spike-protein specific IgG and IgA responses in cellular responders and non-responders to the same antigen. Whereas a positive IgG response could be detected in 92 % of cellular responders, the majority of cellular non-responders was characterized by a lack of detectable anti-spike IgG responses. Whereas this difference was highly significant, we did not observe such interdependence for anti-spike IgA responses (Fig. 2B). Patients who deceased during the study period (that is, within 6 weeks from the time point of cellular analysis) were more frequently cellular non-

responders; however, based on patient numbers, this observation was only significant with respect to responses against spike protein, where 5/10 non-responders deceased as compared to only 1/23 in the cellular responder group (Fig. 2C). We then interrogated whether non-responders were characterized by a distinct degree of acute pneumonia; however, we did not observe a significant association of Pneumonia Severity Index (PSI) with non-responsiveness towards any of the three antigens (Fig. 2D).

Impact of patient age and comorbidities on CoV-2 specific cellular immunity

To decipher which factors influence quantitative and qualitative characteristics of CoV-2 specific T helper responses, frequencies of $CD154^{+}CD137^{+}$ $CD4^{+}$ cells as well as portions of $IFN\gamma^{+}$ cells were correlated with age and comorbidities. Interestingly, advanced patient age and comorbidity significantly correlated with an increased relative magnitude of M-, N- and S responses with the exception of responses to membrane protein that only showed a trend with respect to CCI (Fig. 3A+B).

Intriguingly, as opposed to their impact on the magnitude of specific T cell responses, advanced patient age and higher CCI appeared to be associated with lower frequencies of antigen-specific IFNγ secreting cells after stimulation with membrane protein. This association was similarly pronounced, albeit equally just not reaching significance, for cytokine production towards N antigen stimulation in relation to age (Fig. 3C+D).

CoV-2 specific T cell responses in patients admitted to intensive care

To address whether COVID-19 patients with severe clinical manifestations requiring intensive care (from whom the majority had developed ARDS, Table I) showed distinct antigen-specific T cell responses, we compared them to non-ICU hospitalized individuals and to non-hospitalized donors with mild symptoms on average 26 days after recovery. ICU and non-ICU patients were characterized by significant differences with respect to acute disease based on PSI as well as regarding comorbidities (Table I). We further noted that non-ICU patients responded less frequently to stimulation with M and N antigen than ICU treated

individuals with the latter comparison reaching significance; both patient groups harboured almost identical portions of responders to spike protein stimulation (Fig. 4A).

With respect to cellular responses, ICU treated patients exhibited higher frequencies of antigen-specific T cells as compared to non-ICU patients and recovered individuals; however, only the latter comparison reached significance for M- and S-antigen stimulation (Fig. 4B). Interestingly, we did not observe substantial differences of mean frequencies of specific IFNγ secreting T cells amongst groups (Fig. 4C); however, ICU patients had higher frequencies of ex vivo proliferating $Ki67^+$ cells co-expressing IFNγ and TNFα as compared to non-ICU and recovered individuals with the latter showing significance only for responses to nucleocapsid protein (Fig. 4D). CoV-2 specific responses in ICU patients were subsequently correlated with Acute Physiology And Chronic Health (APACHE) II scores predicting ICU mortality. Importantly, higher APACHE scores were significantly associated with lower frequencies of $IFN\gamma^+$ T cells specific for membrane, but not for nucleocapsid or spike protein (Fig. 5).

To examine whether COVID-19 patients exhibited a general impairment of cytokine production, T cell responses were analysed after activation with the polyclonal stimulus SEB. The overall magnitude of the SEB specific T cell response is largely pre-determined by T cell receptor β chain usage and HLA haplotype for a given individual (18), thereby not allowing specific conclusions (Suppl. Fig. 1A). However, with respect to functionality, we observed significantly higher frequencies of $IFN\gamma^+$ and $IFN\gamma^+TNF\alpha^+$ $CD4^+$ T cells in recovered individuals who experienced a mild disease course as compared to ICU patients and a trend compared to non-ICU patients (Suppl. Fig. 1B).

IL-2 secretion capacity, exhaustion and differentiation status of CoV-2 specific T cells

Since active COVID-19 patients showed increased relative portions of ex vivo proliferating T cells, expression of the pan T cell growth factor IL-2 was examined in a limited number of study subjects. In analogy to our findings for IFNγ secretion, we did not observe differences in frequencies of specific T cells secreting IL-2 between groups (Fig. 6A). However, higher

frequencies of IL-2^{+} T cells significantly correlated with patient age and comorbidity for M- and N-, but not for S-specific T-cells (Fig. 6B+C). Since impaired effector cytokine production, including IFNγ, might be based on functional exhaustion, expression of the co-inhibitory molecule PD-1 was characterized on CoV-2 specific T cells. Of note, active patients, regardless of being in ICU care or not, showed higher PD-1 expression levels as compared to recovered individuals based on mean fluorescence intensity (MFI), reaching significance for M- and S-specific T cells (Fig. 7A). Interestingly, when comparing the differentiation status of antigen-specific T cells according to CD45RO and CD62L expression, we noted a trend towards higher frequencies of CD45RO^{-}CD62L^{-} effector-like T cells specific for M- and N-protein in recovered individuals that reached significance for T cells reactive to S protein (Fig. 7B and Supplemental Fig. 2A). CoV-2 specific T-cells consistently expressed CD28, were largely CD57^{-} and contained only few cells expressing the cytotoxic molecule Granzyme B, as exemplarily demonstrated in Supplementary Fig. 2B and C.

We did not observe significant differences in frequencies of IFNγ^{+}TNFα^{+}IL-2^{+} polyfunctional CoV-2 specific T cells between patient groups and recovered individuals (Supplemental Fig. 3A); furthermore, portions of polyfunctional T cells did neither significantly correlate with COVID-19 patient age or CCI (Supplemental Fig. 3B and C).

Discussion

In this study, by applying a highly sensitive flow cytometry based assay, we characterized fundamental features and clinical correlations of the CoV-2 specific T helper cell response against three viral proteins formerly predicted to contain multiple T cell epitopes (12-15). In addition to providing important new data on the overall relative magnitude and quality of the anti-viral response in acute CoV-2 infection, we identify both advanced patient age and higher comorbidity as patient-related risk factors associated with increasing frequencies of antigen-specific $CD4^+$ T cells, including those secreting IL-2, but decreasing portions of IFNγ secreting T cells, particularly in response to membrane protein stimulation. We further extend the latter observation by demonstrating that ICU patients show significantly reduced frequencies of M-specific $IFN\gamma^+$ T cells with increasing mortality risk based on the APACHE scoring system.

Several lines of evidence point towards an overall impairment of innate and adaptive immunity in COVID-19 patients. In that respect, Th and cytotoxic T cells show significantly higher ex vivo expression levels of PD-1 and TIM-3, suggesting a state of functional exhaustion (19). Consequently, two recent reports verified that COVID-19 patients exhibit a trend towards lower frequencies $IFN\gamma^+$ cells after unspecific stimulation (20, 21), an observation that could be confirmed by us using the polyclonal stimulus SEB. More importantly, our data significantly extend the emerging concept of impaired cellular immunity by demonstrating that CoV-2 specific Th cells in active patients upregulate PD-1, accompanied by diminished frequencies of cells expressing the prototypic Th1 cytokine IFNγ in relation to their predisposing risk factors, entailing possible implications for anti-viral defense. Importantly, the latter observation could be overlooked when solely comparing mean frequencies of $IFN\gamma^+$ cells in ICU- and non ICU-treated individuals. Interestingly, both risk factors overlap with those identified to generally predict mortality in a recent retrospective study (22).

So far, the contribution of Th cells to CoV-2 specific immunity is largely obscure, considering that only few reports addressed specific T cell responses against multiple antigens so far

(23), (24). Although our analysis only relies on a small patient cohort, we identified a significantly increased number of deceased patients within the non-responder group, particularly against spike protein. As in turn, cellular non-responders less frequently developed anti-viral IgG titers, we can only speculate whether T helper cells exert direct effects against CoV-2, e.g. by cytokine release, beyond provision of B cell help for immunoglobulin production. The more detailed characterization of anti-SARS-CoV (2002/03) specific immunity proved that transfer of virus-specific $CD4^+$ T cells into SCID mice mediated protection from lethal SARS-CoV challenge in the absence of humoral immunity (9). To account for the predominance of aged individuals suffering from severe SARS-CoV disease, a mouse model reflecting such senescent state was created where Th cells were equally instrumental for viral control (25). With respect to the precise role of cytokine secretion, a very recent study identified airway-resident, CoV nucleocapsid specific Th cells as a critical source for IFNγ production since its neutralization was associated with significantly decreased survival after viral challenge (11). Assuming that virus-induced IFNγ production by T cells might equally support anti-CoV-2 immunity in humans, it remains to be determined which factors contribute to diminished frequencies of cytokine producers in COVID-19 patients holding risk factors such as advanced age or high comorbidity- and APACHE scores. Pioneering data suggest that lymphocyte functionality might be impaired by the overall inflammatory state prevailing in severely affected COVID-19 patients. This has exemplarily been shown for high IL-6 levels being associated with reduced natural killer (NK) cell functionality that was restored after in vivo IL-6 blockade with tocilizumab (21). Excessive production of innate cytokines, including IL-6, could be synergistically triggered in patients at risk by inflamm-ageing (as reviewed in (26)), bearing e.g. the potential to enhance PD-1 expression on T cells (27), with subsequent implications for altered Th1 cytokine production. Irrespective of the role of IL-6 in this process, the impact of increasing age on impaired IFNγ production has already been demonstrated for human cytomegalovirus- (CMV-) specific T cells (8) and is likely based on interlinked changes in both innate immunity (e.g. due to modifications in pattern recognition receptor signaling as reviewed in (28)) and adaptive

components. However, given that CoV-2 induces acute rather than long-lasting chronic infection, it is comprehensible that we did not detect typical T cell features associated with immunosenescence, such as downregulation of CD28, acquisition of Granzyme B or CD57 expression, being hallmarks of extremely long-lived individuals (29) and potentially resulting from chronic antigen exposure, e.g. caused by persistent CMV infection (30, 31). On that background, future studies need to assess immune components beyond T cells for age-dependent modifications in COVID-19 patients. Innate inflammation as a driver for functional T cell exhaustion might be also fueled by mechanical ventilation (32), being applied to the majority of ICU patients included herein, since experimental data suggest an upregulation of IL-6 by this invasive treatment (33). An initial hyperinflammatory phase is also a hallmark of severe sepsis that is often followed by immuno-depression, resulting e.g. in elevated PD-1 expression (34), along with reduced IFNγ secretion by T cells (35), thus providing a possible link to our findings.

Based on the experimental limitations imposed by human samples, only animal models will allow to decipher at what stage of COVID-19 patients might benefit from pro- or anti-inflammatory interventions, as is currently being controversely discussed (36), including evaluation the PD-1/PD-1L pathway as therapeutic target (37).

Intriguingly, we observed a pronounced positive correlation between the relative magnitude of the CoV-2 specific T cell response and age or comorbidity index. This relative cellular expansion might be mechanistically linked to an increased bioavailibility of the T cell growth factor IL-2, acting e.g. in an autocrine fashion (38), since we uncover a positive correlation of IL-2$^+$ M- and N- specific T cells with both risk factors. Being in line with this, antigen-specific T cells derived from acutely infected patients contained a substantial fraction that proliferated ex vivo as indicated by Ki67 expression; in addition, the small population of proliferating Ki67$^+$ cells co-expressing IFNγ and TNFα was highest in ICU- as compared to non-ICU patients and recovered non-hospitalized individuals.

Data from model infections indicate that the magnitude of the T cell response might reflect initial viral load. This relation has been verified in humans for live attenuated yellow fever

vaccine, where viral load could be easily and repeatedly monitored in vaccinees´ plasma and correlated to specific cellular immunity (39). Although precise viral load was not routinely assessed in our patients, two recent reports indeed observed an elevated viral burden in COVID-19 patients with advanced age (40) and/or severe disease (41), thereby supporting the notion of antigen-load dependent expansion of CoV-2 specific T-cells. Evidence from HIV infected individuals suggest that IL-2 secretion as a co-factor for cellular expansion of specific T cells might be less sensitive to inhibition via the PD-1/PD-1L pathway, as opposed to IFNγ secretion (42), permitting another explanation for the inverse correlation of IL-2 vs. IFNγ with patient risk factors in our study.

In summary, we provide here pioneering quantitative and functional data on the interrelation of CoV-2 specific cellular immunity and patient-immanent features shaping this response. Furthermore, our data on non-responders imply a crucial role of cellular immunity for anti-viral protection. Given the limitations of the cross-sectional design, future studies clearly need to address T cell dynamics in individual patients over time during infection and in the memory phase, along with outcome correlations, thereby more accurately allowing to evaluate the individual impact of specific T cell and humoral responses on host protection. Such monitoring should also assess the role of CoV-2 specific $CD45RO^{-}CD62L^{-}$ effector-type T cells for viral clearance, being accumulated in convalescent individuals and also making a contribution to the anti-SARS (2002/2003) T cell response in recovered individuals (43). Given that lymphopenia was found to be associated with severe CoV-2 infection (1 , 44), and that total CD4 counts can predict disease progression (44), absolute quantification of antigen-specific T cells might allow a deeper understanding of the remarkably different clinical COVID-19 courses observed. We did not conduct such extended examination since absolute counts were not routinely available for the day of specific T cell analysis. Although ICU- and non-ICU patients showed similar absolute $CD4^{+}$ T cell counts ± 3 days around the date of our relative quantifications, a side-by-side comparison of both measures bears the potential to identify patient subgroups as has e.g. been extensively performed in HIV infection (45, 46).

Finally, an analysis of the antigen-specific T cell infiltrate (e.g. from broncho-alveolar lavage samples) will provide important information as to how our observations are representative of the local response against CoV-2.

Methods

Study subjects

Details of individuals enrolled are summarized in Table I. The Charlson Comorbidity Index (CCI) predicting survival of patients with multiple comorbidities was used as described for patients upon hospitalization (47). The Pneumonia Severity Index (PSI) was used to assess the morbidity and mortality risk for community-acquired pneumonia (48); values represent the day of blood sampling. To classify the severity of disease of intensive care unit patients upon admission, the Acute Physiology And Chronic Health Evaluation (APACHE) II score (49), the Sepsis-related organ failure assessment score (SOFA) (50) and the Simplified Acute Physiology Score (SAPS) II (51) were used. SARS-CoV-2 infection was verified by PCR-based detection of viral RNA in nasopharyngeal swabs. SARS-CoV-2 spike protein specific IgG and IgA antibodies were detected in serum samples (collected on the day of T cell analysis) by ELISA (Euroimmun, Lübeck, Germany). A positive humoral response was determined according to the manufacturer's guideline.

SARS CoV-2 Antigens

Stimulations were conducted with overlapping peptide pools consisting of 15-mers with 11 amino acids overlap encompassing the full sequence of the SARS-CoV-2 (GenBank MN908947.3) membrane glycoprotein, nucleocapsid phosphoprotein and partial domains of spike glycoprotein (amino acids 304-338, 421-475, 492-519, 683-707, 741-770, 785-802, and 885-1273), respectively. All peptide pools were designed by and purchased from Miltenyi Biotec ("PepTivator", Bergisch Gladbach, Germany), diluted in sterile water and used at a final concentration of 1 µg/ml for each peptide.

Cell isolation and stimulation

Serum was collected and immediately cryopreserved. Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood by Ficoll-Paque™ density gradient centrifugation and freshly stimulated. For antigen-specific T-cell stimulation, 3-5x10^6 PMBC

were cultured in RPMI1640 media containing 0.3 mg/ml glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 10 % human AB serum (all Biochrom, Berlin, Germany) in the presence of M-, N- or S-peptide pools for 16 h. Staphylococcus aureus enterotoxin B (SEB, Sigma-Aldrich, Darmstadt, Germany) served as positive control and was used at 1 µg/ml. In all stimulations, Brefeldin A (10 µg/ml, Sigma-Aldrich) was added after 2 h, allowing intracellular molecule detection. Based on cell number limitations, SEB stimulation was not conducted for all individuals.

Flow cytometric analysis

For surface stainings, antibodies against CD3 (SK7, Biolegend (BL), Carlsbad, CA, USA), CD4 (SK3, Becton Dickinson (BD), Franklin Lakes, NJ, USA), CD8 (SK1, Ebioscience, San Diego, CA, USA), CD45RO (UCHL1, BL), CD62L (DREG-56, BL), PD-1 (EH12.1, BD), CD28 (CD28.2, BD) and CD57 (QA17A04, BL) were used. Unwanted cells were excluded via a "dump channel" containing $CD14^+$ (M5E2, BL), $CD19^+$ (HIB19, BL) and dead cells (fixable live/dead, BL). After stimulation, cells were fixed in FACS™ Lysing Solution (BD), permeabilized with FACS™ Perm II Solution (BD) and intracellularly stained with anti-CD154 (24-31, BL), anti-CD137 (4B4-1, BL), anti-TNFα (MAb11, BL), anti-IFNγ (4SB3, Ebioscience), anti-IL-2 (MQ1-17H12, BL), anti-Ki67 (B56, BD) and anti-Granzyme B (GB11, BD). Cells were analysed on a FACS™ Fortessa X20 (BD) flow cytometer.

Data analysis

FACS data were analysed with FlowJo 10 (BD). The gating strategy for analysis of antigen-specific T cells is depicted in Figure 1A. A T cell response was considered positive when SARS-CoV-2 peptide mix stimulated cultures contained at least twofold higher frequencies of $CD154^+$CD137 $CD4^+$ T cells as compared to the unstimulated control with at least ten events. Some patients were excluded from sub-analyses when event numbers in the respective gates were low. Co-expression was assessed via Boolean gating.

Statistics

Statistical analysis and graph composition were executed using GraphPad Prism 8.0 (GraphPad, La Jolla, CA, USA). The Kolmogorov-Smirnov test was used to evaluate the distribution of each parameter. Depending on normality distribution, ANOVA (with Holm-Sidak´s post-hoc) or Kruskal-Wallis test (with Dunn post-hoc) were chosen for multiple comparisons. For two-group comparisons, unpaired t test or Mann-Whitney test was used. The relationship between two variables was examined by simple linear regression analysis. For analysis of contingency tables, Fisher's exact or χ^2 test was used. In all tests, a value of $p<0.05$ was considered significant; non-significant results were not annotated unless indicated.

Study approval

The study protocol was approved by the ethics committee of the Charité-Universitätsmedizin Berlin (No. EA2/066/20 and EA2/035/16) and carried out in compliance with its guidelines. All participants gave written informed consent in accordance with the Declaration of Helsinki.

Acknowledgements

This study was supported by a grant from the Sonnenfeldstiftung, Berlin, Germany to A.S. and K.K.

Author contributions

A.S. designed the study, performed research, analyzed data and wrote the manuscript. S.A. designed the study, analyzed data and wrote the manuscript. H.S., K.M.H., D.K. and S.T. analyzed data. F.H. and M.E.K. designed the study. K.K. designed the study and wrote the manuscript.

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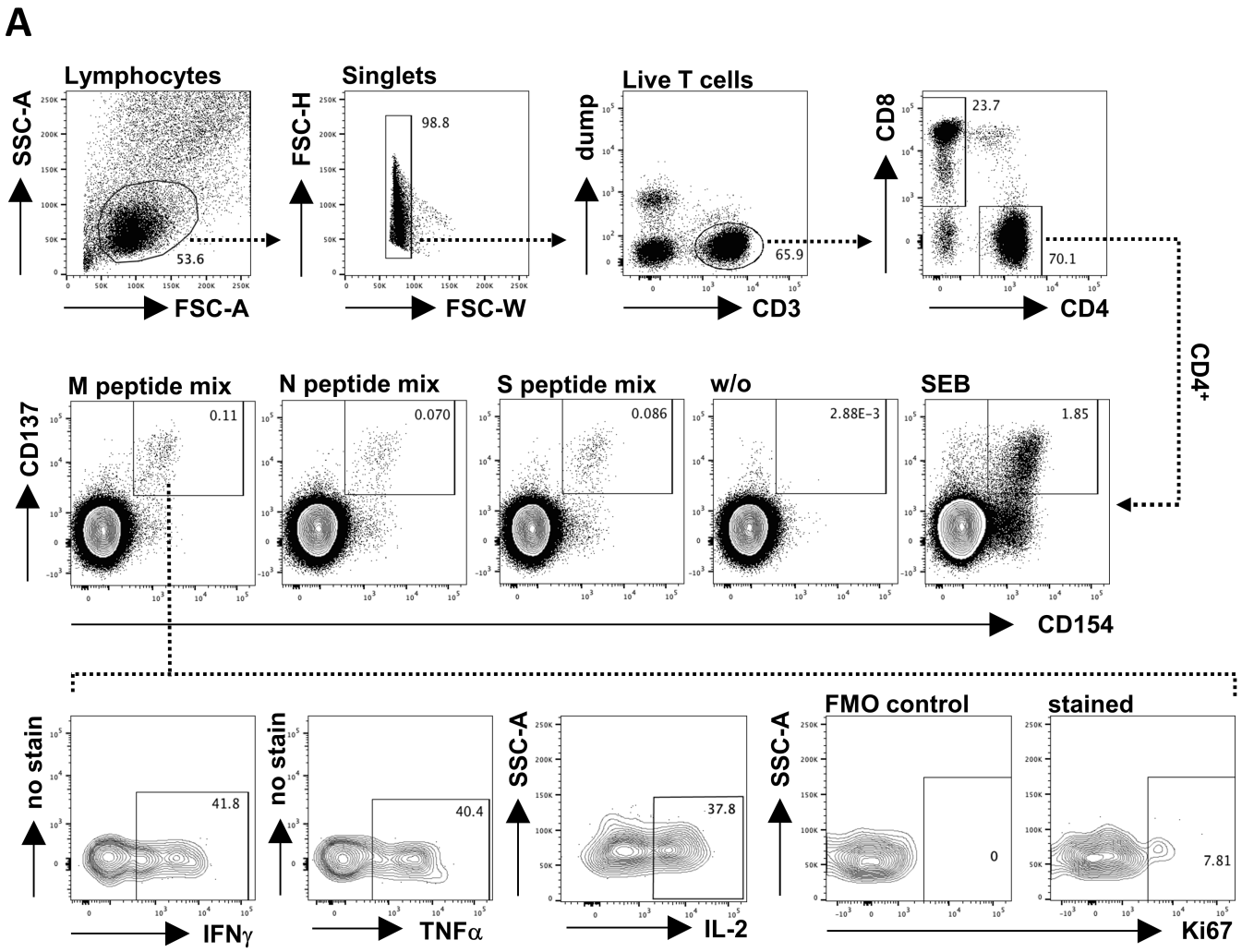
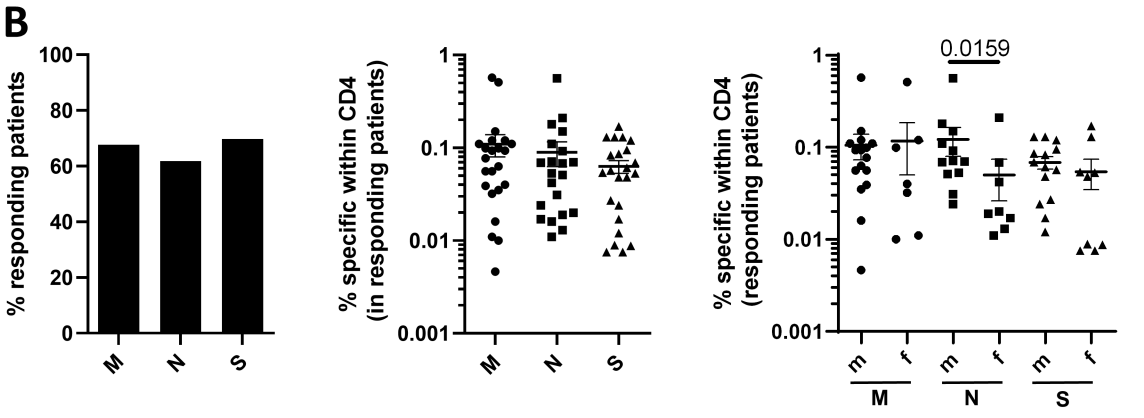
Sattler et al., Figure 1





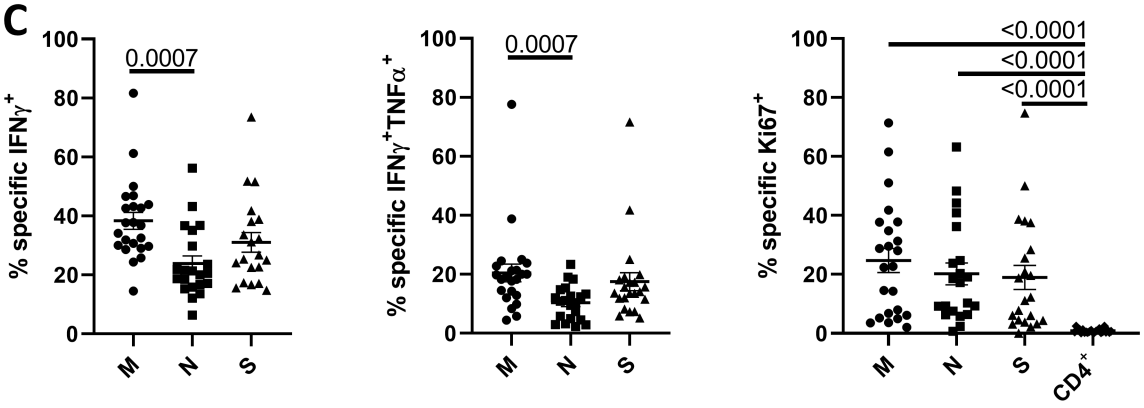


Figure 1

Identification of SARS-CoV-2 specific T cells in COVID-19 patients. (A) PBMC were stimulated or not with M-, N- or S-peptide mix or SEB for 16 h as indicated. Live single $CD14^-$ $CD19^-CD3^+$ specific $CD4^+$ Th cells were identified based on co-expression of CD154 and CD137. Specific $CD4^+CD154^+CD137^+$ T cells were further analyzed for expression of IFNγ, TNFα, IL-2 and Ki67, with the latter including a fluorescence-minus-one (FMO) control. (B) Percentage of all patients with a positive $CD4^+$ T cell response (left; M: n=34, N: n=34, S: n=33), frequencies of antigen-reactive $CD4^+$ T cells in responding patients (middle; Kruskal-Wallis test) and frequency distribution in males vs. females (right; M: n=23, N: n=21, S: n=23; Mann-Whitney test). (C) Frequencies of antigen-specifc $CD4^+$ T cells expressing IFNγ (left; Kruskal-Wallis test), IFNγ+TNFα (middle; Kruskal-Wallis test) or Ki67 (right, in comparison to the total $CD4^+$ population; Kruskal-Wallis test; M: n=23, N: n=21, S: n=20). Where applicable, graphs show means ± SEM.

Sattler et al., Figure 2

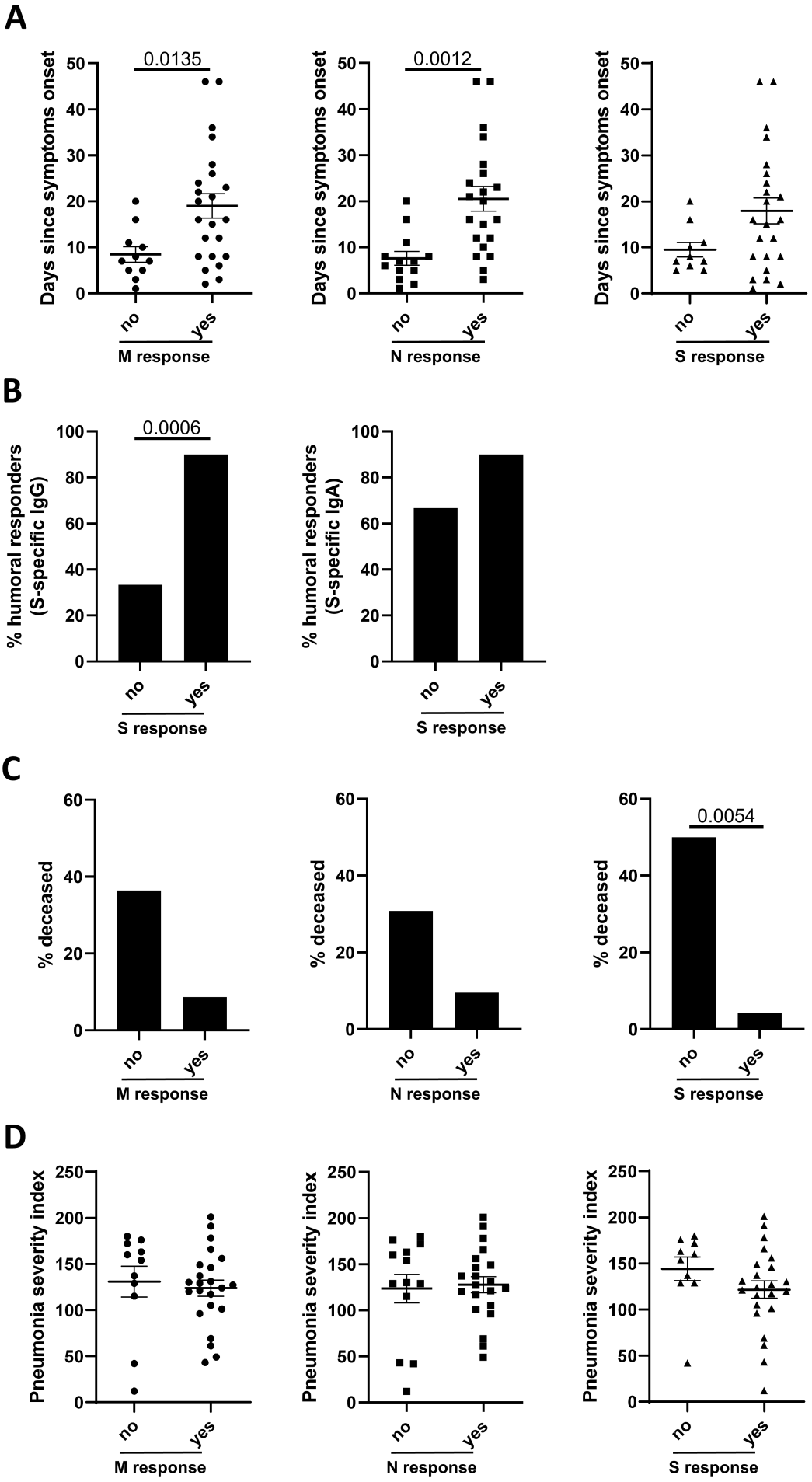


Figure 2

Features of cellular non-responders to CoV-2 specific stimulation. (A-D) All patients were stratified according to their capacity to mount a specific $CD4^+$ T cell response or not after M- (left), N- (middle) or S-protein (right) stimulation with n as described in Fig. 1B. In responders and non-responders, the number of days since symptoms onset was analyzed by t test (A), or (B) the percentage of patients showing spike-protein specific IgG (left; analyzed using Fisher´s exact test) or IgA responses (right; analyzed using Fisher´s exact test). (C) The percentage of individuals who deceased within 6 weeks after analysis (Fisher´s exact test) or (D) the severity of pneumonia (t test) were examined. Where applicable, graphs show means ± SEM.

Sattler et al., Figure 3

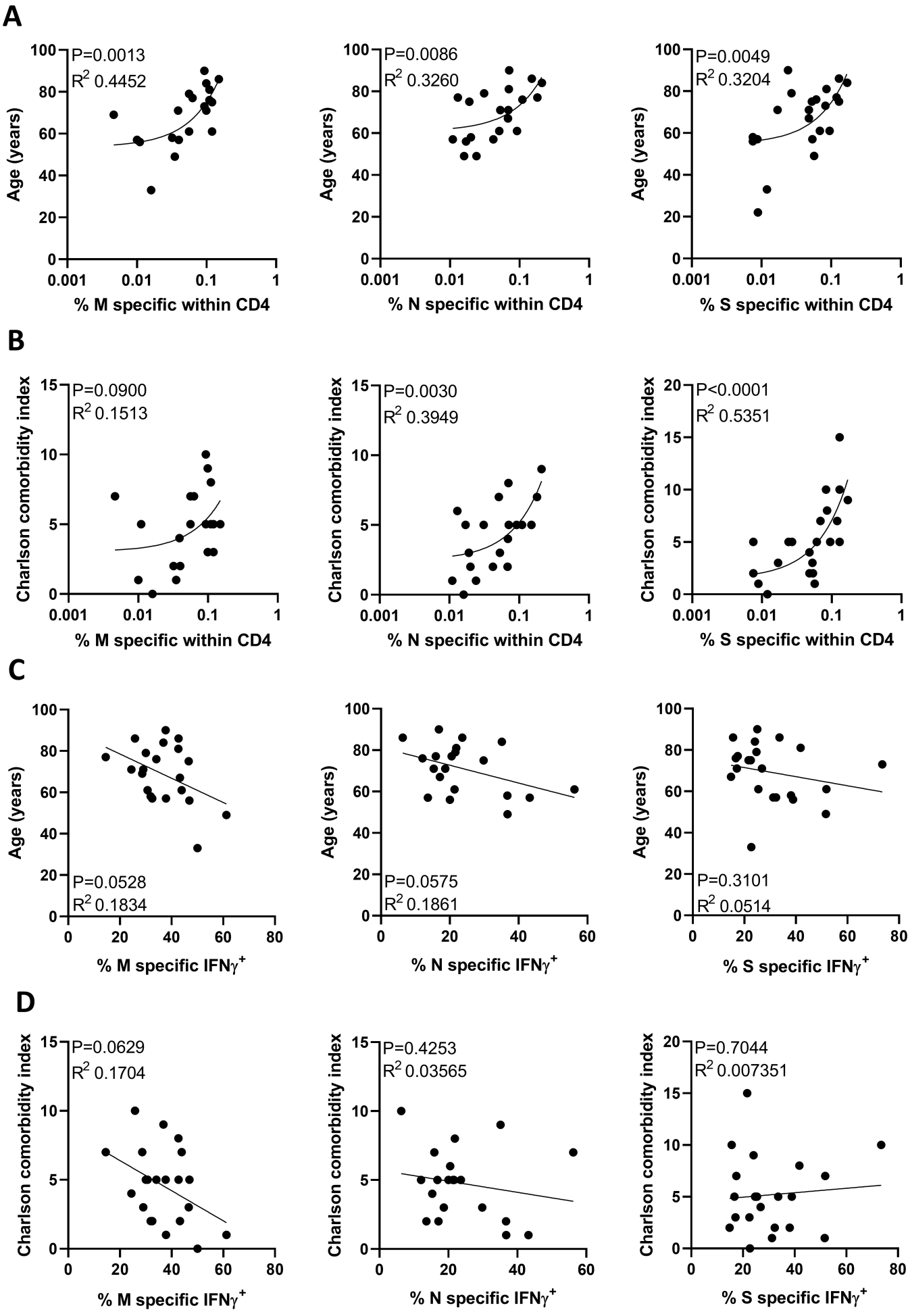


Figure 3

Correlation of the CoV-2 specific $CD4^+$ T cell response with patient age and comorbidities. Frequencies of M-, N- or S-protein specific $CD4^+$ T cells were correlated with patient age (A) or comorbidities (B) with n as in Fig. 1B. Frequencies of M-, N- or S-specific IFNγ secreting T cells with n as in Fig. 1C were correlated with patient age (C) or comorbidities (D). Simple linear regression analysis was performed throughout.

Sattler et al., Figure 4

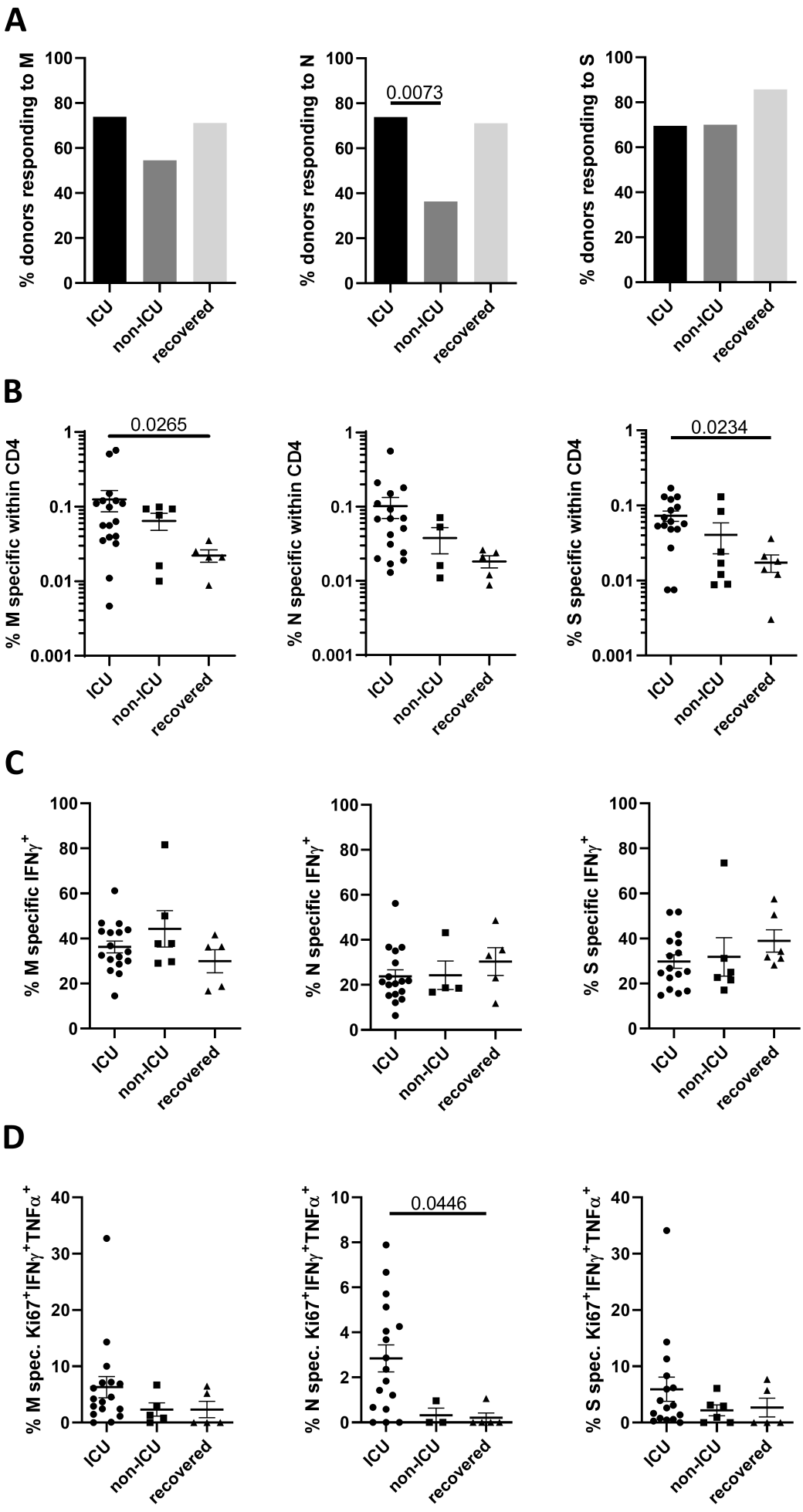


Figure 4

Characteristics of the CoV-2 specific T cell response in patients admitted to intensive care. (A) Patients were stratified according to ICU (M/N/S: n=23, respectively) or non-ICU treatment (M: n=11, N: n=11, S: n=10) and compared to non-hospitalized individuals after recovery (n=7) for the percentage of donors showing specific CD4 responses (χ^2 test), (B) frequencies of antigen-specific $CD4^+$ T cells in cellular responders (ICU - M: n=17, N: n=17, S: n=16; non-ICU - M: n=6, N: n=4, S: n=7; recovered - M: n=5, N: n=5, S: n=6), (C) frequencies of antigen-specific $CD4^+$ T cells secreting IFNγ or (D) ex vivo proliferating $Ki67^+$ co-expressing IFNγ and TNFα (with n as in (B) and analyzed by Kruskal-Wallis test, respectively). Where applicable, graphs show means ± SEM.

Sattler et al., Figure 5

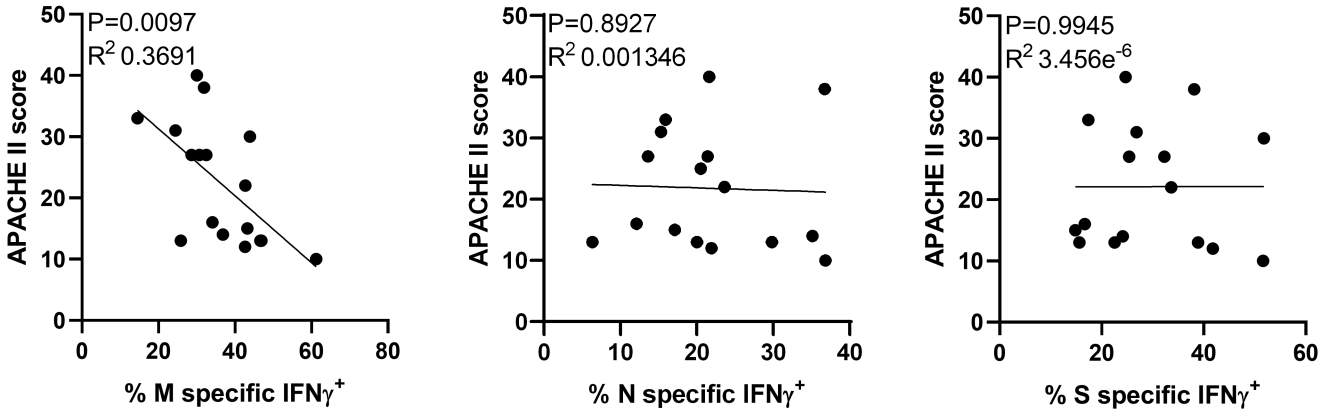


Figure 5

Correlation of CoV-2 specific IFNγ secretion and APACHE II scores in ICU patients. Frequencies of antigen-specific IFNγ expressing $CD4^+$ T cells after M-, N- or S-protein stimulation were determined in responding ICU patients and correlated with the individual APACHE II score values as indicated (M: n=17, N: n=17, S: n=16) by simple linear regression analysis.

Sattler et al., Figure 6

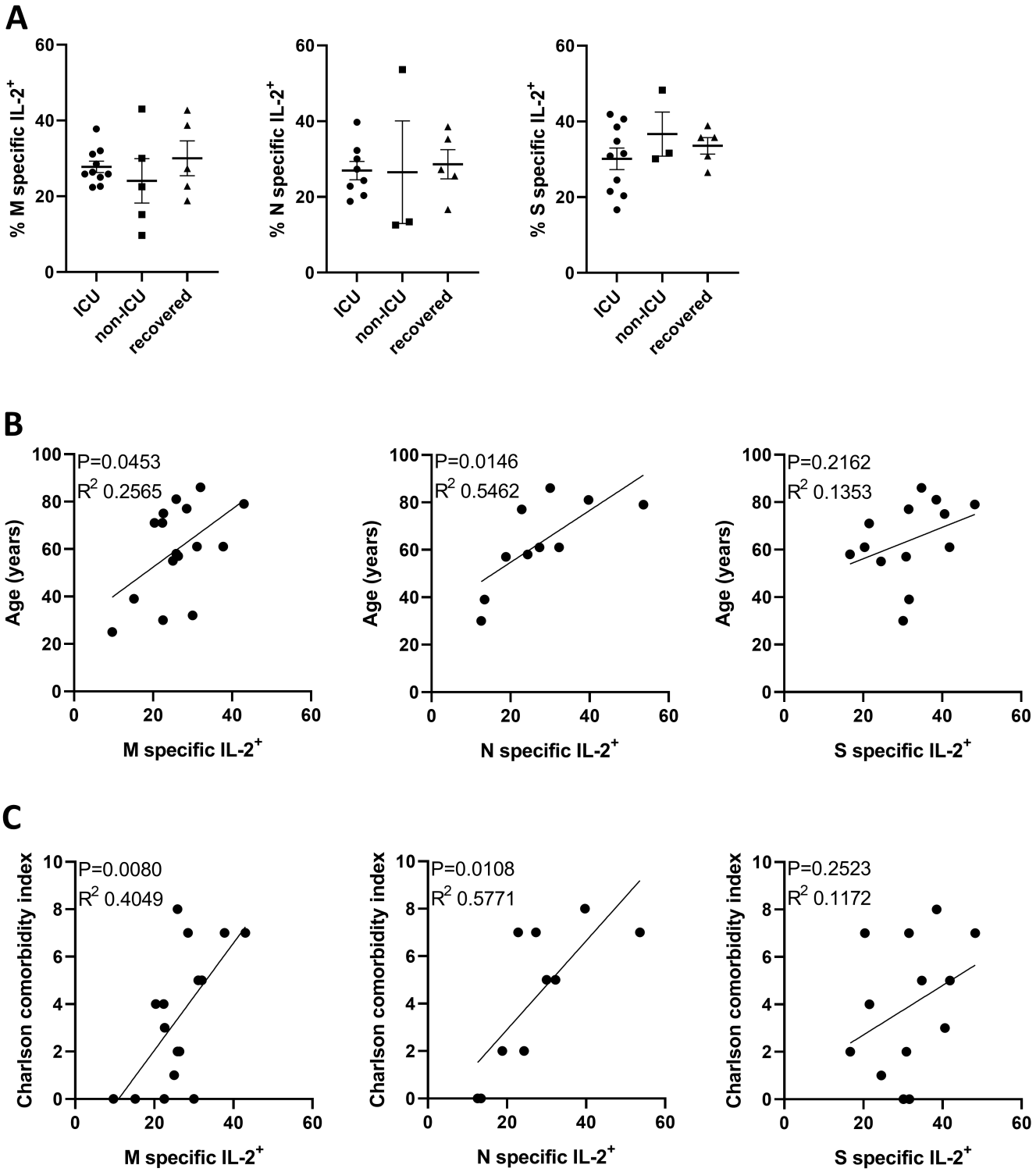


Figure 6

CoV-2 specific IL-2 secretion and its correlation with patient predisposition. Frequencies of antigen-specific IL-2 expressing $CD4^+$ T cells after M-, N- or S-protein stimulation were (A) determined in patients stratified according to ICU- or non-ICU care or in non-hospitalized individuals after recovery (ICU - M: n=10, N: n=8, S: n=10; non-ICU - M: n=5, N: n=3, S: n=3; recovered - M: n=5, N: n=5, S: n=5); analysis by ANOVA, respectively. Frequencies of specific IL-2 secreting T cells were further correlated in active COVID-19 patients with (B) age or (C) comorbidity (M: n=16, N: n=10, S: n=13, respectively) and analyzed by simple linear regression. Bar graphs show means ± SEM.

Sattler et al., Figure 7

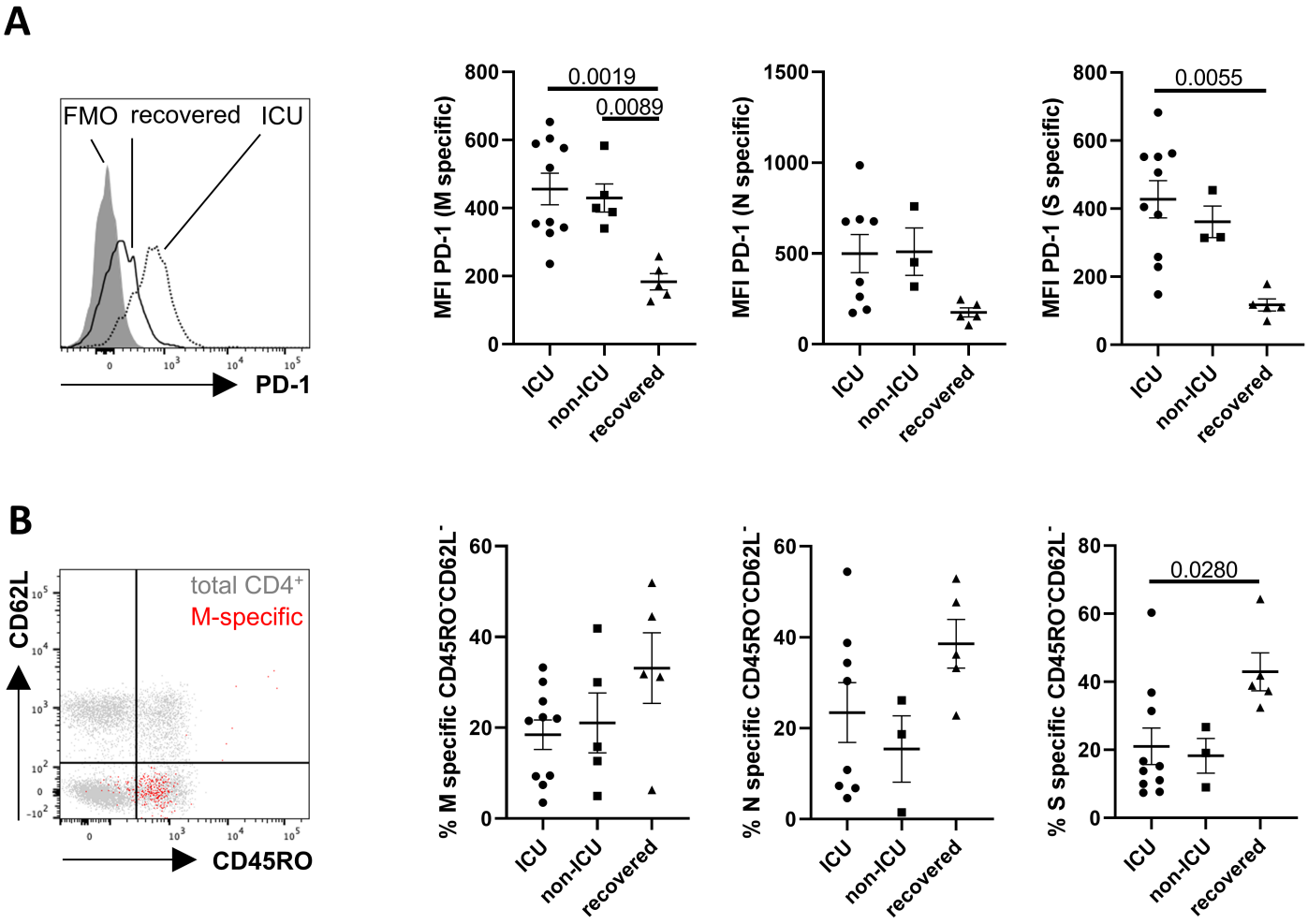
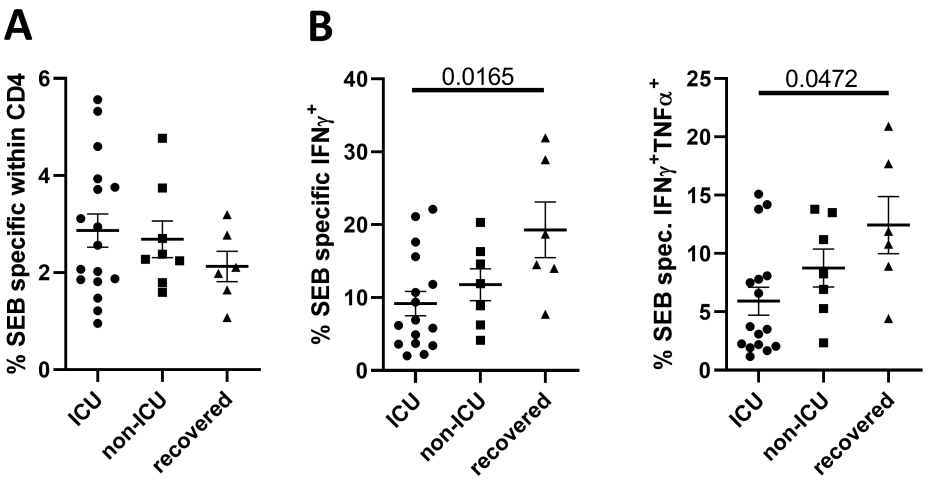


Figure 7

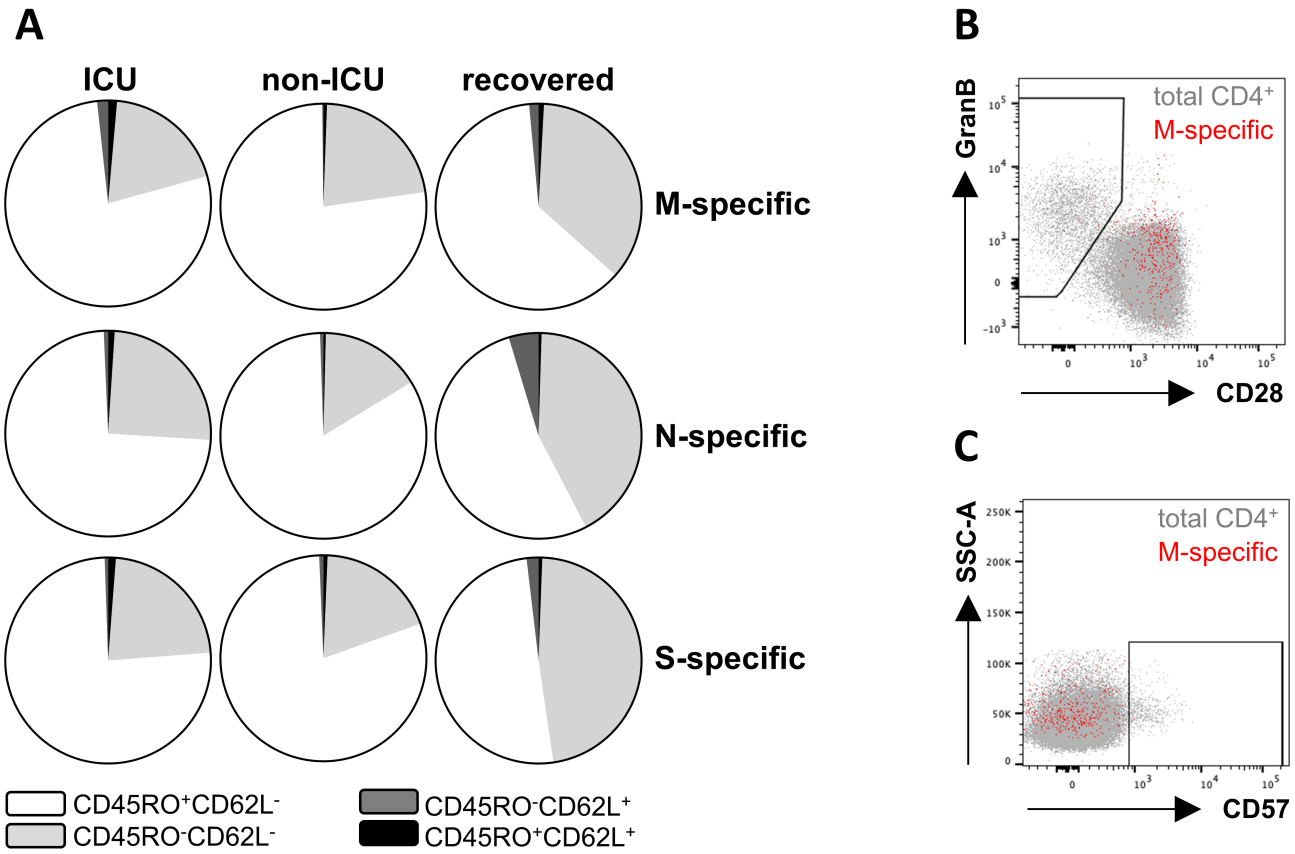
Exhaustion and memory phenotype of CoV-2 specific T cells. (A) Exemplary PD-1 expression in S-specific $CD4^+$ T cells in a recovered individual vs. a patient in ICU care with fluorescence minus one (FMO) control (left) and MFI of PD-1 in M- (ANOVA analysis), N- (ANOVA-analysis) and S- (Kruskal-Wallis analysis) specific T cells in patients stratified as indicated (right). (B) Memory subset distribution of CoV-2 specific T cells was determined based on CD45RO and CD62L expression. Exemplary subset identification in total (grey) and M-specific (red) $CD4^+$ T cells (ICU-patient, left) as well as frequencies of M- (ANOVA analysis), N- (ANOVA analysis) and S- (Kruskal-Wallis analysis) specific $CD45RO^-CD62L^-$ T cells (right) in patients stratified as indicated (ICU - M: n=10, N: n=8, S: n=10; non-ICU - M: n=5, N: n=3, S: n=3; recovered - M: n=5, N: n=5, S: n=5, in (A) and (B), respectively). Bar graphs show means ± SEM.

Sattler et al., Suppl. Figure 1



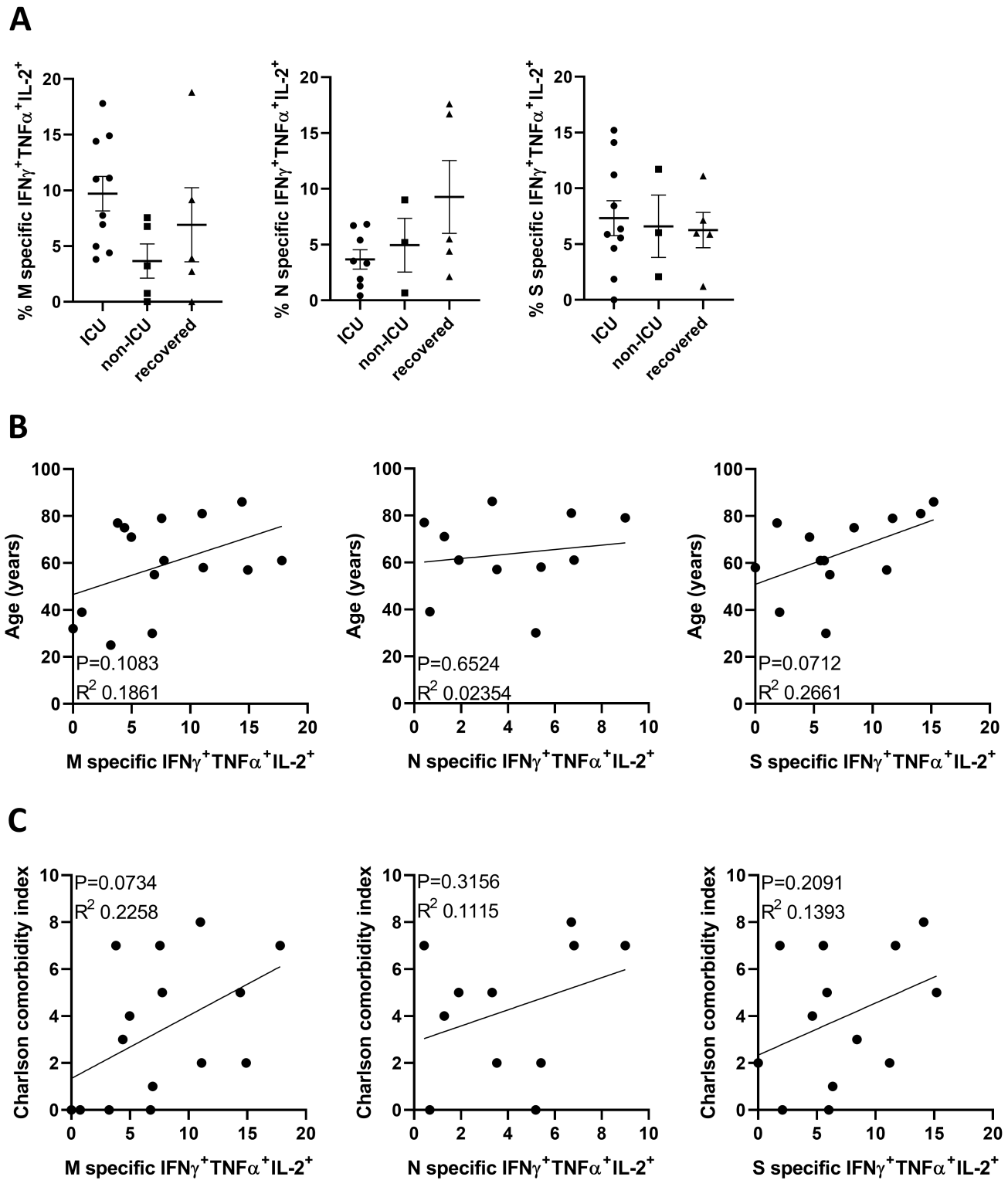
Supplemental Figure 1 *SEB-specific CD4 responses in acute COVID-19 patients and recovered individuals*. (A) Frequencies of $CD154^+CD137^+$ T helper cells after SEB stimulation in ICU (n=17) and non-ICU (n=8) treated patients and recovered individuals (n=6). (B) Analysis of frequencies of IFNγ- (left, by ANOVA) or IFNγ/TNFα coexpressing (right, by Kruskal-Wallis) $CD4^+$ T cells after SEB stimulation with n as in (A). Graphs show means ± SEM.

Sattler et al., Suppl. Figure 2



Supplemental Figure 2 *Phenotype of CoV-2 specific CD4⁺ T cells.* (A) Mean frequencies of the depicted antigen-specific T cell subsets in hospitalized ICU- and non-ICU patients as well as in recovered individuals (ICU - M: n=10, N: n=8, S: n=10; non-ICU - M: n=5, N: n=3, S: n=3; recovered - M: n=5, N: n=5, S: n=5). (B) and (C) Exemplary dot plots showing the distribution of all CD4⁺ T cells (grey) and M-specific T cells (red) according to CD28 and Granzyme B (GranB) (B) or CD57 (C) expression. Representative for all three antigens and patients with n as in (A).

Sattler et al., Suppl. Figure 3



Supplemental Figure 3 *Polyfunctionality of CoV-2 specific CD4⁺ T cells.* (A) Frequencies of polyfunctional IFNγ⁺TNFα⁺IL-2⁺ CoV-2 specific T cells were quantified after Boolean gating in hospitalized ICU- and non-ICU patients as well as in recovered individuals (ICU - M: n=10, N: n=8, S: n=10; non-ICU - M: n=5, N: n=3, S: n=3; recovered - M: n=5, N: n=5, S: n=5; ANOVA, respectively); frequencies in acute COVID-19 patients were further correlated (by simple linear regression analysis) with (B) age or (C) Charlson comorbidity index with patient numbers as in (A). Where applicable, graphs show means ± SEM.

Table I

Characteristics of hospitalized patients and non-hospitalized individuals after recovery

Variable	ICU (n=23)	Non-ICU (n=16)	**p***	Recovered (n=7)
Age (mean yrs ± SD)[$]	72.70 (11.91)	55.6 (24.34)	**0.0059**	32 (3.32)
Females (%)	10/23 (43.5)	3/16 (18.8)	0.1693	4/7 (57.1)
Caucasians (%)	23/23 (100)	15/16 (93.8)	0.4103	7/7 (100)
Days since recovery[$]				25.86 (10.57)
Clinical Parameters				
Days since symptoms onset**,[$]	18.65 (12.46)	11.25 (10.50)	**0.0181**	
Leukocytes/µl**,[$] (Reference range: 3900-10500)	11280 (5510)	6299 (3992)	**0.0043**	
Lymphocytes/µl**,[$$] (Reference range: 1500-3000)	1193 (714)	1456 (599)	0.1658	
$CD4^+$ T cells/µl**,[$$] (Reference range: 500-1200)	578 (426)	544 (298)	0.7892	
CD4:CD8 ratio**,[$] (Reference range: 1.1-3.0)	4.09 (4.03)	2.15 (1.52)	**0.0322**	
PSI**,[$]	144.4 (32.06)	76.31 (49.94)	**<0.0001**	
ARDS (%)[$]	20/23 (86.96)	0/16 (0)	n.a.	
CCI**	5.50 (2.50)	3.43 (4.46)	**0.0202**	
Type 2 diabetes (%)	9/23 (39.1)	2/16 (12.5)	0.0857	
Chronic heart failure (%)	4/23 (17.4)	2/16 (12.5)	>0.9999	
Coronary heart disease (%)	11/23 (47.8)	2/16 (12.5)	**0.0371**	
Chronic lung disease (%)	6/23 (26.1)	3/16 (18.8)	0.7110	
Chronic liver disease (%)	1/23 (4.3)	0/16 (0)	>0.9999	
Chronic kidney disease (%)	5/23 (21.7)	5/16 (31.3)	0.7110	
Obesity (%)	9/23 (39.1)	2/16 (12.5)	0.0857	
Bacterial superinfection (%)[$]	18/23 (78.3)	2/16 (12.5)	**<0.0001**	
Mechanical ventilation (%)[$]	16/23 (69.6)	0/16 (0)	n.a.	
Deceased within 6w post analysis (%)	6/23 (26.1)	0/16 (0)	0.0642	
Deceased due to respiratory dysfunction	6/6 (100)			
Females within deceased (%)	3/6 (50)			
APACHE II**	21.22 (9.53)			
SOFA**	7.55 (4.25)			
SAPS**	45.57 (17.33)			

*Comparison of ICU vs non-ICU patients
**Mean and standard deviation
[$]At day of antigen-specific T cell analysis
[$$]Within ± 3 days around date of antigen-specific T cell analysis
n.a. – not applicable since conditions require ICU admission
PSI – Pneumonia Severity Index, ARDS – Acute Respiratory Distress Syndrome, CCI – Charlson Comorbidity Index, APACHE - Acute Physiology And Chronic Health Evaluation score, SOFA - Sepsis-related Organ Failure Assessment score, SAPS - Simplified Acute Physiology Score