Presence of SARS-CoV-2-reactive T cells in COVID-19 patients and healthy donors

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Summary

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has caused a rapidly unfolding pandemic, overwhelming health care systems worldwide[1]. Clinical manifestations of Coronavirus-disease 2019 (COVID-19) vary broadly, ranging from asymptomatic infection to acute respiratory failure and death[2], yet the underlying mechanisms for this high variability are still unknown. Similarly, the role of host immune responses in viral clearance of COVID-19 remains unresolved. For SARS-CoV (2002/03), however, it has been reported that CD4^{+} T cell responses correlated with positive outcomes[3,4], whereas T cell immune responses to SARS-CoV-2 have not yet been characterized. Here, we describe an assay that allows direct detection and characterization of SARS-CoV-2 spike glycoprotein (S)-reactive CD4^{+} T cells in peripheral blood. We demonstrate the presence of S-reactive CD4^{+} T cells in 83% of COVID-19 patients, as well as in 34% of SARS-CoV-2 seronegative healthy donors (HD), albeit at lower frequencies. Strikingly, S-reactive CD4^{+} T cells in COVID-19 patients equally targeted N-terminal and C-terminal epitopes of S whereas in HD S-reactive CD4^{+} T cells reacted almost exclusively to the C-terminal epitopes that are a) characterized by higher homology with spike glycoprotein of human endemic "common cold" coronaviruses (hCoVs), and b) contains the S2 subunit of S with the cytoplasmic peptide (CP), the fusion peptide (FP), and the transmembrane domain (TM) but not the receptor-binding domain (RBD). In contrast to S-reactive CD4^{+} T cells in HD, S-reactive CD4^{+} T cells from COVID-19 patients co-expressed CD38 and HLA-DR, indivative of their recent *in vivo* activation. Our study is the first to directly measure SARS-CoV-2-reactive T cell responses providing critical tools for large scale testing and characterization of potential cross-reactive cellular immunity to SARS-CoV-2. The presence of pre-existing SARS-CoV-2-reactive T cells in a subset of SARS-CoV-2 naïve HD is of high interest but larger scale prospective cohort studies are needed to assess whether their presence is a correlate of protection or pathology for COVID-19. Results of such studies will be key for a mechanistic understanding of the SARS-CoV-2 pandemic, adaptation of containment methods and to support vaccine development.

Main

The COVID-19 pandemic poses an unprecedented threat to public health and the global economy. On 9th April 2020, the number of confirmed COVID-19 cases worldwide surpassed 1,500,000 cases with over 90,000 COVID-19 related deaths[1]. Diagnosis of COVID-19 is routinely achieved by detection of SARS-CoV-2 RNA in nasopharyngeal swabs via PCR[5], which works reliably in the acute phase of COVID-19[6,7]. However, limited test availability and preferential testing of symptomatic patients has likely lead to significant underestimation of infection burden and overestimation of mortality rates[8]. Whereas serological analysis of SARS-CoV-2-induced humoral immunity could reveal asymptomatic infections, it is not yet widely applied[9,10] and complicated by the fact that coronavirus-induced antibody responses are quite variable and rather short-lived[11,12]. Coronavirus-induced cellular immunity is predicted to be more sustained, but poorly characterized so far. However, a number of T cell epitopes in coronavirus structural proteins have been predicted or identified[11,13–15]. Importantly, T helper cell responses and generation of neutralizing antibodies may be interdependent[11,16]. Studies on the SARS-CoV epidemic in 2002/03 have shown that adaptive immune responses towards spike glycoprotein were protective[11,17,18]. Hence, induction of SARS-CoV-2-specific CD4^{+} T cells is likely to be critical in the instruction of affinity maturated and potentially protective antibody responses[19]. We therefore examined the presence, frequencies and phenotypic characteristics of SARS-CoV-2 spike glycoprotein (S)-reactive T cells in COVID-19 patients compared to SARS-CoV-2 seronegative healthy donors (HD).

Direct identification of SARS-CoV-2 spike glycoprotein-reactive CD4^{+} T cells

SARS-CoV-2 spike glycoprotein (S)-reactive CD4^{+} T cells were identified according to their expression of CD40L and 4-1BB after *in vitro* stimulation of peripheral blood mononuclear cells (PBMCs) using spike glycoprotein peptides. To this end, we designed two overlapping peptide pools (15 amino acids (aa), 11 aa overlaps) spanning the entire S that comprised different amounts of putative MHC-II epitopes according to experimental data for identified epitopes in SARS-CoV[13–15] (Figure 1a). Peptide pool PepMix[TM] 1 (henceforth referred to as S-I) comprised overlapping peptides covering the N-terminal part (aa residues 1-643) including 21 predicted SARS-CoV MHC-II epitopes (Figure 1a, Supplementary Figure 1, Supplementary Table 1). The second peptide pool PepMix[TM] 2 (henceforth referred to as S-II) covered the C-terminal portion (amino acid residues 633-1273) including 13 predicted SARS-CoV MHC-II epitopes (Figure 1a). Peptide pool S-II exhibits a higher homology with human endemic "common cold" coronaviruses (HCoV; 229E, NL63, OC43, HKU1) with regard to the SARS-CoV MHC-II epitopes, as compared to peptide pool S-I (Figure 1a, b). The peptides of the receptor-binding domain (RBD) in the subunit S1, which represents a major target of neutralizing antibodies, are included in S-I[20,21]. For antigen-specific stimulation, PBMCs were isolated from blood samples of COVID-19 patients and HD (see Table 1 and Table 2 for patient characteristics and Supplementary Table 2 for HD characteristics) and stimulated for 16 hours with S-I and S-II peptide pools, respectively. Antigen-reactive CD4^{+} T cells were identified by co-expression of 4-1BB and CD40L, enabling the sensitive detection of S-reactive CD4^{+} T cells re-activated by TCR engagement *ex vivo*[22–24] (Figure 2a, Supplementary Figure 2). Twelve of 18 COVID-19 (67%) patients had detectable S-I-reactive CD4^{+} T cells. Fifteen COVID-19 (83%) patients had detectable S-reactive CD4^{+} T cells against S-II peptide pool (Figure 2b, d). Most non-reactive COVID-19 patients were characterized by critical disease states

114   (Supplementary Figure 3). Remarkably, S-reactive CD4^{+} T cells could also be detected in 23
115   (34%) of 68 HD, albeit at lower frequencies compared with COVID-19 patients (Figure 2e).
116   These HD are henceforth defined as reactive healthy donors (RHD). Thereof, S-I-reactive CD4^{+}
117   T cells could only be detected in 6 of these 23 RHD, i.e. in 8.8% of all HD. All HD were
118   negative for IgG antibodies specific for S subunit 1 (S1) in contrast to COVID-19 patients
119   (Figure 2f). We also ruled out early SARS-CoV-2 infection in 10 RHD by PCR standard
120   diagnosis of nasopharyngeal swabs (data not shown).

121

Spike N-terminal versus C-terminal CD4^{+} T cell reactivity delineates COVID-19 patients
122

123   **from RHD**
124   S-reactive CD4^{+} T cells in COVID-19 equally targeted both N-terminal (S-I) and C-terminal
125   peptide pools (S-II) of S (Figure 2d). In contrast, S-reactive CD4^{+} T cells from HD exhibited a
126   significantly stronger reactivity to the C-terminal peptide pool S-II, characterized by higher
127   homology to spike glycoprotein of HCoV, compared to the N-terminal pool S-I (Figure 2d).
128   The data suggest that S-reactivity among CD4^{+} T cells in SARS-CoV-2-naïve HD originated
129   from previous immune responses against HCoV. Therefore, we additionally tested 18 of the 68
130   HD for the presence of antibodies specific for the four HCoV (229E, NL63, HKU1, OC43).
131   We detected IgG antibodies against the four HCoV in all tested HD, regardless of the presence
132   of measurable S-reactive CD4^{+} T cells (Supplementary Figure 4), showing that S-
133   (cross-)reactive CD4^{+} T cells from HD do not correlate with antibody levels against HCoVs.
134   This is in line with findings from other anti-viral CD4^{+} T cell responses such as in the course
135   of yellow fever vaccination (YFV-17D). Only at very early time points after YFV-17D
136   vaccination CD4^{+} T cell responses correlated with generation of high titers of neutralizing
137   antibodies measured at later time points such as day 14[25].

138

Specific activation signatures of S-reactive CD4^{+} T cells in COVID-19 patients
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140   Recent studies on SARS-CoV-2 serology have raised the question whether antibody studies are
141   sufficient to identify individuals with mild or asymptomatic courses of COVID-19[7,10] or
142   whether additional, cellular signatures are needed to identify acute or past infections. Here, we
143   assessed additional activation marker profiles on S-reactive T cells from COVID-19 patients.
144   Expression of CD38, HLA-DR and Ki-67 has previously been shown to reliably characterize
145   recently *in vivo* activated human T cells during acute and chronic infection[26–30]. Notably, S-
146   reactive CD4^{+} T cells from COVID-19 patients largely expressed CD38, HLA-DR and Ki-67
147   (Figure 3a-d). The majority of S-reactive T cells in COVID-19 patients co-expressed CD38 and
148   HLA-DR (Figure 3e) characteristic for effector T cell responses during acute viral
149   infections[26,28] whereas CD38 and Ki-67 co-expression was more variable (Figure 3f). By
150   contrast, S-reactive CD4^{+} T cells from HD did not express CD38, HLA-DR and Ki-67, or only
151   at low frequencies (Figure 3b-f), and co-expression was not observed (Figure 3e, f). In COVID-
152   19 patients, considerable proportions of peripheral CD4^{+} and CD8^{+} T cells co-expressed CD38
153   and HLA-DR (data not shown), which, however, could not be re-activated with our S peptide
154   pools *in vitro*. These findings are in line with results of a recent study showing refractory T cell
155   signatures in COVID-19 patients[31] and also a proportion of these CD38^{+}HLADR^{+} CD4^{+} T cells
156   may target other structural proteins of SARS-CoV-2. We also show that the presence of S-
157   reactive CD4^{+} T cells and in particular of CD38 expressing cells among S-reactive CD4^{+} T cells
158   exhibited a high variability among patients in the course of COVID-19 disease (Figure 3g,h).

159

160 **Discussion**

161 Our study demonstrates the presence of S-reactive CD4^{+} T cells in COVID-19 patients, and in
162 a subset of SARS-CoV-2 seronegative HD. In light of the very recent emergence of SARS-
163 CoV-2, our data raise the intriguing possibility that pre-existing S-reactive T cells in a subset
164 of SARS-CoV-2 seronegative HD represent cross-reactive clones raised against S-proteins,
165 probably acquired as a result of previous exposure to HCoV. Endemic HCoV account for about
166 20% of "common cold" upper respiratory tract infections in humans. HCoV infections are
167 ubiquitous, but they display a winter seasonality in temperate regions[32–34]. Based on
168 epidemiological data indicating an average of two episodes of "common cold" per year in the
169 adult population, it may be extrapolated that the average adult contracts a HCoV infection on
170 average every two to three years. Protective antibodies may wane in the interim but cellular
171 immunity could remain[15,35]. Although the overall amino acid sequence homology of spike
172 glycoproteins is relatively low among HCoV, there is an overlap of MHC-II epitopes located
173 especially in the C-terminal domain of the here used peptide pools (Figure 1a, Supplementary
174 Figure 1). This may explain the preferential reactivity of CD4^{+} T cells to the C-terminal domain
175 in one third of SARS-CoV-2 seronegative HD.

176 The biological role of pre-existing SARS-CoV-2 S-cross-reactive CD4^{+} T cells in 34% of HD
177 remains unclear for now. However, these cells may represent the key to understanding the
178 vastly divergent manifestations of SARS-CoV-2 disease courses, and particularly the suspected
179 high rate of asymptomatic infections in children and young adults assuming that these S-cross-
180 reactive CD4^{+} T cells have a protective role in SARS-CoV-2 infection. Since children and
181 young adults have on average more frequent social contacts than the elderly, one might expect
182 a higher transmission rate and HCoV prevalence in the former. This assumption would need to
183 be investigated in future longitudinal studies assessing the presence of pre-existing SARS-CoV-
184 2-cross-reactive CD4^{+} T cells and their impact on the susceptibility to SARS-CoV-2 infcetion
185 and age-realted clinical outcomes of COVID-19.

186 SARS-CoV neutralizing antibodies are associated with convalescence, and they have been
187 detected 12 months after disease[11]. However, the durability of neutralizing antibody responses
188 against SARS-CoV-2 currently remains unknown. Antibodies against HCoV can wane within
189 months after infection, although HCoV re-infection is accompanied by low-level and short-
190 lived virus shedding with only mild symptoms of short duration[12] pointing towards residual
191 immunity. Cellular immunity has not yet been studied in this context. The extent to which and
192 how SARS-CoV-2 specific humoral or cellular immunity mediates durable protection against
193 reinfection is unknown but will be one of the critically important fields of research in the
194 coming months. It has been demonstrated in mouse models that CD4^{+} as well as CD8^{+} T cell
195 responses directed against structural proteins such as spike or nucleocapsid protein of SARS-
196 CoV critically contribute to viral clearance[17,36,37].

197 To our knowledge, this study represents the first report of cellular SARS-CoV-2-cross-
198 reactivity in human T cells. Our results provide a decisive rationale to initiate worldwide
199 prospective studies to assess the contribution of pre-existing SARS-CoV-2-cross-reactive
200 immunity (potential regional differences) to clinical outcomes of SARS-CoV-2-infections.
201 Together with currently introduced, novel serology tests, the data generated by such studies
202 may critically inform evidence-based risk evaluation, patient monitoring, adaptation of
203 containment methods, and last but not least, vaccine development.

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213

214 **Materials and Methods**

215

216 **Study subjects**
217 This study was approved by the Institutional Review board of the Charité (EA2/066/20). After
218 providing written informed consent, 68 healthy donors (Supplementary Table 1) and 18
219 COVID-19 patients (Table 1) were included in the study. COVID-19 patients tested positive
220 for SARS-CoV-2 RNA in nasopharyngeal swabs were recruited at Charité Campus
221 Virchowklinikum, Berlin between March 1st and April, 2nd 2020.

222

223 **Serology**
224 Anti-SARS-CoV-2 IgG ELISA was performed using a commercial kit (EUROIMMUN
225 Medizinische Labordiagnostika AG) as described and validated before[38]. Recombinant
226 immunofluorescence assays (rIFA) to determine IgG titers against HCoV was done by using
227 VeroB4 cells expressing cloned recombinant coronavirus spike proteins from HCoV-229E,
228 HCoV-NL63, HCoV-OC43, HCoV-HKU1 as described in Corman et al.[39].

229

230 **Cell isolation and stimulation**
231 Peripheral blood mononuclear cells (PBMC) were isolated from heparinized whole blood by
232 gradient density centrifugation according to manufacturer's instructions (Leucosep tubes,
233 Greiner; Biocoll, Bio&SELL). Stimulation was conducted with $5x10^6$ PBMC in RPMI 1640
234 medium (Gibco) supplemented with 10% heat inactivated AB serum (Pan Biotech), 100 U/ml
235 penicillin (Biochrom), 0.1 mg/ml streptomycin (Biochrom) and PepMix^{TM} SARS-CoV-2 spike
236 glycoprotein (JPT) peptide pool 1 or 2 in the presence of 1 µg/ml purified anti-CD28 (clone
237 CD28.2, BD Biosciences). The PepMix^{TM} SARS-CoV-2 spike glycoprotein pool 1 covering
238 the N-terminal amino acid (aa) residues 1-643 (abbreviated to "S-I (N-term)") contained 158
239 15-mers overlapping by 11 aa. PepMix^{TM} SARS-CoV-2 spike glycoprotein pool 2 covered the
240 C-terminal aa residues 633-1273 (abbreviated to "S-II (C-term)") containing 156 15-mers
241 overlapping by 11 aa and one 17-mer at the C-terminus, i.e. 157 peptides in total. Both peptide
242 pools were used at 1 µg/ml per peptide, respectively. Further details on the peptide pools and
243 predicted MHC-II epitopes are given in Figure 1, Supplementary Figure 1 and Supplementary
244 Table 2. Stimulation controls were performed with equal concentrations of DMSO in PBS
245 (unstimulated) or 1.5 mg SEB/1.0 mg TSST1 (Sigma-Aldrich) and PepMix^{TM} HCMVA (pp65)
246 (>90%) (JPT) in the presence of 1 µg/ml purified anti-CD28 (clone CD28.2, BD Biosciences)
247 as positive controls, respectively. Incubation was performed at 37˚C, 5% CO_2 for 16 h with 10
248 µg/ml brefeldin A (Sigma- Aldrich) added after 2 h.

249

Flow Cytometry

250 Stimulation was stopped by incubation in 20 mM EDTA for 5 min and surface staining
251 conducted for 15 min with the following fluorochrome conjugated antibodies titrated to their
252 optimal concentrations: CD38-PE-Vio770 (clone REA671, Miltenyi), CD69-APC-Cy7 (FN50,
253 Biolegend), HLAD-DR-VioGreen (REA805, Miltenyi), CD4-BrilliantViolet605 (RPA-T4,
254 Biolegend), CD8-PerCP (SK1, Biolegend) with 1 mg/ml Beriglobin (CSL Behring) added prior
255 to the staining. For exclusion of dead cells, Zombie Yellow fixable viability staining (Biolegend)
256 was added for the last 10 min of incubation. Fixation and permeabilization were performed with
257 eBioscience™ FoxP3 fixation and PermBuffer (Invitrogen) according to the manufacturer's
258 protocol and intracellular staining carried out for 30 min in the dark at room temperature with
259 Beriglobin added prior to intracellular staining with 4-1BB-PE (clone 4B4-1, BD), CD40L-
260 APC (5C8, Miltenyi) and Ki-67-AlexaFluor488 (B56, BD). Samples were measured on a
261 MACSQuant® Analyzer 16. Instrument performance was monitored daily with Rainbow
262 Calibration Particles (BD).

264

Data analysis and statistics

265 Flow cytometry data were analyzed with FlowJo 9.9.6 (FlowJo LLC). Prism 5 (GraphPad Inc.)
266 was used for plotting and statistical analysis. Non-parametric testing was used to compare cell
267 frequencies and antibody titers between groups (two-tailed Mann-Whitney U test). n indicates
268 the number of donors.

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Table 1 | Baseline characteristics of hospitalized COVID-19 patients.

*Day after onset of symptoms, ICU: intensive care unit, y: yes, n: no, m: male, f: female,

				COVID-19 patients		
ID	Severity	Gender	Age	Chief complaints at admission	ICU (y/n)	Sampling (day)*
P01	mild	m	21	Fever, dry cough, malaise	n	39
P03	mild	m	45	Fever, dry cough, runny nose	n	25
P10	mild	f	50	Fever, dry cough, myalgia, cephalgia, diarrhea	n	17
P18	mild	f	60	Fever, myalgia, cephalgia, runny nose	n	21
P19	mild	m	52	Dry cough, cephalgia, arthralgia, nausea	n	11
P23	mild	f	44	Fever	n	5
P27	mild	f	41	Fever, dry cough	n	13
P06	severe	m	24	Fever, dyspnea, malaise	y	11
P07	severe	m	33	Fever, dry cough, dyspnea, myalgia	n	5
P11	severe	m	61	Fever, dry cough, dyspnea, sore throat	y	12
P16	severe	m	74	Fever, dry cough, dyspnea, malaise	y	12
P24	severe	m	64	Fever, dry cough, dyspnea	y	19
P08	critical	m	63	Fever, dyspnea	y	2
P12	critical	m	75	Fever, dyspnea, malaise	y	8
P14	critical	m	81	Fever, dyspnea	y	8
P15	critical	m	54	Dry cough, dyspnea	y	6
P20	critical	f	53	Dry cough, dyspnea	y	11
P21	critical	m	52	Dry cough, dyspnea	y	9

Table 2 | Baseline characteristics of COVID-19 patients and healthy donors.

*Day after onset of symptoms, Avg: average, m: male, f: female, ICU: intensive care unit, y: yes, n: no.

| Cohort	m:f	Age Avg (range)	severity		ICU (y/n)	Sampling day Avg (range)
COVID-19 patients	72 : 28 %	52.6 (21-81 yrs)	mild severe critical	38.9% 27.8% 33.3%	n 44.4% y 55.6%	14.9 (2-39)
Healthy donors	31 : 59 %	41.9 (20-64 yrs)	-		-	-

Figure 1
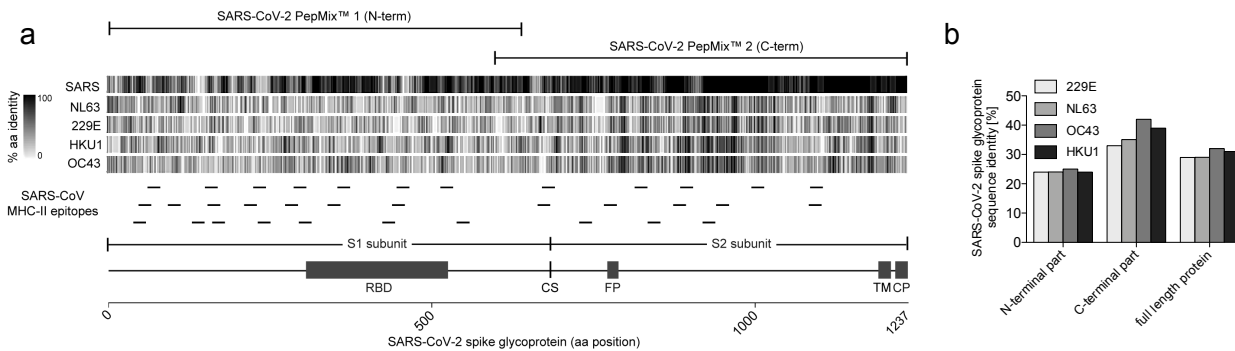
Figure 1: Structural domains, homology and MHC-II epitopes of the SARS-CoV-2 spike glycoprotein. a, SARS-CoV-2 spike glycoprotein (1237 amino acids (aa)) is separated at the cleavage site (CS) into subunit S1 harboring the receptor-binding domain (RBD) and subunit S2 containing the fusion peptide (FP), the transmembrane domain (TM) and the cytoplasmic peptide (CP). Sequence homology of spike glycoprotein of SARS-CoV-2 to SARS-CoV and HCoV strains NL63, 229E, HKU1 and OC43 was calculated as percentage of aa identity in sliding windows of 10 aa and is depicted as grey scale bars. Known SARS-CoV MHC-II epitopes are indicated as small lines below and sequences are listed in Supplementary Table 2. Homology is depicted for each reported MHC-II epitope in Supplementary Figure 1. SARS-CoV-2 PepMix™ 1 (N-term) (refered to as S-I) spans over the N-terminal and SARS-CoV-2 PepMix™ 2 (C-term) (refered to as S-II) over the C-terminal part of S. **b,** Proportion of sequence identity of the N-terminal and C-terminal parts of SARS-CoV-2 spike glycoprotein to the spike glycoproteins of HCoV strains NL63, 229E, HKU1 and OC43.

Figure 2
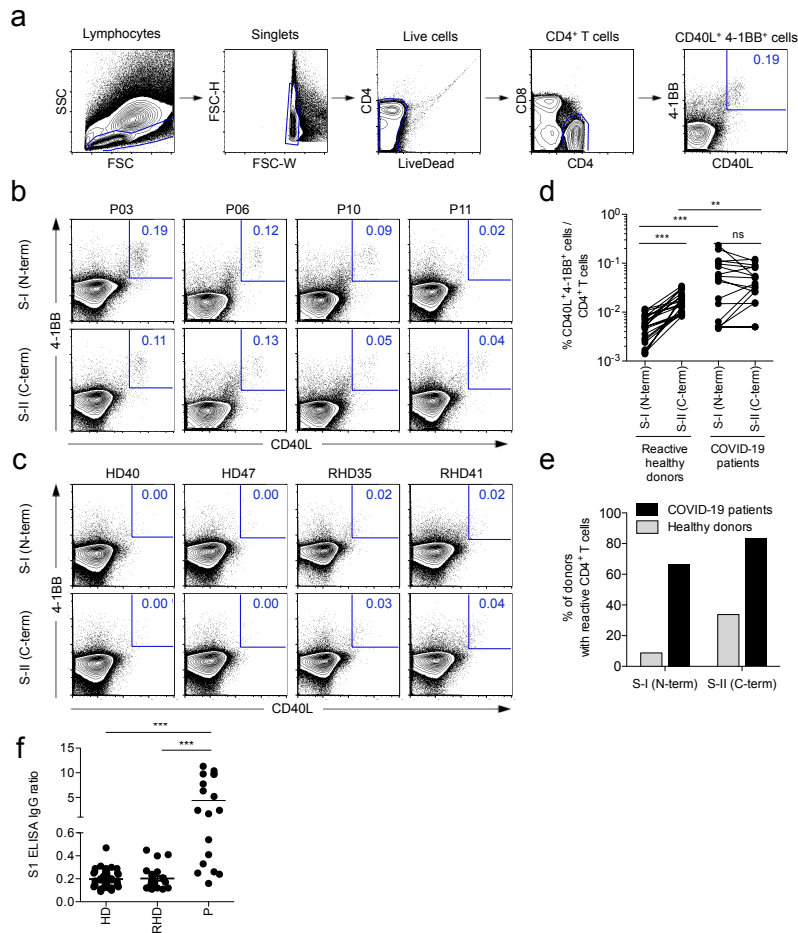
Figure 2: SARS-CoV-2 spike glycoprotein-reactive CD4+ T cells in COVID-19 patients and healthy donors. a, Gating strategy to detect SARS-CoV-2-reactive CD4+ T cells after 16 hours *in vitro* stimulation with PepMix™ SARS-CoV-2 spike glycoprotein peptide pool 1 (S-I (N-term)) and 2 (S-II (C-term)). Representative result of one COVID-19 patient is depicted. **b, c,** Representative plots displaying CD40L and 4-1BB expression on CD4+ T cells of COVID-19 patients (P), healthy donors (HD) and reactive healthy donors (RHD) after 16 hours *in vitro* stimulation with S-I (N-term) or S-II (C-term). Numbers indicate percent of total CD4+ T cells. **d,** Comparison of S-I (N-term)- or S-II (C-term)-reactive CD40L+4-1BB+ frequencies of RHD (*n*=23) and COVID-19 patients (*n*=18). **e,** Ratio of S-I (N-term)- or S-II (C-term)-reactive individuals within the cohort of COVID-19 patients and HD. **f,** Comparison of anti-spike glycoprotein subunit 1 (S1) IgG titers (ratio normalized to calibrator well) of HD (*n*=44), RHD (*n*=23) and P (*n*=18). * p<0.05, ** p<0.01, *** p<0.001 as calculated by two-tailed Mann-Whitney U test.

Figure 3
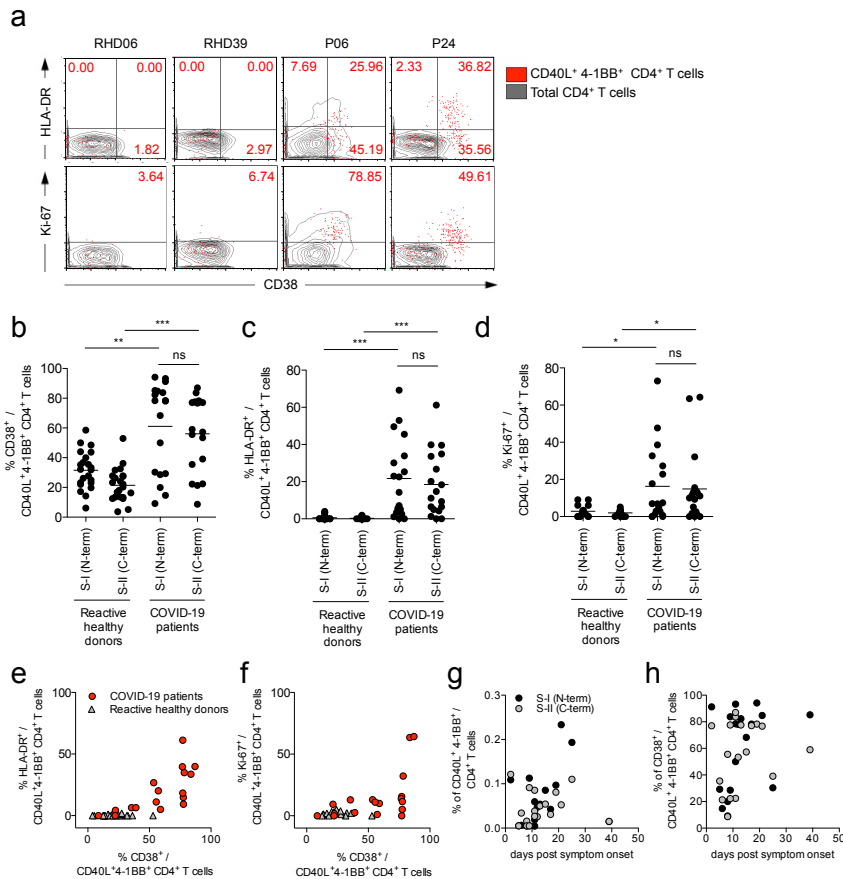
Figure 3: CD38, HLA-DR and Ki-67 expression of SARS-CoV-2 S-I and S-II-reactive CD4$^+$ T cells discriminates SARS-CoV-2 patients from reactive healthy donors. a, Representative examples of HLA-DR or Ki-67 against CD38 expression on S-II (C-term)-reactive CD4$^+$ T cells (red dots) compared to total CD4$^+$ T cells (grey contours) in RHD and COVID-19 patients. **b-d,** Comparison of frequencies of CD38$^+$, HLA-DR$^+$ and Ki67$^+$ cells among S-I (N-term)- and S-II (C-term)-reactive CD4$^+$ T cells in RHD (**b,c,** n=23; **d,** n=17) and COVID-19 patients (n=18). * p<0.05, ** p<0.01, *** p<0.001 as calculated by two-tailed Mann-Whitney U test. **e, f,** Co-expression of HLA-DR$^+$ or Ki-67$^+$ and CD38$^+$ among S-II (C-term)-reactive CD4$^+$ T cells from RHD (**e,** n=23; **f,** n=17) and COVID-19 patients (n=18). **g, h,** Frequencies of S-I (N-term)- and S-II (C-term)-reactive CD40L$^+$ 4-1BB$^+$ CD4$^+$ T cells or CD38$^+$ among S-II (C-term)-reactive cells of COVID-19 patients (n=18) plotted over time (days post symptom onset).